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THE IMPACT OF PHOSPHATIDYLETHANOLAMINE ON THE ATHEROPROTECTIVE FUNCTIONS OF HDL

Novel insights into the role of polyunsaturated fatty acids in the context of
atherosclerotic cardiovascular diseases



SORBONNE
UNIVERSITY

Malik TARADEH

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**The Impact of Phosphatidylethanolamine on the
Atheroprotective Functions of HDL**

*Novel insights into the role of polyunsaturated fatty acids in
the context of atherosclerotic cardiovascular diseases*

Par [Malik TARADEH]

Thèse de doctorat de [Biologie intégrée]

Dirigée par [Dr. Wilfried Le Goff et Dr. Isabelle Guillas]

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Devant un jury composé de :

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Résumé français

Les HDL, ou lipoprotéines de haute densité, sont souvent appelées "bon cholestérol" en jouant un rôle vital dans la réduction du risque de maladie cardiovasculaire athéroscléreuse (ASCVD). Les HDL exercent plusieurs effets athéropotecteurs, y compris au travers de leur capacité d'efflux du cholestérol (CEC) des macrophages artériels, de leurs activités anti-inflammatoires (AIA) et de leurs activités anti-oxydantes (AOX). Parmi les composants des HDL, les phospholipides sont considérés comme des composants majeurs de leurs fonctionnalités. Nous avons cherché à identifier de nouvelles espèces de phospholipides impliquées dans la détermination des fonctions athéropotectives des HDL. Des taux plasmatiques élevés d'espèces de phosphatidyléthanolamines (PE) sont fortement liés à un risque accru d'ASCVD, mais les mécanismes sous-jacents restent flous. Parmi les espèces de PE, le PE (36:5) semble être une signature lipidique prédictive des événements d'ASCVD. Dans le plasma, le PE (36:5) existe sous forme eicosapentaénoïque (EPA, C20:5)-PE et acide arachidonique (ARA, C20:4)-PE. Dans cette thèse de doctorat, nous avons établi une association positive forte entre les teneurs totales en PE et en PE (36:5) associées aux HDL et l'athérosclérose chez les femmes atteintes de syndrome métabolique. En outre, notre étude a ajouté de nouvelles informations à cette association *in vivo*, en utilisant ARA-PE, la forme prédominante de PE (36:5) dans les maladies cardiométaboliques (CMD) et l'ASCVD. En injectant des HDL reconstituées (rHDL) dans un modèle animal d'athérosclérose, nous avons montré une diminution de la capacité de l'ARA-PE rHDL à réduire l'athérosclérose par rapport au rHDL contrôles, indiquant une altération des capacités athéropotectrices de ces rHDL. Nos expériences *in vitro* ont révélé que le PE, et de manière plus prononcée l'ARA-PE, atténuait les fonctions des rHDL et les convertissait en particules pro-inflammatoires. À l'inverse, nos résultats *in vitro* ont mis en évidence l'impact bénéfique significatif de l'EPA-PE sur les fonctions des rHDL. L'EPA-PE a non seulement amélioré la CEC des rHDL, mais a également préservé et amélioré leur AOX et leur AIA, respectivement. En outre, nos résultats révèlent que l'EPA-PE rHDL atténue l'inflammation en réduisant la production d'eicosanoïdes pro-inflammatoires, suggérant un mécanisme nouveau pour les effets cardioprotecteurs de l'EPA.

En conclusion, ce travail de recherche fait progresser notre compréhension du rôle des espèces de PE dans l'athérosclérose en les identifiant comme de nouveaux biomarqueurs pour les fonctions des HDL. Ces résultats pourraient avoir un impact significatif sur la gestion de l'ASCVD, améliorant l'évaluation des risques et le traitement.

English abstract

HDL, or High-Density Lipoproteins, are often called "good cholesterol" by playing a vital role in reducing the risk of atherosclerotic cardiovascular diseases (ASCVD). HDL exert several atheroprotective effects, including their capacity to promote cholesterol efflux (CEC) from arterial macrophages, anti-inflammatory activities (AIA), and anti-oxidative activities (AOX). Among HDL components, phospholipids are considered the major determinants of HDL functions. Here, we aimed to identify new phospholipid species implicated in determining the atheroprotective functions of HDL. Higher plasma levels of phosphatidylethanolamine (PE) species are strongly linked to an increased risk of ASCVD, however, the underlying mechanisms remain unclear. Among PE species, PE (36:5) is recognized as a predictive lipid signature for ASCVD events. In human plasma, PE (36:5) exist as eicosapentaenoic (EPA, C20:5)-PE and arachidonic acid (ARA, C20:4)-PE. In this doctoral dissertation, we established a strong positive association between total PE and PE (36:5) contents in HDL and atherosclerosis in women with metabolic syndrome. Furthermore, our study added new information to this association *in vivo*, using ARA-PE, the predominant form of PE (36:5) in cardiometabolic diseases (CMD) and ASCVD. By infusing reconstituted HDL (rHDL) in an animal model with atherosclerosis, we revealed the inability of ARA-PE rHDL to reduce atherosclerosis compared to control rHDL, indicating an impaired rHDL-mediated atheroprotection. Our *in vitro* experiments revealed that PE, and more profoundly ARA-PE, attenuated the functions of rHDL and converted them into pro-inflammatory particles. Conversely, our *in vitro* findings highlighted the significant beneficial impact of EPA-PE on rHDL functions. EPA-PE not only enhanced the CEC of rHDL, but also preserved and improved its AOX and AIA, respectively. Furthermore, our findings revealed that EPA-PE rHDL attenuated inflammation by reducing pro-inflammatory eicosanoid production, suggesting a novel mechanism for the cardioprotective effects of EPA.

In conclusion, this research work advances our understanding regarding the role of PE species in atherosclerosis by identifying them as novel biomarkers for HDL functions. These findings could significantly impact risk assessment and therapeutic management in the context of ASCVD.

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Abbreviations

I

1-palmitoyl-2-arachidonyl phosphatidylcholine (PAPC)	70
1-palmitoyl-2-linoleoyl PC (PLPC)	58
1-palmitoyl-2-oleoyl phosphatidylcholine (POPC)	70

A

cyl-dihydroxyacetone (DHAP)	79
amino acids (AA)	43
angiopoetin-like 3 protein (ANGPTL3)	18
antisense oligonucleotides (ASO)	18
apolipoprotein (Apo)	9
apolipoprotein-CIII (apo-CIII)	18
arachidonic acid (ARA)	3
atherosclerotic cardiovascular disease (ASCVD)	1
ATP binding cassette G1 (ABCG1)	38
ATP-binding cassette transporter A1 (ABCA1)	36

B

b-cell lymphoma-extra-large (Bcl-xL)	59
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C

cardiometabolic diseases (CMD)	61
cardiovascular diseases (CVD)	4
carotid intima-media thickness (cIMT)	12
chinese hamster ovary (CHO)	78

cholesterol efflux capacity (CEC)	52
cholesteryl ester (CE)	24
cholesteryl ester transfer protein (CETP)	19
chylomicrons (CM)	9
CM remnants (CM-R)	25
common carotid artery (CCA)	12
computed tomographic (CT)	12
coronary artery calcium (CAC)	12
coronary heart disease (CHD)	2, 4
c-reactive protein (CRP)	12
cyclooxygenases (COXs)	87
cytidine diphosphate (CDP)	78

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docosapentaenoic acid (DPA)	87

E

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electrocardiogram (ECG)	12
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F

framingham heart study (FHS)	10
framingham risk score (FRS)	9
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G

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Oral communications

- **Enrichment of high-density lipoproteins (HDL) with phosphatidylethanolamine (36:5) impairs their protective biological activities and is associated with atherosclerosis in women.**

Malik Taradeh, Veronica Dahik, Sophie Galier, Lise Hardy, Eric Frisdal, Hervé Durand, Anatol Kontush, Maryse Guérin, Isabelle Guillas, Wilfried Le Goff.

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Preface

In this preface, I welcome you to my doctoral dissertation, which aims to contribute to the growing body of knowledge in the field of cardiovascular research and lipidomics, focusing on the critical interplay between phosphatidylethanolamine (PE) lipid species, and high-density lipoprotein (HDL) in the context of atherosclerotic cardiovascular diseases (ASCVD).

ASCVD are the major cause of mortality worldwide. The hallmark of ASCVD is the formation of atherosclerotic plaques within the arterial wall, with a progressive narrowing of the arterial lumen over time. The process of atherosclerosis, which is characterized by chronic inflammation, starts with the deposition and oxidation of atherogenic lipids in the arterial wall. This, in turn, triggers a cascade of inflammatory pathways, fostering the accumulation of lipids, as well as cellular components, and proteins, thereby leading to the formation of atherosclerotic plaques. Chronic exposure to several atherogenic risk factors perpetuates plaque growth, ultimately obstructing the arterial lumen and precipitating ASCVD complications such as myocardial infarction (MI) and ischemic stroke.

Plasma lipids, particularly cholesterol, constitute pivotal factors in the pathogenesis of ASCVD and are transported in the blood by lipoproteins. Among these, low-density lipoprotein (LDL) is the major carrier of plasma cholesterol and it is a pro-atherogenic lipoprotein that is often labeled as "bad cholesterol". Several studies have shown that elevated levels of LDL-cholesterol (LDL-C) are strongly associated with increased risk of CVD and related mortality (1). Conversely, the HDL plays an antiatherogenic roles, earning its well-known description as a "good cholesterol." It was in 1976 that the first ground-breaking inverse association was found between plasma levels of HDL-Cholesterol (HDL-C) and the presence of ASCVD (2). Several epidemiological studies further solidified this association, highlighting that diminished HDL-C levels are associated with an increased incidence of ASCVD (3–5). This association is believed to be deeply rooted in the key biological function of HDL, namely facilitating the cholesterol efflux from the cholesterol-overloaded macrophages within the arterial wall. This key function of HDL was found to be adversely linked to the progression of atherosclerosis development (6), MI (7), and ASCVD events (8).

In addition to their pivotal role in promoting cellular cholesterol efflux, HDL particles exhibit several atheroprotective functions. These include anti-inflammatory, anti-oxidative,

antiapoptotic, vasodilatory, and anti-thrombotic effects. Nonetheless, despite these promising protective effects of HDL against ASCVD, therapeutic efforts aimed at raising HDL-C levels have failed to yield a reduction in the risk of major cardiovascular events. Intriguingly, a "U-shaped" relationship has recently emerged between HDL-C levels and ASCVD, with both low and extremely high plasma levels of HDL-C being linked to an elevated risk of cardiovascular mortality (9). Furthermore, several studies have demonstrated the notion that HDL-C levels "quantity" do not always predict accurately the full spectrum of HDL's atheroprotective functions. Conversely, an emerging body of evidence is pointing toward the pivotal role of HDL composition and structure "quality" as the primary determinants of the atheroprotective functions of HDL.

Plasma HDL is a group of particles that exhibit remarkable heterogeneity in their protein and lipid composition, a diversity that significantly impacts their structural and functional aspects. Previous proteomic and lipidomic studies have reported hundreds of individual proteins and lipids in HDL particles. Notably, within the HDL lipidome, phospholipids emerge as prominent constituents, accounting for 35-50% of the total HDL lipidome. Among the major phospholipid classes found in HDL are phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol. In this regard, several structural-functional analytical studies have demonstrated that HDL phospholipids play a significant role in determining HDL functionality, based on their quantity, chemical, and physical properties (10,11).

Previous studies have shed light on the dynamic nature of both HDL's structure and composition, revealing their susceptibility to alterations during pathological conditions. Such alterations often attenuate or lead to a complete loss of the atheroprotective nature of HDL. Indeed, several pathological conditions marked by chronic oxidative stress and inflammation such as ASCVD, obesity, dyslipidemia, diabetes mellitus type 2 (T2D), and metabolic syndrome are often associated with the transformation of physiologically functional HDL into a dysfunctional state. This altered form of HDL exhibits marked structural and compositional changes that have been linked with the conversion of HDL into proatherogenic lipoprotein (12).

In this regard, several studies have highlighted the altered abundance of specific phospholipid and sphingolipid molecular species as a key contributor to HDL dysfunctionality (13,14). Advances in lipidomic research have enabled the identification and quantification of several phospholipid molecular species to be implicated in the pathogenesis of ASCVD, with special attention directed toward the phosphatidylethanolamine (PE) species. Noteworthy, in a

previous lipidomic analysis comparing plasma from patients with stable coronary heart disease (CHD) to that of healthy individuals, 16 PE species out of 20 exhibited a positive association with stable CHD (15). Subsequently, the population-based Bruneck study validated these results, highlighting the significant positive association of 9 PE species out of 18 with the incidents of CVD. Most notably, PE (36:5) molecular species has emerged as a potent predictor for ASCVD by improving risk prediction and classification when was added to traditional risk factors (16). However, the precise molecular mechanisms by which plasma PE species, and especially PE (36:5), promote the development of ASCVD remain largely unknown.

Based on the total number of carbon atoms and double bonds in PE (36:5), six molecular species could exist in human plasma. Among these, two major molecular subspecies of PE (36:5) including PE (C16:1/20:4), and PE (C16:0/20:5) have been consistently detected in human plasma (17). In this regard, arachidonic acid (ARA, C20:4) claims attention, as it is classified as an omega-6 polyunsaturated fatty acid (PUFA) that is largely known to play a deleterious role in ASCVD. Conversely, eicosapentaenoic acid (EPA, C20:5) is an omega-3 PUFA with pleiotropic beneficial effects against ASCVD. The significance of ARA and EPA in ASCVD positions both PE (C16:1/20:4) and PE (C16:0/20:5) subspecies of PE (36:5) at the forefront, raising questions about the roles of their PUFA constituents, namely ARA and EPA, respectively, in determining the established strong association between PE (36:5) and ASCVD.

In blood circulation, about 60% of plasma PE species are carried by HDL, thus constituting a major phospholipid class within the HDL phospholipidome. Previous studies have illuminated the capacity of the total PE content of HDL particles to influence their structural characteristics (18), suggesting that PE play a role in shaping the physical properties of HDL. In addition, total PE content of HDL has been inversely correlated with relative fractional cholesterol efflux to human serum (19), suggesting a potential negative impact of PE on HDL functionality.

My research work is focused on studying the impact of the presence of PE, especially PE (36:5) species, in HDL particles on their atheroprotective functions and atherosclerosis development. Moreover, my work extends to explore the roles undertaken by PUFA, including omega-6 ARA and omega-3 EPA, in determining the impact of PE on HDL functionality. This comprehensive investigation aims to identify the molecular mechanisms by which plasma PE species, specifically PE (36:5), might promote the development of ASCVD.

INTRODUCTION

- 1.1 ATHEROSCLEROTIC CARDIOVASCULAR DISEASES
- 1.2 LIPOPROTEINS STRUCTURE, COMPOSITION AND METABOLISM
- 1.3 HDL ATHEROPROTECTIVE FUNCTIONS
- 1.4 ALTERED HDL FUNCTIONALITY AND DYSFUNCTION
- 1.5 THERAPEUTIC TARGETING OF DEFECTIVE HDL FUNCTIONALITY
- 1.6 MOLECULAR LIPID BIOMARKERS IN ASCVD
- 1.7 PHOSPHATIDYLETHANOLAMINES

1

"In science, there are no shortcuts to truth." - Louis Pasteur

"If I have seen further, it is by standing on the shoulders of giants." - Isaac Newton

Chapter 1. Introduction to literature

1.1 Atherosclerotic cardiovascular diseases

Atherosclerotic cardiovascular diseases, abbreviated as ASCVD, include a broad spectrum of conditions characterized by the narrowing or obstruction of arterial blood vessels as a result of plaque buildup within their walls. This buildup progressively diminishes or completely blocks the blood supply (and thus oxygen) to the heart or other organs, leading to ischemia and potentially tissue infarction in the affected region.

1.1.1. Epidemiological context

Cardiovascular diseases (CVD) remain the leading cause of mortality and morbidity worldwide (20). According to the World Health Organization (WHO), 17.9 million people died from CVD in 2019, accounting for 32% of all global deaths. Of these deaths, 8.9 million were due to coronary heart disease (CHD), and 6.2 million were due to stroke (WHO, 2021). In Europe, CVD contribute to 45% of all deaths, and they cost the European Union about €210 Billion annually (21,22). The number of CVD-related deaths is expected to reach about 32,3 million in 2050 (WHO, 2018), which is largely linked to the global pandemic of metabolic diseases, such as T2D, obesity, and metabolic syndrome (23). Despite advances in ASCVD prevention and early detection using traditional risk factors, the high incidence rates indicate a huge challenge and burden on the current risk factors scores. Therefore, efforts are required to understand the underlying pathological mechanisms that drive the onset and progression of ASCVD, which could lead to better prevention and treatment options.

1.1.2. Clinical context

The term ASCVD includes (i) CHD, the most prevalent form, and it affects the coronary arteries that nourish the heart; (ii) cerebrovascular disease or stroke, which affects the arteries in the brain; (iii) peripheral vascular disease; and (iv) aortic atherosclerotic disease, which both affect arteries rather than the one that supply the heart or the brain; (24,25). The common clinical symptoms of CHD are chest pain, shortness of breath, and general fatigue. Symptoms can be stable (i.e. angina pectoris) which increases with physical activity, or unstable, manifesting as sudden attacks at rest and ranging from unstable angina to MI, or sudden cardiac

death (26). The main pathological process underlies the development of ASCVD known as atherosclerosis (27). The development of atherosclerotic plaque can be seen as a continuum with a normal artery at one end and a restricted or occluded artery at the other. Persistent exposure to one or more atherogenic stimuli has been proposed to be the key factor that ensures the initiation and progression of the atherosclerosis process over time. ASCVD are considered as multifactorial diseases with a well-known cluster of risk factors that have been largely linked to CVD (28,29). Traditional ASCVD risk factors include advanced age, male gender, hypertension, T2D, obesity, dyslipidemias, metabolic syndrome, smoking, family history of premature CHD, and elevated markers of inflammation. Other risk factors include physical inactivity, unhealthy diet, stress, and low socio-economic status (30,31).

1.1.3. Atherosclerosis-pathogenesis

Atherosclerosis is a progressive chronic inflammatory process that affects the arterial blood vessels and is considered the primary cause of ASCVD. Atherosclerosis is defined as the formation of atherosclerotic plaque within the inner layer of the arterial wall due to the deposition of lipids, cells, calcium, and extracellular matrix components, accompanied by asymmetrical wall thickening and lumen narrowing (32). Over time, the growing plaque causes partial or total arterial occlusion, which manifests clinically as angina, MI, stroke, or sudden cardiac death (33). As shown in **Figure 1.1**, the evolution of atherosclerosis is envisaged in three stages including initiation, progression, and complications (34).

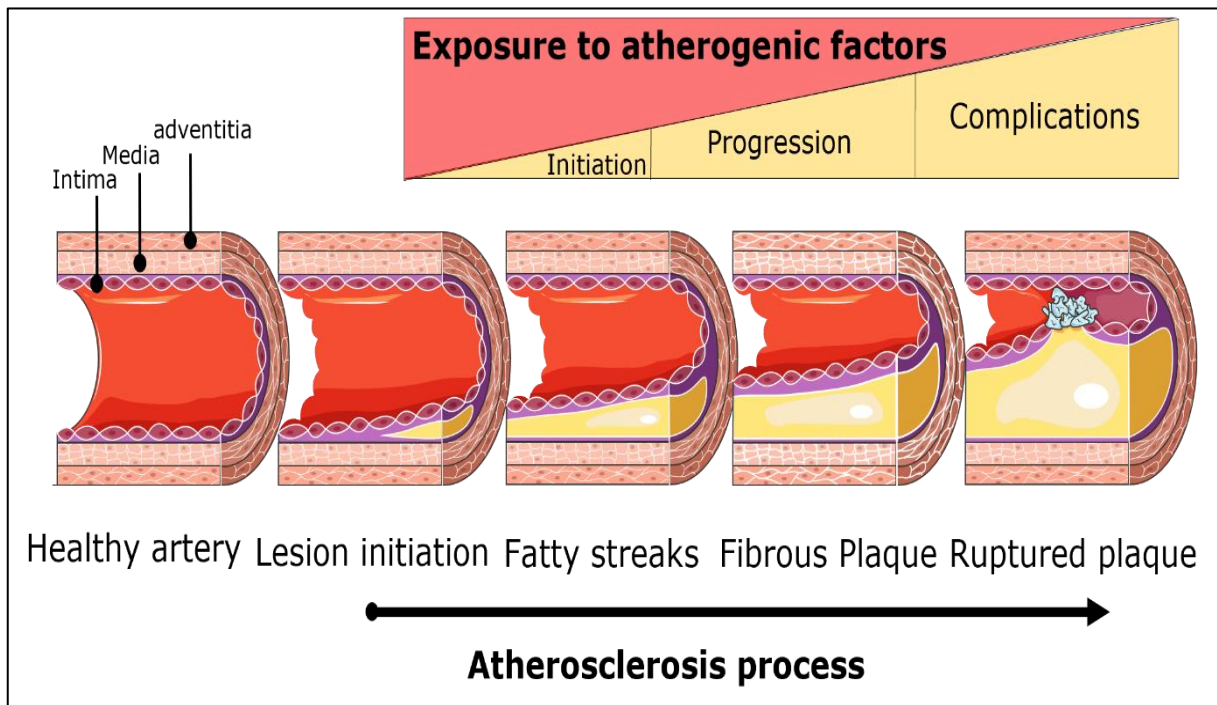


Figure 1.1: Evolution of atherosclerosis. The artery wall comprises three layers including intima, the innermost layer; media, the intermediate layer; and adventitia, the outermost layer. Atherosclerosis initiates within the arterial intima as a result of vascular endothelial injury induced by several atherogenic factors. The plaque starts as fatty streaks and grows overtime causing progressive arterial lumen narrowing. The growing plaque eventually ruptures and causes blood clot formation, which reduces or obstructs the blood vessel resulting in a vascular accident. [Adapted from Jebari - Benslaiman et.al 2022 (34)]

As illustrated in **Figure 1.2**, the process of atherosclerosis is thought to begin in the arterial wall when plasma LDL particles extravasate through leaky and dysfunctional endothelial cells (characterized mainly by impaired nitric oxide production) into arterial intima, where they undergo oxidation and become cytotoxic, proinflammatory, chemotactic and pro-atherogenic (35,36). Oxidized LDL (ox-LDL) initiates a cascade of events by activating endothelial cells. Consequently, the activated endothelial cells enhance the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin, and P-selectin. This response aims to facilitate the recruitment of blood-borne cells, predominantly monocytes, into the arterial intima. The ultimate goal of this process is to facilitate the uptake of ox-LDL through endocytosis by these recruited cells (37,38). Activated endothelial cells also up-regulate the expression of monocyte

chemotactic protein-1 (MCP-1) chemokine to promote the transendothelial migration of monocytes into the arterial intima after their adhesion to the endothelium (35). Within the arterial intima, the monocytes differentiate into macrophages and internalize the pro-atherogenic ox-LDL particles through specific receptors referred to as scavenger receptors (SR) (e.g. SR-A and CD36). The phagocytosis of ox-LDL results in the formation of lipid-loaded macrophages, filled with massive amounts of cholesteryl esters (CE), referred to as foam cells, and the hallmark of both early and late atherosclerotic lesions.

The development of foam cells initiates the formation of small yellowish atherosclerotic lesions called fatty streaks (39). In addition, foam cells induce inflammation by releasing pro-inflammatory cytokines, and reactive oxygen species (ROS), to recruit more monocytes, resulting in a sustained inflammatory response within the arterial intima (40). In addition, oxidized phospholipids from ox-LDL can be transferred into cellular membranes, serving as seeding molecules for promoting further oxidative stress (41). Continuous retention of atherogenic ox-LDL and its subsequent phagocytosis by macrophages results eventually in macrophage death, mainly by apoptosis, as a result of cellular cholesterol accumulation. This contributes to the formation of a white stable plaque with a lipid-rich-cell free necrotic core covered by a fibrous cap made of smooth muscle cells (SMC) to stabilize the plaque (33,35). Among the special features of atherosclerotic plaques is focal calcification, which is an active process of calcium deposition within the arterial intima. Plaque calcification is to some extent an active process that is mainly mediated by continuous lipid oxidation and related inflammation (35,42). The persistent exposure to atherogenic factors increases the size of the necrotic core, while softening the fibrous cap, making the plaque more vulnerable and prone to rupture. Eventually, the plaque either ruptures and initiates blood thrombus formation, causing an artery occlusion or thromboembolism, or expands sufficiently to cause arterial stenosis, thereby diminishing the arterial blood flow, and resulting in a wide range of clinical complications depending on the site and the level of arterial obstruction (43).

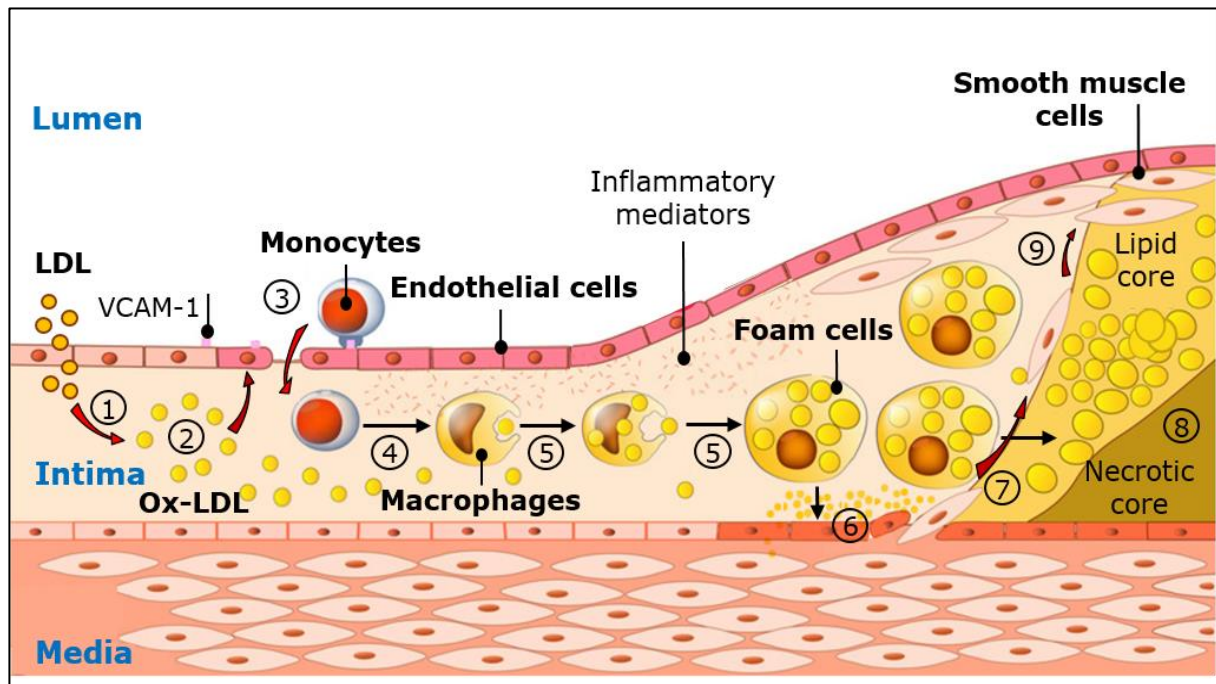


Figure 1.2: Pathogenesis of atherosclerosis. Atherosclerosis starts by: **1.** Infiltration and oxidation of low-density lipoprotein (LDL) in the arterial intima. **2.** Oxidized LDL (Ox-LDL) activates the endothelial cells, which respond by increasing the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1). **3.** VCAM-1 and other adhesion molecules facilitate the recruitment and migration of monocytes. **4.** Monocytes differentiate into macrophages. **5 and 6.** Macrophages take up the Ox-LDL and become foam cells, which also secrete inflammatory mediators to maintain an immune-inflammatory response. **7 and 8.** The foam cells die and their lipid content and cellular debris form the necrotic and lipid cores of the atherosclerotic plaque. **9.** Foam cells also promote the proliferation and recruitment of smooth muscle cells to stabilize the plaque. The growing plaque progressively expands the arterial wall, causing the arterial lumen to narrow overtime. [Adapted from Minelli et.al. 2020 (36)].

1.1.4. Dyslipidemias

Currently, traditional ASCVD risk factors are largely used to predict the future risk of developing CHD. The identification of these risk factors led to the development of multivariable risk assessment tools such as Framingham Risk Score (FRS), which is widely used to assess the risk of developing ASCVD and to identify candidates for risk factor modifications (44). The FRS considers six risk factors, including age, gender, total cholesterol (TC), high density lipoprotein (HDL)-cholesterol (HDL-C), systolic blood pressure (SBP), and smoking habits to calculate the risk of developing a CHD within 10 years (45). In this regard, FRS is proportional to age, male gender, higher TC levels, higher SBP, lower HDL-cholesterol and smoking. A 10-year risk for CHD is considered low if the FRS is less than 10%, moderate if it is between 10% to 19%, and high if it is 20% or higher (46). Given the importance of plasma lipids (i.e. TC and HDL-C levels) in defining the risk of developing a CHD, the focus will be directed toward them.

Dyslipidemias are among the most common findings in patients with ASCVD and they are key components of the metabolic syndrome (MetS), which include a group of metabolic conditions including abdominal obesity, dyslipidemia, hypertension, insulin resistance, and hyperglycaemia (45,47). Dyslipidemias include hypercholesterolemia (i.e. elevated LDL-C) and/or hypertriglyceridemia, and/or low plasma levels of HDL-C, with hypercholesterolemia being the most prevalent. Several studies have identified dyslipidemias as major risk factors for ASCVD. According to WHO, more than one-third of CHD and stroke-related deaths are related to hypercholesterolemia (48). Thus, dyslipidemia-related research is growing with the purpose of providing new insights into therapeutic interventions that could prevent or treat ASCVD (49).

Plasma lipids such as cholesterol and triglycerides (TG) are carried by apolipoprotein-containing particles called lipoproteins, and are closely related to ASCVD. Plasma lipoproteins include mainly chylomicrons (CM), very low-density lipoprotein (VLDL), LDL, Lipoprotein (a), and HDL particles. CM and VLDL are TG-rich particles and they mainly deliver TG to liver and peripheral tissues, respectively. LDL and Lipoprotein (a) are cholesterol-rich particles, and they mainly deliver cholesterol to peripheral tissues. HDL are protein and phospholipid-rich particles and they mainly deliver cholesterol from peripheral tissues to the liver for excretion (50). CM, VLDL, LDL, and Lipoprotein (a) are all apolipoprotein (Apo) B - containing lipoproteins and they contribute to ASCVD development, whilst HDL is the only

anti-atherogenic lipoprotein that protects against ASCVD and it is an ApoA-I containing lipoproteins (49).

1.1.4.1. Total cholesterol and LDL-Cholesterol

The majority of the plasma cholesterol is carried by LDL particles. Multiple large-scale epidemiological studies have established a strong association between both elevated plasma levels of TC as well as LDL-C and the risk of ASCVD (51). According to the Framingham Heart Study (FHS), TC levels ≥ 200 mg/dL or LDL-C levels >130 mg/dL were linked to an increased risk of CHD (52). In line with this data, the atherosclerosis Risk in Communities (ARIC) study indicated that every 38.8 mg/dL increase in LDL-C levels above the median values of 88 mg/dl (in men) and 95 mg/dl (in women), was associated with about 40% increase in the relative risk of CHD (53). The opposite is also true, LDL-C lowering has been associated with a dose dependent reduction in CVD risk and mortality as revealed by several clinical trials, in which reducing LDL-C by 38.6 mg/dL was associated with a 20-25% reduction in CVD mortality (54). Therefore, LDL-C is largely known as the "bad cholesterol".

1.1.4.2. Lipoprotein (a)

Lipoprotein (a) is a cholesterol-rich particle, with similar structural but distinct metabolic, and pathogenic properties compared to LDL. Several studies have found that lipoprotein (a) plays a causal role in the development of ASCVD. Recent large population based studies such as Copenhagen City Heart Study have reported that increasing plasma levels of lipoprotein (a) are associated with marked, independent, stepwise increase in CHD risk, with plasma concentrations ≥ 42 mg/dL being considered high (55). Mechanistic studies have revealed the involvement of lipoprotein (a) in the main steps of atherosclerosis process including foam cell formation, inflammation, SMC proliferation, and plaque instability. Therefore, measurement of lipoprotein (a) are recommended in individuals at high risk for ASCVD (49,55).

1.1.4.3. Triglycerides

The majority of plasma TG is carried by CM and VLDL particles, both known as TG rich lipoproteins (TGRL). The TG content of CM and VLDL is subjected to hydrolysis by lipoprotein lipase (LPL), which reduce their TG content by releasing free fatty acids and glycerol, converting them into smaller remnant particles (56–58). Several studies have

demonstrated that extremely elevated TG levels (>1000 mg/dL) typically do not develop ASCVD, whilst mild-moderately elevated TG levels potentially lead to ASCVD (59–63). This was explained by the inability of large CM and VLDL to penetrate the arterial wall at extremely high TG levels, while mild to moderately elevated TG levels feature the remnants of CM and VLDL (59–61,64,65), which are small enough to infiltrate the arterial wall and provoke the formation of atherosclerotic lesions (56–58). Multiple epidemiological studies including Copenhagen City Heart Study, Women’s Health Study, and Emerging Risk Factors Collaboration study have revealed that elevated plasma levels of non-fasting TG (from 89 to 443 mg/dL) were consistently associated with increased risks of MI, CHD, stroke and all-cause mortality (66–69).

1.1.4.4. HDL-Cholesterol

In the early 1950s, it was demonstrated that patients with CHD frequently exhibit reduced plasma levels of HDL-C as compared to healthy individuals (70,71). Thereafter, low HDL-C levels were linked to insufficient clearance of excess plasma cholesterol, and were proposed to accelerate the development of CHD (72,73). In 1977, it was originally demonstrated that plasma HDL-C levels were inversely related to CVD risk (74). Data from epidemiological studies including FHS and ARIC study have revealed that subjects with subnormal HDL-C levels displayed the highest frequency of cardiovascular event (53,75). Moreover, low HDL-C levels were linked with an accelerated progression of angiographically defined CHD, and plasma HDL-C levels <40 mg/dL in men and <45 mg/dL women have become widely accepted as markers of elevated ASCVD risk, independent of LDL-C levels, as illustrated in **Figure 1.3** (76–78). In line with this data, multiple perspective studies have revealed that 1 mg/dL increase in HDL-C levels was associated with a 2% - 3% reduction of CVD risk, and 3.7% - 4.7% reduction of CVD mortality (79–81). Therefore, HDL-C is largely known as the “good cholesterol”.

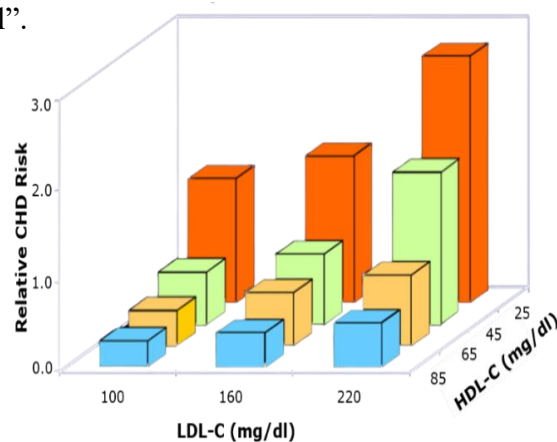


Figure 1.3: Framingham Heart Study (FHS)-10-Year Risk for CHD event. Low HDL-C levels predict CHD independent of LDL levels. [Adapted from Castelli W. 1988 (78)].

1.1.5. Diagnostic and management

Diagnosis of ASCVD is defined as the detection of any vascular accident caused by plaque build-up within the coronary, cerebral, or peripheral arteries. Along with clinical presentation, several tests are currently used to diagnose ASCVD, including blood tests measuring plasma cholesterol, plasma TG, inflammatory markers such as C-reactive protein (CRP), biomarkers of cardiac tissue injury such as troponins, as well as electrocardiogram (ECG), and imaging tests such as cardiac catheterization, and echocardiogram (Mayoclonic, 2022). Since subclinical atherosclerosis is considered a precursor for a clinically evident ASCVD, several guidelines recommend the measurement of coronary artery calcification and carotid wall thickness as non-invasive tests to evaluate early atherosclerosis development (82).

Coronary artery calcium (CAC) score has emerged as a highly accurate surrogate marker of CHD. CAC score indicates the presence of calcium deposits in the coronary arteries. Computed tomographic (CT) scanning is currently used for the detection, localization, and quantification of CAC score. A positive (non-zero) CAC score demonstrates the presence of atherosclerotic plaque within coronary arteries, and higher values indicate an increased CHD risk (83). According to several population-based observational studies, CAC scoring is considered among the most consistent, and reproducible tools to predict the risk for major cardiovascular events, especially in asymptomatic individuals, and can be utilized for periodic assessment of ASCVD (84,85). Carotid intima-media thickness (cIMT) and the presence of atherosclerotic plaque in carotid arteries are also well-known surrogate markers of CHD and stroke. cIMT testing is an ultrasound imaging technique used to measure the area between the intimal and medial layers of the carotid artery (86). The common carotid artery (CCA), which is the first segment of the carotid artery, is the most common and accurate site to measure the cIMT with values of ≥ 0.9 mm considered high (82,87). The cIMT assessment includes either measuring cIMT alone in a plaque-free region or the cIMT with plaque assessment included, the latter being a more powerful predictor of cardiovascular risk. Current guidelines recommend the evaluation of the CAC score, cIMT, and the presence of carotid plaque in asymptomatic adults at intermediate risk of ASCVD, along with the assessment of the traditional risk factors (30,86).

As mentioned before, numerous studies have established dyslipidemia as a major contributor to ASCVD. As a matter of fact, the management of plasma lipid disturbances has

become a central objective in the primary and secondary prevention of ASCVD events. The cornerstone in the management of ASCVD is adopting a heart-healthy lifestyle, which should be recommended for all patient. Besides that, a personalized therapeutic approach is tailored according to the patient's individual risk profile, clinical characteristics, and convenience to pharmacotherapy in terms of efficacy, safety, and cost effectiveness (88).

1.1.5.1. Non-pharmacological management

Healthy lifestyle includes maintaining normal weight, regular physical activity, and healthy diet, which all help to improve plasma lipid disturbances and can significantly reduce the increased risk for CHD (89,90). Guidelines from American College of Cardiology, and American Heart Association recommend healthy diet patterns such as Mediterranean diet, and the Dietary Approaches to Stop Hypertension (DASH) diet, which include reducing the intake of sodium, refined sugar, and saturated fats, and replacing them with complex sugar, vegetables, fruits, whole grains, legumes, and polyunsaturated fats (91,92). Evidence from randomized clinical trials demonstrated that replacing saturated fats such as butter, lard, cheese, and red meat, with polyunsaturated fats such as nuts, olive oil, flaxseed oil, soybean oil and fish significantly reduced total ASCVD events by 31%, which is nearly equivalent to the reduction caused by statins, the first-line lipid-targeting medications (91).

1.1.5.2. Pharmacological management

ASCVD correlate with elevated levels of LDL, lipoprotein (a), TGRL, and decreased HDL levels. In the last decade, several lipid therapies have emerged for preventing or reducing the progression of ASCVD, mainly by lowering atherogenic lipoprotein particles such as LDL, lipoprotein (a), and TG-rich VLDL and their remnants.

LDL lowering agents

Niacin, bile acid sequestrants, and fibrates are among the first established LDL-C lowering agents before statins, however, they are not routinely prescribed (93). Although niacin was shown to reduce hepatic TG and VLDL synthesis, and eventually LDL-C levels (94), clinical trials showed that niacin was not effective in reducing ASCVD events (95,96). Bile acid sequestrants work by inhibiting the reabsorption of bile acids (i.e. cholesterol derivatives) in small intestines. Although bile acid sequestrants showed a small reduction in ASCVD risk in clinical trials by reducing LDL-C levels, their use is limited due to their side effects,

particularly constipation (97–99). Fibrates are peroxisome proliferator-activated receptors-alpha (PPAR- α) agonists and they work by increasing cellular uptake and catabolism of fatty acids, thereby reducing the available fatty acids for TG synthesis, and thus VLDL synthesis. While fibrates have been demonstrated to mildly reduce LDL-C and increase HDL-C levels, they have failed to demonstrate ASCVD benefits in population studies (96,100,101). Statins were the first lipid-lowering agents to consistently reduce the risk of ASCVD (102) by inhibiting the rate-limiting step of cholesterol synthesis in the liver (103). Clinical trials have shown that statins are effective for both primary and secondary prevention of ASCVD events, with a 22% risk reduction for each 38.7mg/dl decrease in LDL-C levels (104–107). However, using statins for primary prevention remains controversial due to their safety profile (108). Other therapeutic approaches to lowering LDL-C levels include ezetimibe, and proprotein convertase subtilisin-kexin type 9 inhibitors (PCSK9i). Ezetimibe inhibits the intestinal absorption of cholesterol and its addition to statins was shown to provide an additional 18% to 22% reduction in LDL-C levels and to reduce the risk of major events in adults with established CHD (109,110). PCSK9i inhibit the degradation of LDL receptor (LDLR) in the liver and thus enhance the clearance of LDL-C from circulation. Several clinical trials demonstrated an effective lowering of LDL-C with PCSK9i up to 47% alone, and up to 59% when combined with statins (111–113). Both ezetimibe and PCSK9i are well tolerated and both are recommended for patients intolerant to statins or with severely elevated LDL-C levels. Due to cost, PCSK9i are considered third line add-on agents after statins and ezetimibe (114,115). Next to statins, ezetimibe and PCSK9i antibodies, several novel LDL-C lowering therapies are currently emerging. **Table 1.1** summarizes the available LDL lowering agents, including their pharmacological, clinical, and side effects while **Figure 1.4** illustrates their working mechanisms (88,96,116).

A mounting evidence suggests that a significant residual ASCVD risk persists after controlling the LDL-C levels, especially in high risk patients with elevated TG, and low HDL-C levels (117–119). On the other hand, the safety issues of the available lipid therapies such as statins intolerance remain a barrier for an optimal management of ASCVD (49). Thus, additional lipid related therapies with good safety profile are still required.

Table 1.1: LDL lowering agents. ApoB100: apolipoprotein B100; ANGPTL3: Angiopoetin-like 3 protein, BAS: bile acid sequestrants; CM: chylomicrons; DM: Diabetes mellitus; GI: gastrointestinal; LDL-C: low-density lipoprotein-cholesterol, VLDL: very-low-density lipoprotein, LDLr: LDL receptor; LPL: lipoprotein lipase; PCSK9: proprotein convertase subtilisin-kexin type 9; TG: triglyceride. [Adapted from (88,96)].

Agents	Pharmacological effects	Clinical effects	Side effects
Niacin	Reduce hepatic TG and VLDL synthesis	Reduce LDL-C levels	Skin rash, GI symptoms
BAS Cholestyramine, Colesevalam, Colestipol	Reduce hepatic bile acid pool, enhance LDLr expression, and LDL clearance	Reduce LDL-C levels	Constipation
Fibrates Bezafibrate, Gemfibrozil, Fenofibrate	Increase cellular uptake and catabolism of fatty acids, and thus reduce hepatic TG and VLDL synthesis	Mildly reduce LDL-C levels Reduce TG levels Increase HDL-C levels	Muscle pain, GI symptoms, elevated liver enzymes, elevated creatinine levels
Statins Atorvastatin Fluvastatin, Lovastatin, Rosuvastatin, Simvastatin	Inhibit cholesterol synthesis, enhance hepatic LDLr expression, and thus LDL clearance	Reduce LDL-C around 20% to 50% Reduce TG levels around 9% to 31%	Myopathy, DM, Memory disturbances, confusion, sexual dysfunction
Ezetimibe	Reduces the intestinal absorption of cholesterol	Reduces LDL-C levels by 18% to 22% when combined to statins	Injection site reactions
PCSK9 inhibitors Alirocumab, Evolocumab	Inhibit degradation of hepatic LDLr, thus increase the number of LDLr and enhance the clearance of LDL-C from circulation	Reduce LDL-C levels up to 47% alone Reduce LDL-C levels up to 59% when combined with statins Reduce lipoprotein (a) levels by 25% to 30%	
Mipomersen	Inhibits production of apoB100 protein, and thus reduces VLDL production	Reduces LDL-C levels by mean of 26%	liver toxicity, injection site reactions, and flu-like symptoms
Lomitapide	Inhibits CM, and VLDL production	Reduces LDL-C levels up to 50%	liver toxicity, and GI symptoms

Bempedoic acid	Inhibits the hepatic synthesis of cholesterol by reducing the production of acetyl-CoA	Reduces LDL-C levels up to 21%	Increase uric acid levels, and risk for gout
Inclisiran	Inhibits the degradation of hepatic LDLr	Reduces LDL-C levels up to 50%	Injection site reactions
ANGPTL3 inhibitor Evinacumab	Enhance the hydrolysis of TG by LPL and thus the conversion of VLDL to LDL increases the hepatic clearance of LDL	Reduces LDL-C levels up to 49%	Elevated liver enzymes

Lipoprotein (a) lowering agents

Despite the direct association between lipoprotein (a) levels and the risk of ASCVD, there is still no clinically effective therapy that reduces CVD risk by specifically reducing lipoprotein (a) levels up to date. Among the LDL lowering agents, the PCSK9i reduce the lipoprotein (a) levels by 25% - 30%, with evidence to improve the prognosis in CHD patients as a result of this effect (120,121). In addition, Mipomersen, an antisense oligonucleotide (ASO) designed to bind apoB100 mRNA, was reported to reduce lipoprotein (a) levels by 75% in mice (122). Another promising agent is IONIS-APO (a)-L_{Rx}, an ASO designed to bind apo (a) mRNA, forming a stable ASO/mRNA duplex, thus preventing the protein translation. This innovative approach has led to a mean reduction in lipoprotein (a) levels, up to 92%, and is currently in phase 3 trials (123,124). In addition, Muvalaplin, an oral small molecule inhibitor of lipoprotein (a) formation by interacting with apo (a), and preventing it's binding to apoB100. Recently, Muvalaplin was shown to lower lipoprotein (a) levels up to 65% in a randomized controlled trial (125).

Triglyceride lowering agents

Mild to moderate increase of TG levels is consistently associated with increased ASCVD risk in several epidemiological, and clinical studies (126–128). Fibrates are commonly known for their TG-lowering effects. Fibrates reduce TG levels by enhancing their LPL-mediated lipolysis. Such effect is achieved by inhibiting the synthesis of apolipoprotein-CIII (apo-CIII), an inhibitor of LPL, while stimulating the gene expression of LPL (129). Although old fibrates can lower TG levels and increase HDL-C levels, they are not recommended for clinical use due to their inconsistent results in ASCVD outcome studies, and their side effects such as elevated levels of liver enzymes, and creatinine levels, as well as myopathy (129,130). Currently under investigations, a highly selective PPAR α agonist called pemafibrate showed higher TG reduction up to 50% from baseline at very low doses and less frequent side effects when compared to old fibrate (i.e. fenofibrate) (131,132). Other approaches to lower TG levels include angiopoetin-like 3 protein (ANGPTL3) and apo-CIII inhibitors. ANGPTL3 and Apo-CIII inhibit the hydrolysis of TG by LPL and thus the conversion of VLDL to LDL (121). Evinacumab, is a monoclonal antibody directed against ANGPTL3, which was shown to reduce plasma TG and surprisingly LDL-C levels in clinical studies (133). In addition, vupanorsen is an ASO targeting the ANGPTL3 mRNA, which is currently under clinical evaluation (134). Furthermore, targeting the mRNA of Apo-CIII by volanesorsen, an ASO, has dramatically

decreased the levels of apo-CIII up to 74.83%, TG levels by 67.9%, and VLDL-C by 72.9%, without significant impact on LDL-C, in patients with familial chylomicronemia syndrome (135–137).

The most recent breakthrough in the treatment of hypertriglyceridemia has been the unexpected cardiovascular effectiveness of omega-3 fatty acids. Omega-3 PUFA preparations such as EPA alone or with docosahexaenoic acid (DHA) are well known to reduce VLDL production and enhance VLDL clearance from plasma (138). The REDUCE IT trial (Reduction of Cardiovascular Events with Icosapent Ethyl Intervention Trial) has reported 25% reduction of major ASCVD events in high risk patients with controlled LDL levels (75mg/dl) by statins and elevated TG levels who received 4g daily of Icosapent Ethyl (highly purified form of EPA) compared to placebo (139). However, analyses of REDUCE-IT and meta-analyses have suggested that the reduction in cardiovascular risk with Icosapent Ethyl is greater than what can be achieved by triglyceride reduction alone, suggesting additional pleiotropic effects of EPA, such as anti-inflammatory and antithrombotic effects. In a recent EVAPORATE trial which involved 80 patients with CHD given a similar dose of Icosapent Ethyl as in the REDUCE IT trial, coronary CT scans showed that treatment with Icosapent Ethyl reduced the volume of atherosclerotic plaque by 17% compared to placebo (140). These data indicate that EPA holds promise in future recommendations for managing ASCVD, particularly in patients with residual risk despite controlled LDL levels (88).

Therapeutic targeting of HDL-C levels

Epidemiological studies have shown that plasma HDL-C levels have an inverse relationship with the occurrence of ASCVD, making HDL-C a strong predictor of ASCVD (5,141,142). Thus, therapeutic raising HDL-C levels (i.e. HDL-C quantity) might provide a novel treatment option for ASCVD. The main HDL-C raising therapies that have been tested in large scale clinical trials include cholesteryl ester transfer protein (CETP) inhibitors, niacin, and fibrates. Other HDL-targeting approaches are discussed in details in **section 1.5**.

CETP is a plasma enzyme produced by the liver, mainly bound to HDL, and plays a key role in mediating the transfer of cholesterol esters from HDL to CM, VLDL, and LDL in exchange for TG (143). Large scale observational studies have demonstrated that genetic CETP deficiency (i.e. total mass, and activity) increases HDL-C levels while mildly reduce LDL-C, and is associated with lower incidence of CVD (144,145). The first CETP inhibitor was

torcetrapib, which showed a substantial rise in HDL-C up to 70%, and reduced LDL-C by 25% in phase 3 clinical trials. However, the ILLUMINATE trial was terminated as a result of increased cardiovascular and all-cause mortality for patients in the torcetrapib treatment group, mostly resulted from increases in blood pressure, aldosterone, and cortisol levels (146–148). Other CETP inhibitors, such as dalcetrapib and evacetrapib were shown to increase the HDL-C levels by 30% and 133%, respectively, without affecting the blood pressure. However, the dal-OUTCOMES trial for dalcetrapib, and the ACCELERATE trial for evacetrapib were terminated due to lack of efficacy on cardiovascular outcome (149,150). The most recent CETP inhibitor, anacetrapib, showed 104% increase in HDL-C levels, while reduced the LDL-C by 17% and apoB by 18%. In the REVEAL study, 9% reduction in ASCVD events was reported with anacetrapib, largely due to reducing the Apo B - containing lipoproteins levels rather than increasing the HDL-C (151,152). Obicetrapib, is another CETP inhibitor that is currently under investigations for its LDL-C lowering effects. In a very recent double-blind, randomized, phase 2 clinical trial, obicetrapib was shown to lower LDL-C levels up to 50% and increase HDL-C levels up to 165% in dyslipidaemic patients with acceptable safety profile (153). All mentioned clinical trials involved ASCVD patients who were receiving a standard medical therapy, including statins, with or without CETP inhibitors.

Niacin generally increases the HDL-C levels up to 40% (154), by inhibiting the hepatic uptake and catabolism of ApoA-I, the major protein constituent of HDL (155). Although two meta-analysis of clinical trials have demonstrated a reduction in the incidence of CHD events with niacin (156,157), two clinical studies including AIM-HIGH trial and HPS2-THRIVE, in which patients with established ASCVD with controlled LDL-C levels (by statin ± ezetimibe) assigned to receive niacin or placebo, were terminated after 3-4 years, once it became evident that there was no incremental clinical benefit from the addition of niacin to statin therapy (158,159). Furthermore, in the HPS2-THRIVE study, the main reason of stopping the trial was the excess of myopathy in the niacin treated group. Therefore, the value of niacin in CHD remains unclear, with limited clinical use due to its side effects (160).

Fibrates affect the lipoprotein metabolism in different ways such as increasing the activity of LPL and the synthesis of ApoA-I and ApoA-II (the second major protein constituent of HDL), resulting in reducing plasma TG and raising HDL-C, respectively (148). In HELSINKI heart, and VA-HIT studies, gemfibrozil treatment reduced the incidence of CHD by 34% and 22%, respectively (161,162). Similar effects were reported with Bezafibrate in BIP

trial, with 39.5% reduction in the cumulative probability of incidence CVD events (163). Although those trials reported an increased HDL-C levels, none of them tested the effect of raising the HDL-C in particular, since the TG levels were concomitantly reduced. On the other hand, the ACCORD and FIELD trials reported that the addition of fenofibrate to statin in diabetic patients with low TG levels did not reduce the incidence rate of CVD events, as compared to statin alone (164,165), while in a general population with high TG levels $\geq 150\text{mg/dl}$, the combination of fenofibrate with statin for at least one year reduced the incidence rate (IR) of all-cause death and CVD death, compared to non-fenofibrate users (IR: 4.81 vs 5.35 for all cause death and 6.28 vs 6.42 for CVD per 1000 person-years) (166). These studies suggest that the beneficial effects of fibrates on CVD is related more to TG reduction rather than raising the HDL-C levels. In addition, the common side effects of fibrates including gastrointestinal, hepatic, renal, and muscle toxicity limit their clinical use and efficacy.

Beside the failure of the majority of HDL-C raising therapies to reduce the ASCVD risk, several epidemiological studies have demonstrated an increased mortality with elevated HDL-C levels (167). In the CANHEART study which involved 631,762 individuals without pre-existing CVD, low and very high levels of HDL-C were linked to a higher risk of CV and non-CV mortalities (168). This last observation was confirmed by two prospective population-based studies, the CCHS and the CGPS, which found that extremely high levels of HDL-C levels are paradoxically associated with elevated mortality, and the association between HDL-C levels and all-cause mortality was U-shaped for both men and women (169). In the same time, several studies found that HDL-C levels are influenced by several factors such as unhealthy diet, smoking, alcohol consumption, obesity, diabetes, gender, and low-income (168,170,171). Thus, the effectiveness of targeting the HDL-C levels to improve the ASCVD outcomes has raised doubts (172). Such doubts led to suggestion of reconsidering the evaluation of HDL functionality (i.e. quality) rather than just HDL-C levels (i.e. quantity) to better determine the risk of ASCVD and to improve therapeutic targeting of HDL to treat or prevent CVD.

Conclusion

While LDL-C, TG and HDL-C levels have been extensively studied and targeted in ASCVD management, HDL functionality is emerging as a promising target for more effective prevention and treatment. In this regard, HDL exhibits several atheroprotective functions as illustrated in **Figure 1.5**, with cholesterol efflux capacity (CEC) from arterial macrophages serving as the main function (173). The CEC of HDL has been inversely associated with

atherosclerosis development and ASCVD events (6–8), and thus it was proposed as a better predictor for ASCVD risk compared to HDL-C levels (6). Therefore, evaluating HDL functionality could better identify individuals at high risk of ASCVD despite unremarkable lipid disturbances (174–176). The concept of HDL dysfunctionality (resulting from structural and compositional changes under certain pathological conditions) reinforces the importance of focusing on HDL functionality (i.e. quality) beyond HDL-C levels (177–180). While HDL-C levels do not predict HDL functionality or composition (181), understanding the role of HDL components in determining its functional state offers insights for enhancing its atheroprotective functions beyond increasing its cholesterol cargo (176,182).

1.1.6. Bridging a gap of knowledge in the ASCVD

ASCVD remain the leading cause of mortality worldwide and they are considered as multifactorial diseases with a well-known cluster of risk factors. The identification of individuals at increased risk for ASCVD could help to reduce incidence ASCVD and related mortality. While the FRS has been established as an independent and reproducible model for identifying individuals at an elevated risk of ASCVD, there are still notable challenges to accurately predicting the disease's occurrence. Notably, several studies have shown that 10–15% of patients presented with CHD events were lacking the major traditional risk factors, pointing to the limitations of the existing risk assessment tools (183,184). In addition, Akosah et al. found that about 70% of young adults diagnosed with MI were initially classified as low-risk individuals (185). Furthermore, several studies have found that FRS score has limitations in estimating the risk among young people and women (144).

To bridge these gaps in risk assessment, attention has been devoted toward investigating additional markers that could enhance the prediction of CVD risk. In this context, the emerging field of plasma lipidomics has gained significant attention for its potential to identify the implication of specific molecular lipid species in ASCVD. Aligned with this evolving research landscape, the central focus of this doctoral dissertation will be on plasma lipoproteins, with a specific emphasis on HDL. The major objective of this work is investigating the potential of specific molecular lipid species to serve as novel biomarkers for HDL atheroprotective functions and their implications in the context of ASCVD.

For that, the next sections of this chapter will delve extensively into the lipoproteins composition, metabolism, and biological functions, with a particular focus on HDL. This

literature review aims to establish a deeper understanding of how the composition of HDL particles influences their functionality, shedding the light on mechanisms by which such interrelationship is implicated in the context of ASCVD pathogenesis.

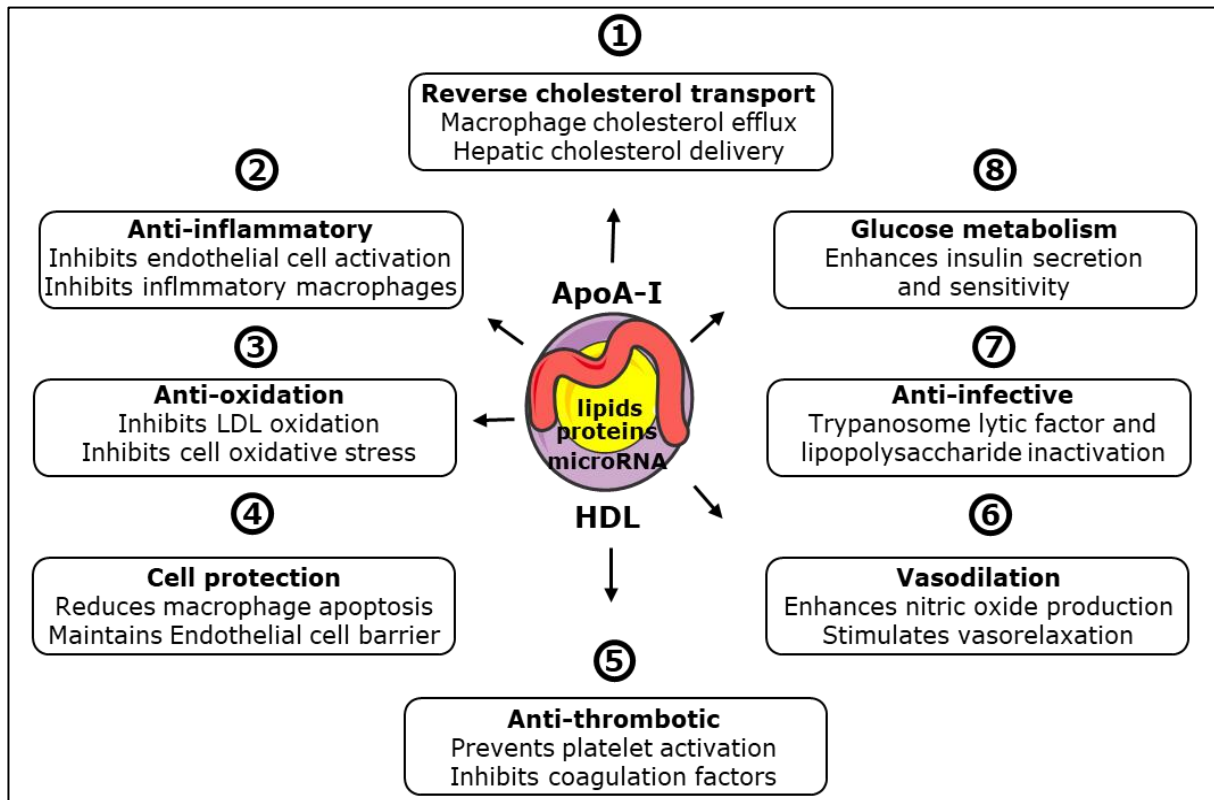


Figure 1.5: HDL vascular protective functions. HDL: High Density lipoprotein, ApoA-I: apolipoprotein A-I, microRNA: micro Ribonucleic Acid. [Adapted from Linton MRF et al. 2019- https://www.ncbi.nlm.nih.gov/books/NBK343489/figure/lipid_athero.F10/]

1.2 Lipoproteins structure, composition and metabolism

Lipids serve as crucial building blocks for living cells and they are essential nutrients alongside with carbohydrates and proteins (186). Major lipid classes are fatty acids, TG, phospholipids, cholesterol, and fat-soluble vitamins. Lipids are hydrophobic, rendering them insoluble in water (187). Given that blood is a water-based media, lipids are transported in the blood complexed to special proteins called apolipoproteins, which are amphiphilic and can accommodate lipids while effectively interact with water-based media. Such lipid-protein complexes are referred to as lipoproteins (188,189).

1.2.1. Lipoproteins structure and classification

Lipoproteins are macromolecular complexes encompassing a diverse protein and lipid composition, responsible mainly for the transportation of lipids throughout the body. In a general context, Lipoproteins adopt a spherical structure as illustrated in **Figure 1.6** with a central core rich in hydrophobic neutral lipids, basically CE and TG, encircled by amphiphilic shell comprising PL, free cholesterol (FC), and one or more apolipoproteins. The latter serve a dual purpose, stabilizing the structure of lipoprotein particles and facilitating their metabolism (190,191).

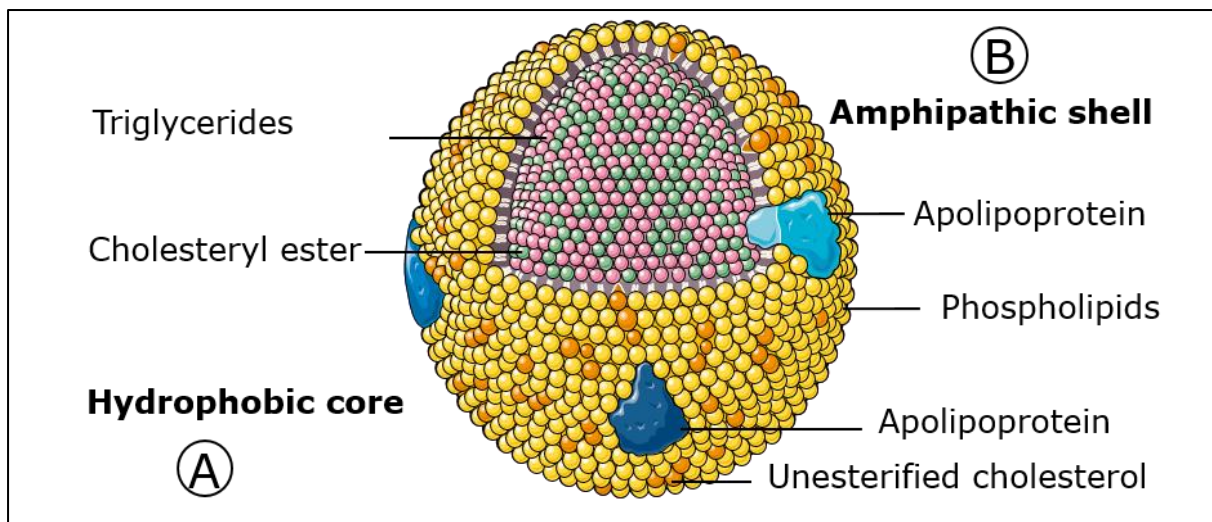


Figure 1.6: Lipoprotein structure. Lipoprotein is composed of. **A)** Central core made of hydrophobic lipids including cholesteryl esters and triglycerides. **B)** Amphipathic shell made of phospholipids, free unesterified cholesterol and apolipoproteins. The amphipathic nature (i.e. having both a hydrophobic and hydrophilic properties) of the outer shell allows the lipoproteins to function in the aqueous environment of blood. [Adapted from Lampasas et.al 2023 (191)].

Plasma lipoproteins are divided into seven classes based on their size, lipid composition, and apolipoprotein content, and they include CM, CM remnants (CM-R), VLDL, VLDL remnants (VLDL-R) (or intermediate-density lipoprotein (IDL)), LDL, Lipoprotein (a), and HDL (50,192). Based on the utilized separation techniques (e.g. gradient ultracentrifugation, electrophoresis, and immunoprecipitation), each lipoprotein class constitutes a continuum of particles that differs slightly in their density, and size, as a result of their continuous remodeling in the circulation as illustrated in **Figure 1.7**. The main structural and compositional features of each lipoprotein class as well as their major sources and functions are listed in **Table 1.2**.

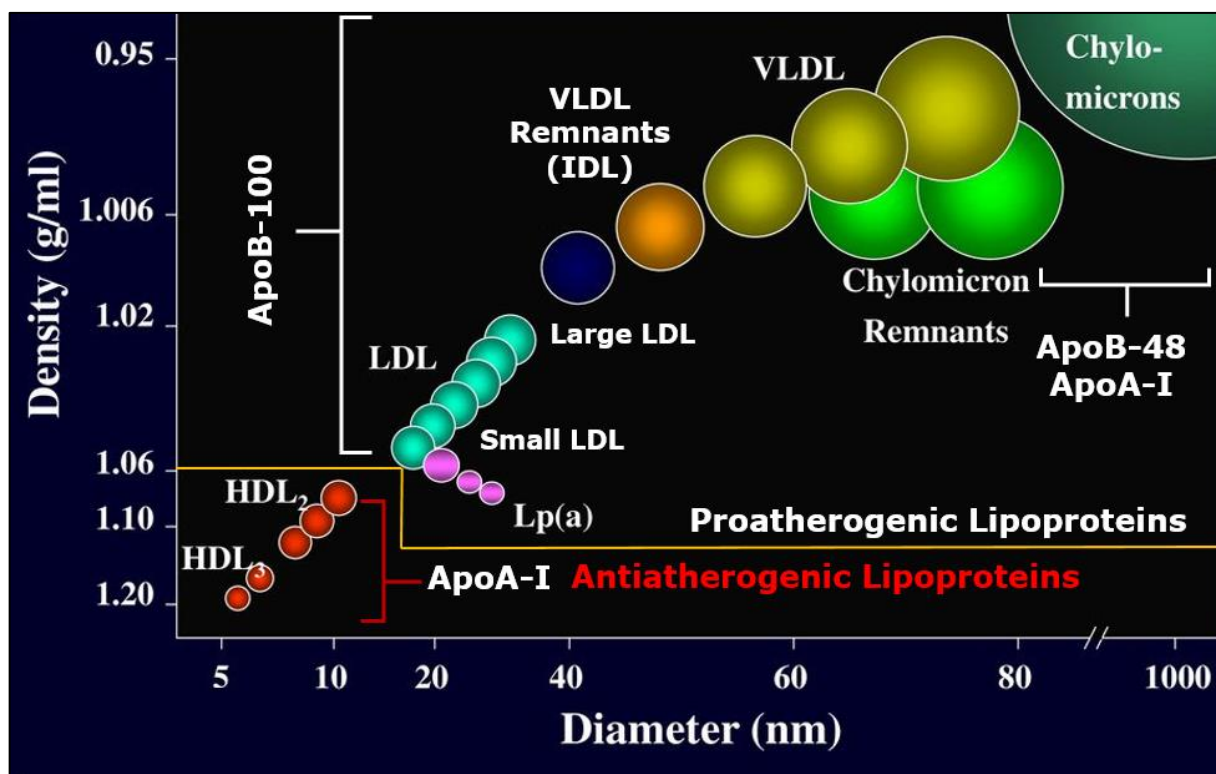


Figure 1.7: Classes of Lipoproteins. Plasma lipoproteins are divided into seven classes based on size, lipid composition, and apolipoprotein. Including chylomicrons (CM), chylomicron remnants (CM-R), VLDL, VLDL remnants (VLDL-R), IDL, LDL, Lp (a), and HDL. CM, VLDL, LDL, and Lipoprotein (a) are all apolipoprotein (Apo) B - containing lipoproteins and they are considered proatherogenic, thus contribute to ASCVD development, whilst HDL is the only anti-atherogenic lipoprotein that protects against ASCVD. [Adapted from Feingold, KR. et al. 2021: <https://www.ncbi.nlm.nih.gov/books/NBK305896/>]

As illustrated in **Figure 1.7**, CM represent large lipoprotein complexes that originate from dietary lipids within the intestinal cells as a means to transfer dietary lipids (referred to as exogenous lipids) to the rest of the body. These particles are primarily composed of TG with a lesser quantity of cholesterol. The size of CM varies depending on the amount of TG ingested. The major apolipoprotein found in CM is ApoB-48, along with additional apolipoproteins such

as ApoAs, ApoCs, and ApoE (192). Within the circulation, the hydrolysis of TG from CM leads to the formation CM-R. The CM-R are smaller in size compared to CM, with their primary components being the cholesterol, and less TG. The major apolipoproteins components found in CM-R are ApoB-48, and ApoE (192–194).

VLDL are large lipoprotein complexes that originate in the liver and are mainly involved in the transport of liver-derived lipids (endogenous lipids) to extrahepatic tissues. In addition to TG as the primary lipid component, VLDL also contain some cholesterol. The major apolipoproteins found in VLDL are ApoB-100, ApoC, and ApoE. VLDL are similar to CM in the fact of being enriched in TG, however, they are smaller in size compared to CM. The hydrolysis of TG in VLDL gives rise to VLDL-R (or IDL). These remnants are primarily composed of cholesterol, along with ApoB-100 and ApoE (50,192). LDL particles are generated by the gradual removal of TG from IDL, and they are even further enriched in cholesterol, with minor amount of TG. As a result, LDL is considered the major carrier of plasma cholesterol (192). According to “Davidson LDL proteomic watch”, few proteomic studies up to date have showed that LDL contains 22 proteins, including mainly ApoB-100, ApoC, and ApoE, with ApoB-100 being the major one and is fundamental for the uptake of LDL by the liver and peripheral tissues (195,196). LDL is smaller in size compared to VLDL and CM, and it consists of a spectrum of particles range from large to small LDL. Very close to LDL, lipoprotein (a) is an LDL particle that has apolipoprotein (a) glycoprotein tail that is covalently attached to ApoB-100 (197–199). The size of lipoprotein (a) varies according to the size of Apo (a), which is largely vary and is determined genetically. Like LDL, lipoprotein (a) is enriched in cholesterol, and is a major carrier of oxidized PL (49,50).

Plasma HDL is a group of relatively small lipoprotein complexes compared to other lipoproteins. HDL particles are enriched in cholesterol, and phospholipids, and composed of ApoA-I as the core structural apolipoprotein (200,201). HDL particles are synthesized in the liver and small intestines, as protein-rich disc shaped pre-beta particles, which are nearly empty of cholesterol and TG. These particles circulate through the bloodstream and mainly mediate the export and transport of cholesterol from peripheral tissues to the liver for excretion (50,192). Plasma HDL particles are very heterogeneous and can be categorized according to their density, size, charge, or apolipoprotein composition into several subparticles. HDL subpopulations, and their heterogeneity are discussed in the following section.

Table 1.2: The main features of each lipoprotein classes and their major functions. CH: cholesterol; CM: chylomicrons; CM-r: CM remnants; HDL: high-density lipoprotein; IDL: intermediate-density lipoprotein; LDL: low-density lipoprotein; LP: lipoprotein; LP (a): lipoprotein (a); PL: phospholipids; TG: triglycerides; VLDL: very-low-density lipoprotein; VLDL-r: VLDL remnants [Adapted from (50,192,195,202,203)].

Lp class	Structure		Composition (%) by weight		Major Apolipoproteins (Apo)	Major lipid	Source	Function
	Density (g/ml)	Size (nm)	Protein	Lipids				
CM	<0.930	75-1200	1-2	98-99	A-I, A-II, A-IV, B-48, C-I, C-II, C-III, E	TG	Intestine	Transport dietary fat
CM-r	0.930-1.006	30-80	6-8	92-94	B-48, E	TG, CH	CM	
VLDL	0.930-1.006	30-80	7-10	90-93	B-100, C-I, C-II, C-III, E	TG	Liver	Transport hepatic fat
IDL VLDL-r	1.006-1.019	25-35	11	89	B-100, E	TG, CH	VLDL	
LDL	1.019-1.063	18-25	21	79	B-100, A-I, C-II, C-III, E	CH	VLDL	Transport CH to periphery
LP(a)	1.055-1.085	~30	34	66	B-100, (a)	CH	Liver	
HDL₂	1.063-1.125	10-20	33	67	A-I, A-II, A-IV, C-I, C-II, C-III, D, E	PL, CH	Liver, intestine	Reverse Transport of CH from periphery to liver
HDL₃	1.125-1.210	5-10	57	43				

1.2.2. Protein and lipid components of Lipoproteins

Plasma lipoproteins contain large number of proteins (e.g. VLDL~90 protein; LDL~22 proteins; and HDL~285 proteins) that are crucial for their biological functions (195,204). Apolipoproteins, in particular, are pivotal for lipoproteins, influencing their metabolism, structure, and functions. Generally, apolipoproteins serve four major roles: 1) guiding lipoprotein synthesis and assembly, 2) stabilizing the micellar structure of lipoproteins by interacting with the phospholipid monolayer to maintain the hydrophilic surface of lipoproteins, 3) facilitating lipid transport and distribution among various tissues through specific interactions with lipoprotein receptors, and 4) modulating enzymatic processes involved in

lipoprotein metabolism (205,206). **Table 1.3** outlines the key apolipoproteins found in various lipoprotein categories, along with their major metabolic functions.

Table 1.3: Major Apolipoproteins and their metabolic roles. Apo: Apolipoprotein; CM: chylomicrons; CM-r: CM remnants; IDL: intermediate-density lipoprotein; LDL: low-density lipoprotein; LP: lipoprotein; LP (a): lipoprotein (a); HDL: high-density lipoprotein; MW: Molecular weight; VLDL: very-low-density lipoprotein [Adapted from (50,192,207)].

Apo	Lipoprotein association	Metabolic functions	MW (Da)	Synthesis
ApoA-I	CM, HDL	Major structural and functional protein for HDL, Activates lecithin: cholesterol acyltransferase (LCAT) enzyme	28,000	Liver, intestine
ApoA-II	CM, HDL	Enhances hepatic lipase (HL) activity	17,400	Liver
ApoA-IV	CM, HDL	CM assembly and secretion	46,000	Intestine
ApoA-V	CM, VLDL, HDL	Promotes intravascular hydrolysis of TG mediated by lipoprotein lipase (LPL)	39,000	Liver
ApoB-48	CM, CM-r	CM assembly and secretion	264,000	Intestine
ApoB-100	VLDL, IDL, LDL, Lp(a)	Assembly and secretion of VLDL, Ligand for LDL receptor	512,000	Liver
ApoC-I	CM, VLDL, HDL	Activates LCAT	6600	Liver
ApoC-II	CM, VLDL, HDL	Activates LPL	8900	Liver
ApoC-III	CM, VLDL, HDL	Inhibits LPL, Inhibits hepatic uptake and catabolism of apoB-containing lipoproteins, enhances the catabolism of HDL, activates inflammatory pathways	8800	Liver
ApoD	HDL	Closely associated with LCAT	33,000	Liver
ApoE	CM, CM-r, VLDL, IDL, HDL	ligand for LDL receptor, ligand for LDL-related protein (LRP) receptor, Role in reverse cholesterol transport	34,000	Liver
apo(a)	Lp(a)	delivering cholesterol to sites of vascular injury	300,000-800,000	Liver

Similar to apolipoproteins, lipids are also fundamental to lipoproteins. The major lipid classes found in plasma lipoproteins are phospholipids, sphingolipids, TG, and cholesterol (11,208). **Figure 1.8** illustrates the approximate lipid and apolipoprotein composition of the major plasma lipoproteins. In the context of plasma lipids, CM and VLDL serve as the primary carriers of plasma TG, while LDL and HDL are the main carriers of plasma cholesterol, and phospholipids, respectively. However, it's important to note that circulating lipoproteins are in a dynamic state, continually exchanging components among one another, and thus changing in their size.

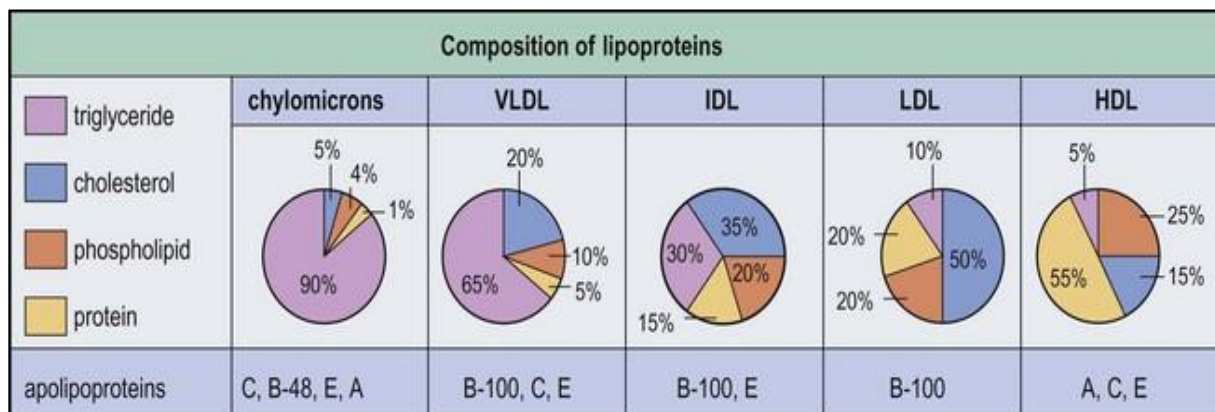


Figure 1.8: Estimated lipid and protein composition of the major plasma lipoproteins. Although the composition in each class is similar, the composition within the particles of each class is heterogeneous, so the percentages given are approximate. In the case of HDL, the figure is shown for HDL3, while HDL2 particles contain less proteins and more lipids. [Adapted from <https://basicmedicalkey.com/lipids-lipoproteins-and-cardiovascular-disease/>]

Phospholipids or glycerophospholipids (GPL) play a critical role in maintaining the structural integrity and functionality of cellular membranes and plasma lipoproteins. As illustrated in **Figure 1.9A**, phospholipids consist of fatty acids linked to a glycerol backbone with a phosphate group. Each phospholipid molecule has two fatty acids, termed lipid tails, and linked to the first (sn-1) and second (sn-2) carbon atoms of the glycerol via ester linkage ($C-COO-CR'$), except for ether GPL (plasmalogens), where the sn-1 fatty acid is linked via ether ($C-O-C-CR'$) or vinyl-ether linkage ($C-O-C=CR'$). In lysophospholipids (e.g. Lyso-phosphatidylcholine (LPC), and Lyso-phosphatidylethanolamine (LPE)), only one hydroxyl group of the glycerol backbone is acylated with a fatty acid chain. The third carbon atom (sn-3) of the glycerol in phospholipids is occupied by a phosphate group linked to an alcoholic group, known as the head group (209). Phospholipids exhibit amphipathic properties, featuring polar, hydrophilic head groups and nonpolar, hydrophobic lipid tails (210,211). In aqueous

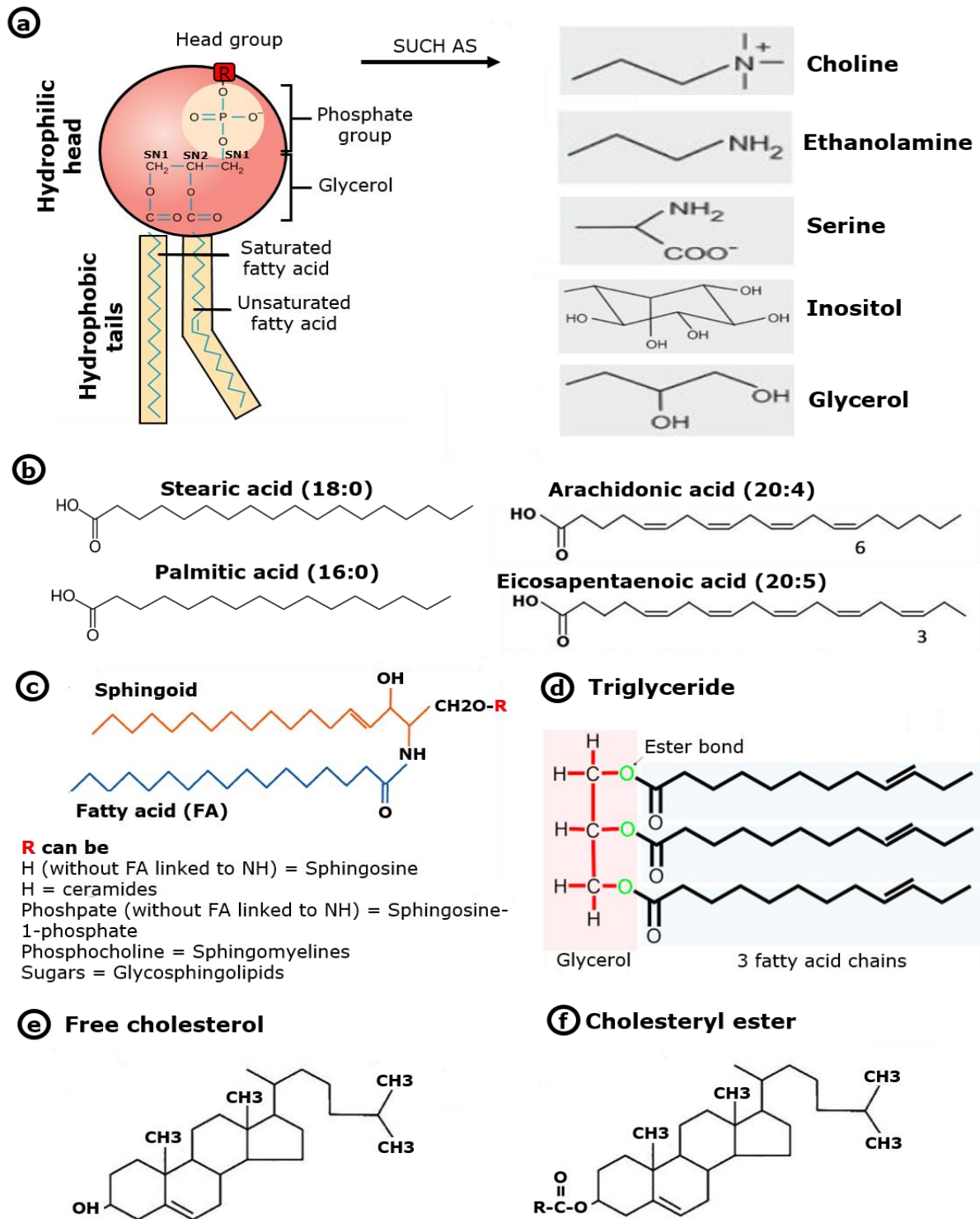


Figure 1.9: Major lipid classes. **A)** Glycerophospholipids are composed of phosphate head group and lipid tails made of two fatty acids (saturated or unsaturated) linked together through a glycerol molecule. Examples of phospholipid' head groups include choline, ethanolamine, serine, and inositol. **B)** Saturated and unsaturated fatty acids. **C)** Sphingolipids are composed of sphingosine, N-fatty acid, and a head group which determines the sphingolipid class. **D)** Triglycerides are composed of glycerol molecule linked to 3 fatty acids. **E)** Free cholesterol. **F)** Cholesteryl ester. [Adapted from LibreTexts BIOLOGY, Harayama and Riezman, et al. 2018 (209), Li et al. 2015 (211), Aktas et al. 2014 (214)]

environments, phospholipids assemble as monolayers in lipoproteins or bilayers in cellular membranes, with their head groups facing the aqueous phase and lipid tails oriented towards the inner aspect of the cell membrane or the lipid core of lipoproteins (209–211). The arrangement of phospholipid's head groups and lipid tails, based on their physicochemical properties, significantly affects the lipoproteins' surface and core fluidities, thereby impacting their ability to transport, exchange and store different lipids (212,213). Major GPL classes are phosphatidylcholine (PC), LPC, phosphatidylethanolamine (PE), LPE, phosphatidylinositol (PI), phosphatidylserine (PS), PE-plasmalogens, phosphatidylglycerol (PG), phosphatidic acid, and cardiolipin (214). While the head group defines the GPL's class, the fatty acid composition (i.e. length and the unsaturation level, **Figure 1.9B**) of the lipid tails determine the molecular species of each phospholipid class.

Sphingolipids is another major class of plasma lipids, and they consist of sphingoid base (which is both the backbone and hydrophobic tail) with an N-acyl fatty acid chain and a head group (**Figure 1.9C**). The degree of unsaturation and hydroxylation defines the sphingoid base type (i.e. sphinganine or hydroxyl-sphinganine, or sphingosine), while the head groups (i.e. hydroxyl, phosphocholine, phosphoethanolamine, glucose, galactose, oligosaccharide, and phosphate) defines the sphingolipids class. Major classes of sphingolipids include ceramides, sphingomyelin (SM), ceramide-phosphoethanolamine, cerebroside (i.e. galactocerebroside, glucocerebroside), ceramide-1-phosphate, lysosphingolipids, and complex glycosphingolipids (i.e. gangliosides) (209,211,215).

In plasma, phospholipids and sphingolipids are unevenly distributed across plasma lipoproteins. GPL such as PC, LPC, PE, and PE-plasmalogens are mainly found in HDL, while SM and ceramides are mainly found in LDL as shown in **Table 1.4** (208).

Other major classes of plasma lipids are TG and cholesterol. TG consist of a glycerol molecule esterified with three fatty acids (**Figure 1.9D**), while cholesterol is a steroid lipid, consists of a central sterol nucleus made of four hydrocarbon rings, a hydrocarbon tail, and a hydroxyl group. Cholesterol presents as free cholesterol (unesterified, **Figure 1.9E**) or CE (esterified, **Figure 1.9F**), the latter form is reserved for cellular storage and transport in plasma (209).

Table 1.4: Distribution of major classes of phospholipids among lipoproteins. HDL: high-density lipoprotein; LDL: low-density lipoprotein; PE: phosphatidylethanolamine; VLDL: very-low-density lipoprotein [Adapted from (208)].

Lipid	Total-serum Concentration (μM)	Distribution among lipoprotein classes in percent		
		VLDL (%)	LDL (%)	HDL (%)
Phosphatidylcholine	1986 \pm 727	8.1 \pm 6.4	29.9 \pm 6.6	62 \pm 10.3
Sphingomyelin	415 \pm 141	7.2 \pm 6.8	50.4 \pm 7.5	42.6 \pm 10.7
Lysophosphatidylcholine	330 \pm 168	1.7 \pm 1.5	11 \pm 4.5	87.3 \pm 5.4
Phosphatidylethanolamine	35.6 \pm 20.8	18.6 \pm 9.4	21.3 \pm 5.6	60.1 \pm 11.4
PE-plasmalogens	31.9 \pm 13.9	11.6 \pm 5.3	28.5 \pm 6.5	59.9 \pm 8.8
Ceramide	8.1 \pm 3.4	15.6 \pm 9.9	60.3 \pm 6.7	24.1 \pm 7.4

1.2.3. Lipoprotein metabolism

The Metabolism of plasma lipoproteins is divided into two distinct pathways: exogenous and endogenous pathways. The exogenous pathway involves the transportation of dietary lipids from small intestines to peripheral tissues and liver. In this pathway, CM and CM-remnants lipoproteins act as carriers for dietary lipids. The endogenous pathway involves the synthesis and subsequent distribution of lipids originating from liver to peripheral tissues through VLDL, IDL, and LDL lipoproteins. These pathways are crucial to ensure the balance between dietary lipid intake, liver lipid synthesis, and the essential distribution of lipids throughout the body (188,216,217).

1.2.3.1 Exogenous pathway

The exogenous pathway (as illustrated in **Figure 1.10A**) of lipid metabolism initiates within the small intestines, where the dietary lipids such as TG are digested into fatty acids and monoacylglycerol (MAG), which are then emulsified with bile acids, and dietary cholesterol to form micelles. The absorption of cholesterol by the enterocytes is facilitated by NPC1L1 transporter, while fatty acids and MAG are likely absorbed through fatty acid transporters and passive diffusion (50). Within the enterocytes, both fatty acids, and cholesterol are re-esterified into TG and CE, respectively, which are then combined with phospholipids, and ApoB-48 to form CM particles. The assembly process of CM is facilitated by microsomal TG transfer protein (MTP). After their assembly, CM particles access the systemic circulation via the lymphatic system, where they acquire ApoC-II and ApoE from HDL particles, resulting in the maturation of CM (188).

In the circulation, ApoC-II serves as a co-factor for the activation of LPL enzyme, which is responsible for breaking down the TG within CM into fatty acids and glycerol. The released fatty acids are then taken up by muscles for energy usage and adipose tissues for storage in form of TG, while glycerol is transported to liver. The removal of TG from CM leads to the formation of CM-remnants, which still retain both ApoB-48 and ApoE. These CM-remnants are rapidly endocytosed by the hepatocytes via LDLR or LDL receptor-related protein (LRP), a process that requires ApoE recognition. Inside the hepatocytes, CM-remnants undergo breakdown into components such as TG, CE, PL, fat soluble vitamins, and amino acids derived from apolipoproteins (189,218–220). On the other hand, CM can also be modified within the

circulation through their interaction with other plasma lipoproteins. In this interaction, some TG from CM are transferred to HDL and LDL through the action of CETP in exchange for CE.

1.2.3.2 Endogenous pathway

The endogenous pathway (illustrated in **Figure 1.10B**) of lipoprotein metabolism initiates within the liver. In this pathway, TG, CE, phospholipids, vitamin E, and apoB-100 are assembled together, under the action of MTP, to form VLDL. The hepatic secretion and the size of VLDL is regulated by both the activity of MTP and the availability of TG from various sources. The amount of TG present in the secreted VLDL depends on the fatty acids availability. Major sources of fatty acids in liver include *de novo* synthesis, hepatic uptake of triglyceride-rich lipoproteins (TGRL), and fatty acids derived from adipose tissues (50,221,222). In the context of cholesterol content in VLDL, about 10-20% is derived from diet and about 75% is derived from *de novo* biosynthesis, via a pathway whose key enzyme is HMGCR enzyme (223). As VLDL particles enter the circulation, they acquire ApoC-II and ApoE from the HDL before reaching all body tissues where LPL facilitates the hydrolysis of their TG into fatty acids and glycerol (194,224). During this process, VLDL loses the ApoC-II (which returns back to HDL) and transforms into VLDL-remnants (also referred to as IDL). About 50% of VLDL-remnants are cleared from circulation by the liver via LDLR or LRP, in a manner similar to the CM-remnants removal. The remaining VLDL-remnants is converted to cholesterol-rich LDL particles, through the hydrolysis of their residual TG via the hepatic lipase on the surface of liver cells. Due to its high cholesterol content, LDL predominantly deliver cholesterol to the liver and peripheral tissues via LDLR. The main functions of cholesterol is to maintain the integrity and fluidity of cellular membranes and to serve as a precursor for the synthesis of several vital substances such as steroid hormones, bile acids, and vitamin D (50,225).

To maintain lipid homeostasis, mechanisms like reverse cholesterol transport (RCT) and reverse remnant-cholesterol transport (RRT) pathways control lipid distribution, particularly cholesterol, to peripheral tissues, mainly via HDL particles. Such pathways involve the removal of cholesterol from peripheral tissues (i.e. RCT), and from circulating lipoproteins-remnants (i.e. RRT) by HDL, followed by cholesterol transport to liver for excretion (50,217,226). These pathways prevent excessive cholesterol accumulation in peripheral tissues including arterial wall. Intravascular HDL metabolism, as well as the RCT and RRT pathways, are detailed in the following section.

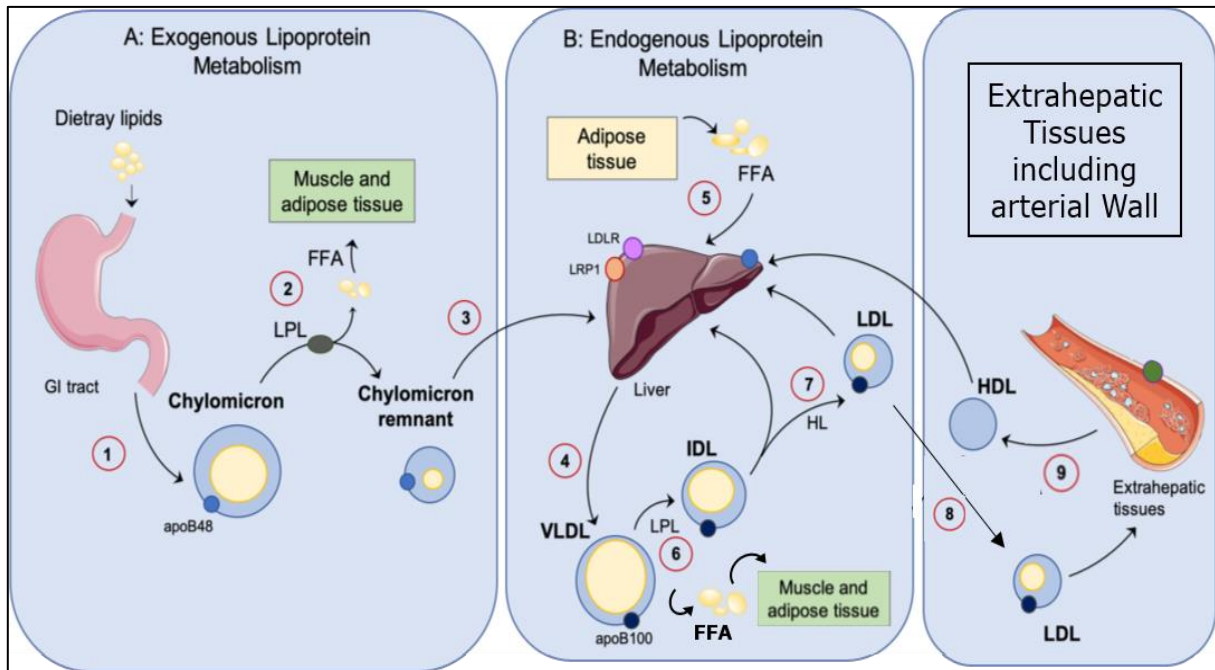


Figure 1.10: Exogenous and Endogenous lipoprotein metabolic pathways. **A)** Exogenous Lipoprotein Pathway. **1.** Digested lipids enter the intestinal enterocytes where they are re-assembled into chylomicron particles and subsequently secreted into lymphatic vessels and released to circulation. **2.** In the capillary beds of the adipose tissue and muscles, TG in chylomicrons are hydrolysed by lipoprotein lipase (LPL) into free fatty acids (FFA), which are then used to produce energy in muscle or for the storage of TG in adipose tissue. Such de-lipidation process produces cholesterol-rich chylomicron remnants. **3.** Chylomicron remnants are rapidly endocytosed by hepatocytes via LDL receptor (LDLR) or LDL receptor-related protein (LRP). **B)** Endogenous Lipoprotein Pathway. **4.** Hepatic VLDL secretion is controlled by the activity by the availability of hepatic lipids from varying sources. Under fasting conditions, FFA are released from adipocytes. **5.** Then FFA are delivered to the liver for re-esterification and secretion as VLDL. **6.** In circulation, VLDL lose TG due to LPL-catalysed hydrolysis and thus become intermediate-density lipoproteins (IDL). **7.** In a second step, IDL are further hydrolysed by hepatic lipase (HL) to produce cholesterol enriched LDL particles. These LDL particles deliver cholesterol to the liver as well as peripheral tissues primarily via the LDLR, where cholesterol serve as substrate for membrane or steroid hormone synthesis in distant cells. **8.** When LDL (particularly small LDL) undergo oxidation, they can be retained in the peripheral tissues and taken up by peripheral cells like macrophages resulting in the formation of foam cells. **9.** High density lipoprotein (HDL) removes excess LDL cholesterol from peripheral cells such as arterial macrophages, for hepatic clearance, preventing excessive cholesterol accumulation in macrophage foam cells. [Adapted from Stemmer. et al. 2020 (217)]. The RCT pathway part shown in the original graph was modified accordingly to present this pathway separately in the context of HDL metabolism.

1.2.3.3 Intravascular metabolism of HDL

The intravascular metabolism of HDL (**Figure 1.11**) initiates with the *de novo* generation of lipid free/lipid poor ApoA-I particles, from the liver or small intestines (227–230). These small precursors of mature HDL are unstable and readily acquire FC and phospholipids from peripheral cells via ATP-binding cassette transporter A1 (ABCA1) lipid transporter. This process leads to the formation of small, discoidal pre- β HDL particles. These particles, in turn, acquire FC and phospholipids from peripheral tissues via ABCA1. Concomitantly, the acquired FC is converted to CE by lecithin-cholesterol acyltransferase (LCAT) enzyme, by transferring a fatty acid from the sn-2 position of PC to the hydroxyl group of free cholesterol, thereby forming CE and LPC (refer to **Figure 1.9E and F** for structure) (231). Phospholipids for LCAT activation are mainly provided via the phospholipid transfer protein (PLTP), by mediating transfer of phospholipids from VLDL into HDL (232). As they form, CE sequester into the core of discoidal HDL particles due to their extreme hydrophobic nature. This effect gradually converts the discoidal HDL into less dense, and larger spherical particles, including small HDL3 and large HDL2 particles. These particles can undergo additional remodeling via particle fusion and surface remnant transfer mediated by PLTP (232). Small HDL3 particles continue to acquire FC from peripheral cells mainly via ABCA1, while large HDL2 particles acquire FC mainly via ATP binding cassette G1 (ABCG1) and scavenger receptor class B Type I (SR-BI) transporters. Large HDL2 can be converted into small HDL3 upon CETP-mediated CE transfer from HDL to VLDL and LDL, selective CE uptake by the liver and steroidogenic organs mediated via SR-BI, and hydrolysis of the core TG by hepatic lipase (HL) as well as of TG and phospholipids by endothelial lipase (EL) (233). These processes promote reduction in HDL size, resulting in the formation of lipid-poor apoA-I or pre- β HDL, which can be recycled for the next lipidation cycle (234,235).

The intravascular metabolism of HDL is intimately linked to that of TGRL (i.e. CM and VLDL). Among the pathways linking HDL and TGRL are the CETP-mediated heteroexchange of CE and TG, and the transfer to HDL of surface TGRL remnants produced during TGRL lipolysis by LPL (236,237). The LPL-mediated hydrolysis of TG within the TGRL result in the shrinkage of their hydrophobic core, leading to the formation of smaller, denser remnant TGRLs, also referred to as core remnants (238–240). Consequently, the redundant molecules of the surface monolayer surrounding the hydrophobic core are shed from the particles in form of surface remnants, which include apolipoproteins, phospholipids, and FC (241). Following

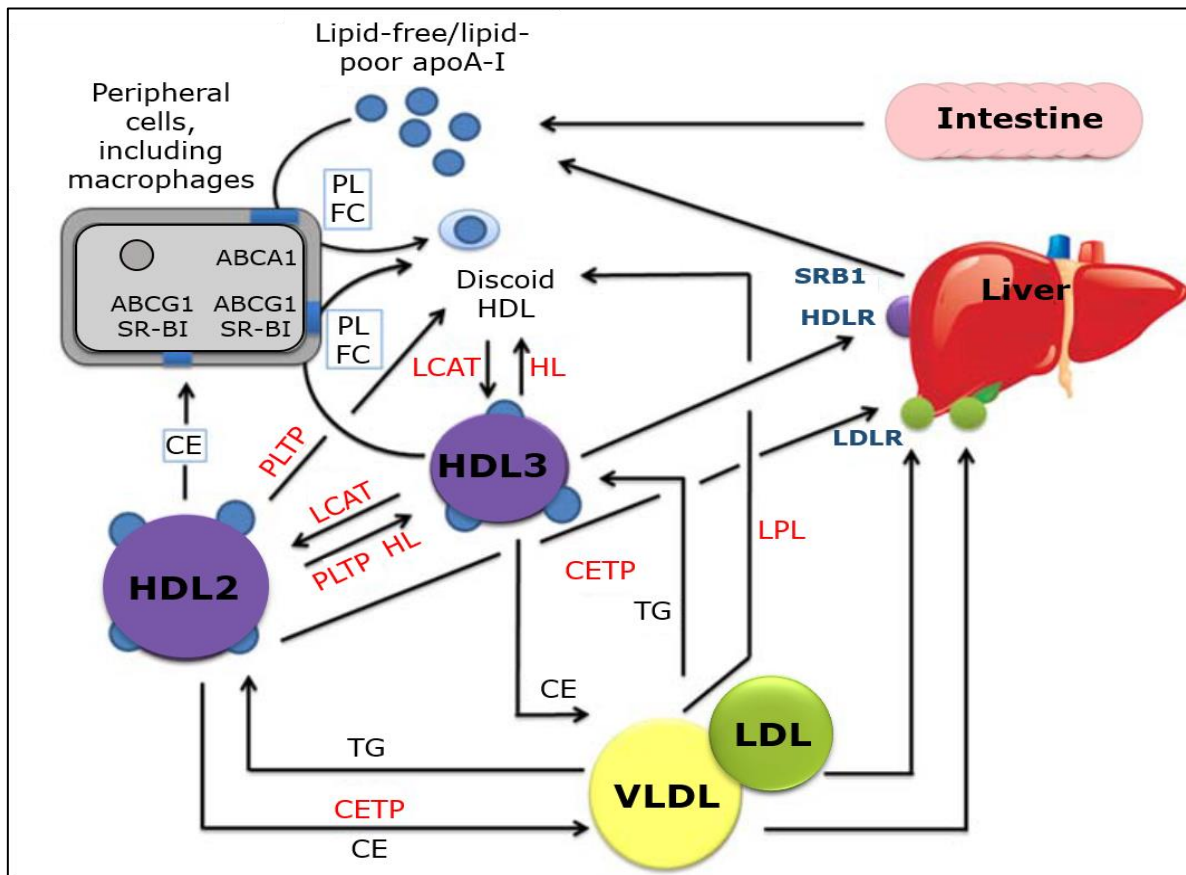


Figure 1.11: Intravascular HDL metabolism. Apolipoprotein A-I (apoA-I) generated by the liver and intestine acquire free cholesterol (FC) and phospholipids (PL) from cells lipids via the ATP binding cassette AI (ABCA1) transporter to form small, discoid pre-beta HDL. Lipoprotein lipase (LPL)-mediated lipolysis of VLDL is another important source for the formation of pre-beta HDL from the redundant surface components of VLDL following triglycerides (TG) hydrolysis. Acting on pre-beta discoid HDL, lecithin: cholesterol acyltransferase (LCAT) esterifies the FC into cholesteryl esters (CE), and subsequently generates a group of small HDL (referred to as HDL3) and large spherical HDL (referred to as HDL2) particles. The HDL2 particles undergo further remodelling via particle fusion and surface remnant transfer mediated by phospholipids transfer protein (PLTP). Small HDL3 particles further combine with FC effluxed via ABCA1, ATP binding cassette G1 (ABCG1) transporters and scavenger receptor class B Type I (SR-BI), while large HDL2 particles acquire FC via ABCG1 and SR-BI. Under the action of cholesteryl ester transfer protein (CETP), the CE in small and large HDL particles is exchanged for TG from apoB-containing lipoproteins (VLDL and LDL), resulting in the formation of TG-rich HDL. Plasma lipases including hepatic lipase (HL) and endothelial lipase (EL, not shown) hydrolyse HDL phospholipids and TG, promoting the reduction in HDL size, formation of lipid-poor HDL particles and shedding from HDL of lipid-free apoA-I, which can interact with ABCA1 in the next lipidation cycle. HDL lipids are catabolized primarily in the liver, either separately from HDL proteins by selective uptake via SR-BI, or via CETP-mediated transfer to apoB-containing lipoproteins, or as holoparticles together with HDL proteins primarily in the liver via uptake through receptors for HDL holoparticles (HDLR). [Adapted from Camont et al. 2011 (173) and Rached et al. 2015 (231)]

their production, the surface TGRL remnants predominantly fuse with HDL, adding material to plasma HDL pool. HDL can efficiently acquire both polar (FC, phospholipid) and non-polar (CE, TG) lipids (226). In this regard, small, dense lipid-poor HDL3 particles represent preferential acceptors for surface lipids released during TGRL lipolysis (242). The transfer of phospholipid and FC to HDL3 enhances LCAT-mediated formation of CE, facilitating conversion of HDL3 into HDL2 (243). Remnant-derived cholesterol can then be transferred from HDL to the liver, either directly via SR-BI or through VLDL and LDL and their hepatic receptors (244,245). HDL's removal of excess surface remnants also facilitate the hepatic uptake of the core remnants (246). This accelerates the removal of surface and core remnants from circulation, which can otherwise influx and accumulate within the extrahepatic tissues, including the arterial wall (241).

HDL lipids are catabolized primarily in the liver. To do so, HDL either selectively delivers its cholesterol to the liver through SR-B1, or to VLDL and LDL through CETP with subsequent uptake through LDLR. HDL particles can also be endocytosed via holoparticle HDL receptors (i.e. cubulin and ectopic β -chain of ATP synthase), and LDLR (for apoE-containing HDL), where they are degraded and their cholesterol is excreted in form of bile acids, or assembled into lipoprotein particles that are secreted back into the circulation. Lipid free ApoA-I particles are primarily eliminated and catabolized in the kidneys (50,188,231,232).

HDL actively participates in lipid metabolic pathways, including RCT and RRT as shown in **Figure 1.12**, to maintain lipid balance and prevent excess cholesterol accumulation in peripheral tissues, by facilitating its transport to the liver for excretion. In RRT, HDL acquires FC from surface TGRL remnants, produced through LPL-mediated TGRL lipolysis, while in RCT, HDL takes up FC effluxed from peripheral tissues. In both RRT and RCT, after accepting FC, HDL undergoes LCAT-mediated FC esterification, resulting in gradual enlargement of HDL, and ultimately, hepatic uptake of cholesterol from HDL for excretion (226,247). Throughout these processes, HDL particles continuously remodel as they interact with peripheral cells, cell receptors, lipoproteins and enzymes. Such interactions lead to the formation of a heterogeneous group of small discoid and spherical HDL particles that differ in size, density, and composition (i.e. proteins and lipids) (173,231). The next section discusses in details the structural and compositional heterogeneity of HDL particles.

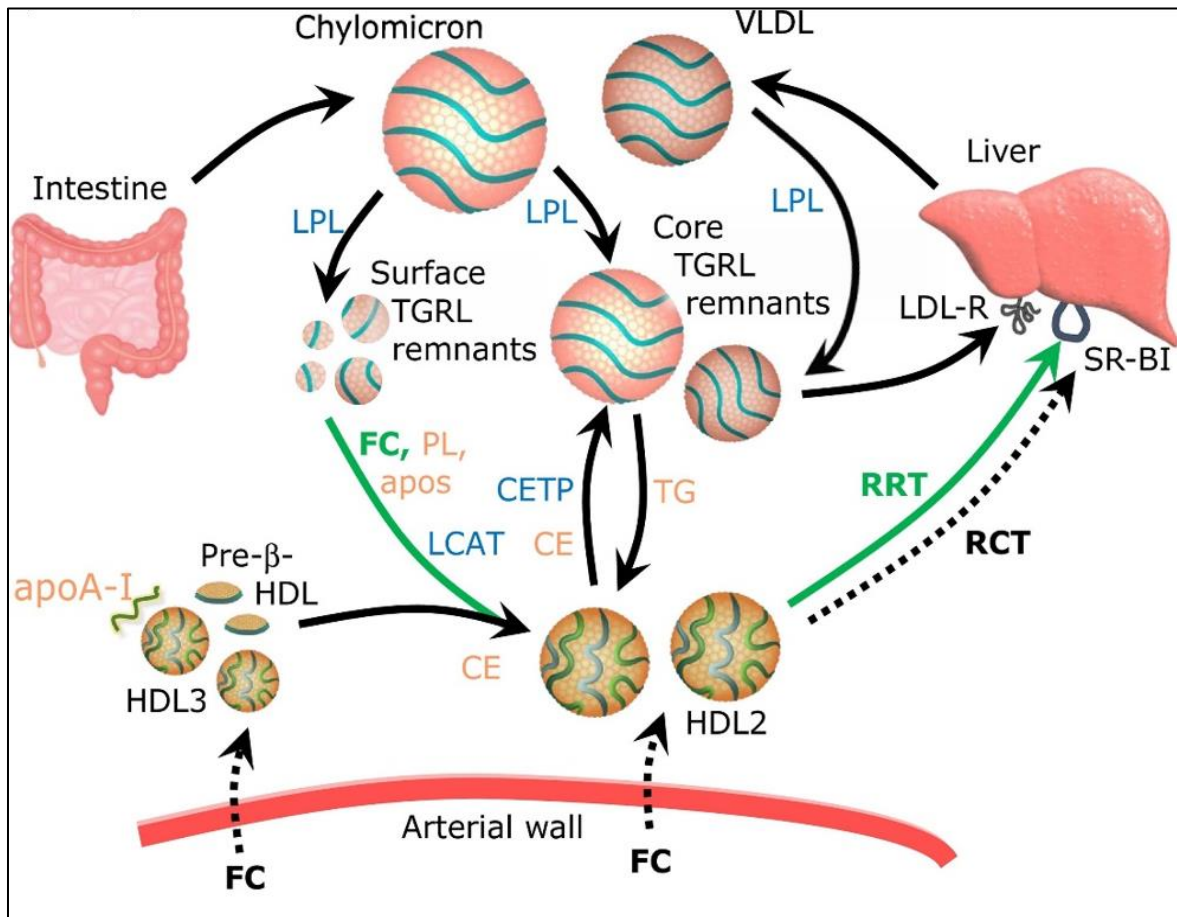


Figure 1.12: RRT and RCT pathways. Following triglyceride (TG) lipolysis by lipoprotein lipase (LPL), triglyceride-rich lipoproteins (TGRL) are converted into core and surface remnants. The core remnants are effectively cleared by hepatic low-density lipoprotein (LDL) receptors (LDL-R). Surface remnants contain free cholesterol (FC), phospholipid (PL), and apolipoproteins (apos). These components combine with the plasma high-density lipoprotein (HDL) pool, leading to the esterification of FC by LCAT and the subsequent enlargement of HDL particles. Both bound FC and cholesteryl esters (CE) are transported to the liver directly via SR-BI (in green) or indirectly via apoB-containing lipoproteins. The traditional reverse cholesterol transport (RCT) pathway from the arterial wall is shown as broken lines. Lipoprotein components are in orange, and enzymes and lipid transfer proteins are in blue. CETP: cholesteryl ester transfer protein; LCAT: lecithin: cholesterol acyltransferase; LPL: lipoprotein lipase; SR-BI: scavenger receptor BI; TG: triglycerides. [Adapted from Kontush A. 2020 (226)].

1.2.4. HDL structural and compositional heterogeneity

Plasma HDL comprises a diverse group of protein rich, small, dense particles, with an average size of 7-12nm and density of 1.063-1.21 g/ml (248). HDL particles are complex structures, primarily composed of polar lipids complexed with apolipoproteins, forming plurimolecular, quasi-spherical or discoid pseudomicellar complexes. Due to high diversity in their protein and lipid composition, HDL particles exhibit significant heterogeneity in both their structural, and functional properties (248,249).

1.2.4.1. Structural heterogeneity

HDL particles are characterized by the elevated degree of heterogeneity in their physicochemical, and biological properties (250–253). This heterogeneity is primarily due to ApoA-I's dynamic structure and unique flexible phospholipid-scavenging capacity, which allow ApoA-I to adapt distinct conformations based on the amount of bound lipids, and particle size (254,255). ApoA-I-phospholipid interactions drive specific motions of ApoA-I molecules such as sliding around their axis, rotation of the protein chains, and out of plane deformation around proline residues. These rules control the formation of HDL particles with different size and conformation according to their phospholipids content (248,256). In addition, the capacity to HDL to carry various proteins, allow the formation of specific protein-protein complexes, contributing markedly to their heterogeneity (257).

The most characteristic structural heterogeneity between HDL particles is in their physical properties. Based on shape, HDL particles are typically discoidal or spherical (**Figure 1.13A**), with discoidal HDL being small (≤ 8 nm) and lipid-poor (minor amounts of phospholipids and FC), while spherical HDL being larger (> 8 nm) and have a hydrophobic core of CE and TG (11,248). Based on density, HDL can be fractionated by sequential flotation ultracentrifugation into large, light, lipid-rich HDL₂ (1.063-1.125 g/ml) and small, dense, protein-rich HDL₃ (1.125-1.21 g/ml) (258,259). Using density gradient ultracentrifugation, HDL₂ and HDL₃ are further subdivided into five subpopulations of decreasing size: large, light HDL_{2b} (9.7-12.9nm), and HDL_{2a} (8.8-9.7nm) and small, dense HDL_{3a} (8.2-8.8nm), HDL_{3b} (7.8-8.2nm), and HDL_{3c} (7.2-7.8nm) (**Figure 1.13B**) (249,260). Another electrophoretic approach involves two-dimensional electrophoresis which separate based on the particle charge and size (**Figure 1.13C**). HDL particles are first separated in agarose gel by charge into pre-beta (β), alpha (α), and pre- α particles, which are then separated in polyacrylamide gel by size into very

small discoidal pre- β 1 HDL (~5.6 nm), very small discoid α 4 HDL (~7.4 nm), small spherical α 3 HDL (~8 nm), medium spherical α 2 HDL (~9.2 nm), large spherical α 1 HDL (~10 nm). Minor HDL subpopulations observed by two-dimensional electrophoresis include pre- α HDL particles (pre- α 1, pre- α 2, pre- α 3), large pre- β 2 HDL, and very large pre- β 3 HDL (261). Using selected-affinity immunosorption in series, with antibodies against apo A-I and apo A-II, HDL can be also classified into LpA-I which contains only ApoA-I, and LpA-I: A-II which contains both ApoA-I, and ApoA-II (**Figure 1.13D**) (173).

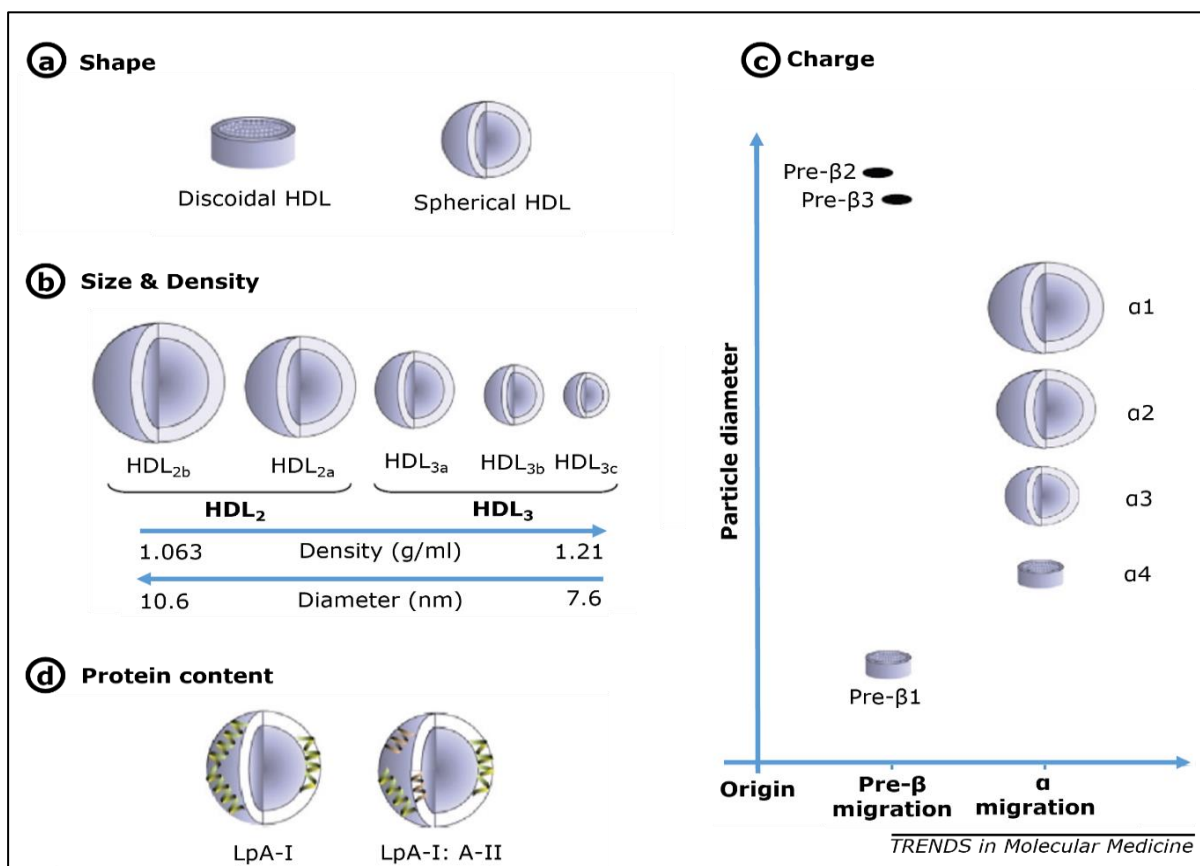


Figure 1.13: Heterogeneity of HDL particles. [Adapted from Schaefer EJ et al. 2010 (262) and Rye KA et al. 2009 (263)].

1.2.4.2. Compositional heterogeneity

HDL particles comprise two major constituents: proteins and lipids. Such components vary in their relative abundance among different HDL subfractions, contributing significantly to the structural and functional characteristics of HDL particles.

Proteins composition and heterogeneity in HDL

Starting with HDL proteome, the heterogeneity of HDL-associated proteins was first established in 1968-1969 (264–266). Besides ApoA-I and ApoA-II, current proteomic studies have identified 285 individual proteins in HDL particles (204), which can be classified into six subgroups, including apolipoproteins, enzymes, acute-phase proteins, complement regulation components, proteinase inhibitors, and minor proteins such as minerals and hormones-binding proteins (249). This diverse proteome enables HDL particles to play multiple roles in lipoprotein metabolism, host defense, blood coagulation, inflammation, and mineral/vitamin transport (267). Of note some of the proteins associated to HDL are glycosylated (248,268). Major proteins in HDL and their main functions are listed in **Table 1.5**. Furthermore, it has been shown that micro-RNAs, which contribute to their biological activities, are also transported by HDLs (248,268). Since they constitute about 90-95% of total HDL proteins, ApoA-I and A-II will be discussed in detailed.

ApoA-I is an essential protein component of HDL particles accounting for about 70% of total HDL proteins and is found in all HDL particles (269). About 80% of plasma ApoA-I are synthesized and secreted by the liver while the remaining 20% are produced by the intestines (248,270). Mature ApoA-I is a single polypeptide composed of 243 amino acids (AA) (MW: 28 kDa) comprising eleven amphipathic α -helical domains accounting for 90% of its structure (**Figure 1.14A and B**), with no glycosylation and disulfide linkages and is encoded by ApoA-I gene located on the 11th chromosome (271,272). The elevated amphipacity of ApoA-I facilitates HDL biogenesis, and underlies its capacity to form stable micellar structures with lipids aiding in their solubilization, and to move between HDL and other lipoproteins such as CM and VLDL (249). The main roles of ApoA-I in HDL include acting as a structural scaffold for the activation of LCAT enzyme, interaction with a range of cellular receptors, and endowing HDL with several atheroprotective functions (refer to **Figure 1.5**) (270,273).

ApoA-II (MW: 17kDa) is the second major apolipoprotein of HDL accounting for about 15-20% of total HDL proteins, and is found in about 50% of HDL particles (274). ApoA-II is primarily synthesized in the liver, but it has also been detected in the intestines (275). Mature, circulating ApoA-II is composed of two identical polypeptide chains (**Figure 1.14C**), each contains 77 amino acids and bound together by a disulfide bridge (276) and is encoded by ApoA-II gene located on the 1st chromosome (277–279). As compared to ApoA-I biophysical properties, ApoA-II protein is less amphipathic, and considered more hydrophobic (275). ApoA-II contributes to HDL maturation into large spherical particles, which increase the quantity of HDL-C “good cholesterol”, but not necessarily the capacity of HDL to promote cholesterol efflux in the RCT pathway as ApoA-I (280).

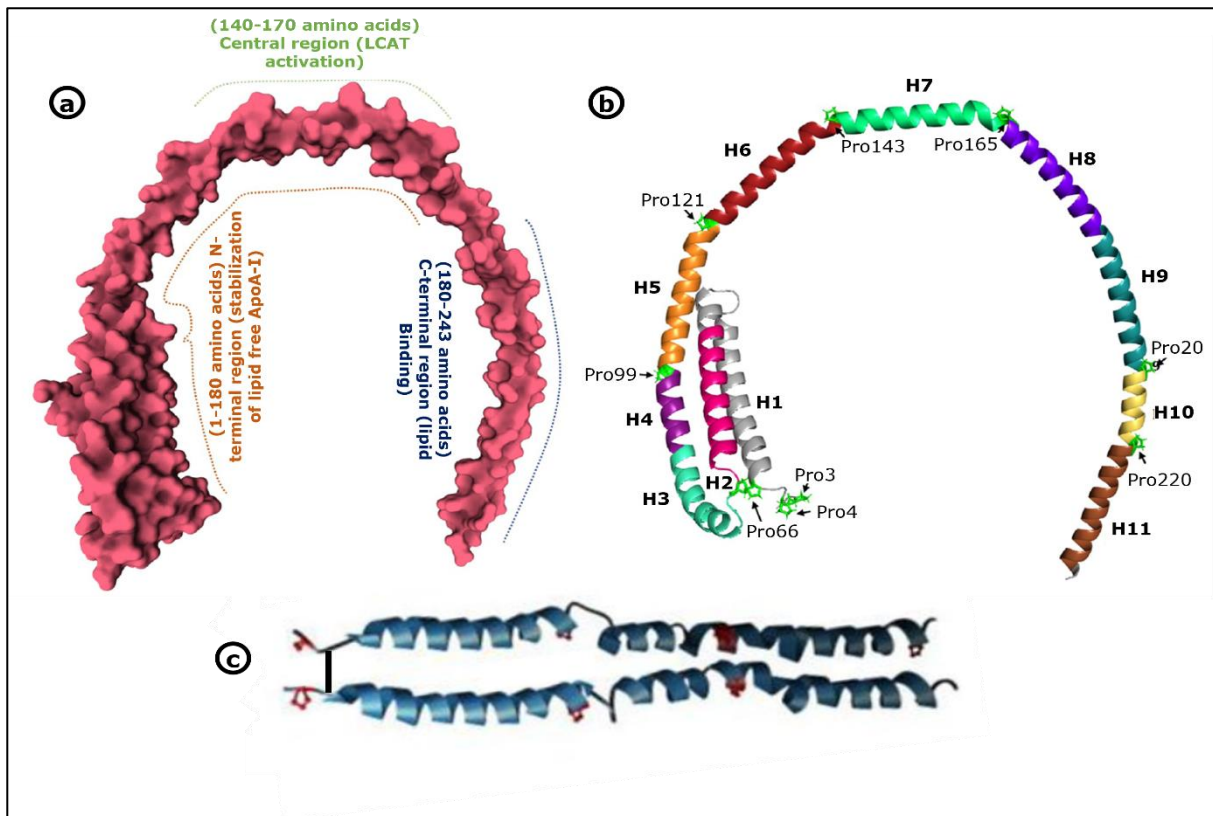


Figure 1.14: Model structures of ApoA-I and ApoA-II. A) Structural regions of ApoA-I protein and their major functions. B) Eleven α -helices (each shown in different color) make up ApoA-I molecule with proline residues at the junctions between the helices result in the curved appearance of ApoA-I molecule. C) Two polypeptide chains linked by disulfide linkage, make up each ApoA-II molecule. [Adapted from Bhale et al. 2022 (270)]

Table 1.5: The major proteins of HDL and their main functions. GSPx-3: Glutathione Selenium Peroxidase-3; Hrp: handle region peptide; LDL-R: LDL-receptor; LRP: LDL receptor-related protein. PAF-AH: platelet-activating factor-acetylhydrolase; PLTP: Phospholipid Transfer Protein; [Adapted from (248,249)].

Protein	Major function
Apolipoproteins (Apo)	
ApoA-I (28 kDa)	Major structural and functional apolipoprotein, lecithin-cholesterol acyltransferase (LCAT) activator
ApoA-II (17 kDa)	structural and functional apolipoprotein
ApoA-IV (46 kDa)	Structural and functional apolipoprotein, most hydrophilic apolipoprotein
ApoA-V (39 kDa)	Activator of lipoprotein lipase (LPL), inhibitor of hepatic production and secretion of triglyceride (TG)
ApoC-I (6.6 kDa)	Inhibitor of Cholesteryl ester transfer protein (CETP), LCAT activator
ApoC-II (8.8 kDa)	Activator of LPL
ApoC-III (8.8 kDa)	Inhibitor of LPL, decreases the hepatic uptake of Chylomicrons
ApoC-IV (11 kDa)	Regulates TG metabolism
ApoD (19 kDa)	Binding of small hydrophobic molecules, high affinity for arachidonic acid
ApoE (34 kDa)	structural and functional apolipoprotein similar to ApoA-I and ApoA-II, LDL-R and LRP ligand
ApoF (29 kDa)	Inhibitor of CETP
ApoH (38 kDa)	Binding of negatively charged molecules such as heparin and dextran sulfate, prevent intrinsic coagulation cascade
ApoJ (70 kDa)	Binding of hydrophobic molecules, interaction with cell receptors
ApoL-I (44-46 kDa)	Trypanolytic factor of human serum, lipid binding protein with high affinity to Phosphatidic acid and Cardiolipin
ApoM (25 kDa)	Binding of small hydrophobic molecules
Enzymes	
LCAT	Esterification of cholesterol to cholesteryl esters
Paraoxonase 1	Calcium-dependent lactonase
PAF-AH (LpPLA ₂)	Hydrolysis of short-chain phospholipids
GSPx-3	Reduction of hydroperoxides by glutathione

PLTP	Conversion of HDL into larger and smaller particles, transport of lipopolysaccharide
CETP	Heteroexchange of cholesteryl ester (CE) and TG, and homoexchange of phospholipids (PL) between HDL and ApoB-lipoproteins
Acute-phase proteins	
Serum amyloid A1	Acute-phase reactant
Serum amyloid A4	Acute-phase reactant
Alpha-2-HS-glycoprotein	Negative acute-phase reactant
Fibrinogen alpha chain	Precursor of fibrin, cofactor in platelet aggregation
Complement components	
Complement 3	Complement activation
Proteinase inhibitors	
Alpha-1-antitrypsin	Inhibitor of serine proteinases
Hrp	Decoy substrate to prevent proteolysis
Other proteins	
Transthyretin	Thyroid hormone binding and transport
Serotransferrin	Iron binding and transport
Vitamin-D-binding protein	Vitamin D binding and transport
Hemopexin	Heme binding and transport

The dynamic exchange of the HDL's molecular components as they travel in the circulation results in significant compositional diversity among HDL subpopulations (11,248). In the context of HDL proteins, all HDL subpopulations contain ApoA-I (259,281), with LpA-I and LpA-I: A-II contain approximately 35% and 65% of plasma ApoA-I, respectively (282). On the other hand, about half of HDL subpopulations contain ApoA-II, which all presents in LpA-I: A-II particles (283). The number of ApoA-I molecules in LpA-I particles increases from two to four with increasing the particle size (284). On the other hand, all the LpA-I: A-II particles contain two ApoA-I molecules, while the number of ApoA-II molecules increases from one to three with increasing the particle size (248). The distribution patterns of ApoA-I and ApoA-II across five HDL subpopulations are shown in **Table 1.7**. HDL subpopulations may also vary in the content of other proteins. For instance, ApoJ, ApoL-I, ApoF, PON1/3, PLTP and PAF-AH predominate in small, dense HDL_{3C} particles (285). In line with these data, ApoJ, ApoL-I, ApoF, PON1, CETP, Apo-VI, ApoM, ApoD, and SAA1/2 preferentially co-isolate with small-dense HDL₃ particles (248). In contrast, ApoE, ApoC-I, ApoC-II, and ApoC-III preferentially occur in large HDL₂ particles (240,257,286,287).

Lipid composition and heterogeneity in HDL

Lipids are essential components of HDL particles, constituting about 35-65% of their total mass (11,281). Major classes of HDL's lipids include free cholesterol, CE, phospholipids, and TG (refer to **Figure 1.9**). Among these classes, phospholipids predominate in terms of quantity, along with SM, accounting for about 40-60 wt% of total HDL lipid content (11). Phospholipids form the surface lipid monolayer of HDL, whereas TG and CE form the hydrophobic lipid core, with unesterified cholesterol found mostly in the surface monolayer, and partially penetrating the core (11,248). Recent advances in mass spectrometry (MS) techniques have allowed lipidomic studies to unravel the complexity of HDL lipidome through the identification and quantification of more than 200 individual molecular species of lipids within HDL particles (11,208,288). Consequently, a novel perspective in research has emerged, focusing on exploring the impact of individual lipid species rather than major lipid classes on HDL functionality. In line with the subject of this work, HDL lipids will be explored in details.

Glycerophospholipids (GPL) constitute the main lipid class of HDL, accounting for about 37.4-49.3% of total HDL lipids (refer to **Table 1.6**). Phospholipids build up the surface lipid monolayer of HDL, ensuring specific HDL structural conformation and regulating its fluidity (248). Major GPL subclasses in HDL include PC, PI, LPC, PE, PC- and PE-derived plasmalogens (refer to **Table 1.4**) (208,289,290). HDL also contain minor amounts of PS, PG, phosphatidic acid and cardiolipin (288,291,292). PC is the key structural phospholipid of cell membranes and lipoproteins (11). PC accounts for about 32-35 % mol of total HDL lipids, and about 70% of total HDL phospholipids (208,248). Among PC species in HDL, PC (16:0/18:2), (18:0/18:2), (16:0/20:4), and (16:0/18:1) account for about 63 mol % of total PC in HDL (208,249,289). As compared to other lipoproteins, HDL is enriched in PC containing polyunsaturated fatty acids (PUFA) moieties in their lipid tails (208). LPC is an important phospholipid in HDL, constituting about 1.4-8.1 mol % of total HDL lipids, and is derived from the hydrolysis of sn-2 acyl chain of PC by LCAT (289). Major molecular species of LPC in HDL are LPC 16:0 and LPC 18:0, representing about 75 mol % of total LPC in HDL (208,249). PC-plasmalogens accounts for about 2.2-3.5 mol % of total HDL lipids, about 43% of which being PC (38:5), (36:2) and (36:4) species (293).

PE which account for 0.7-0.9 mol % of total HDL lipids is a moderately abundant phospholipid in HDL. About 47 mol % of total PE in HDL are PE (34:2), PE (36:2), and PE (38:4) species (208). PE-plasmalogens also account for 0.6-0.9 mol % of total HDL lipids and

noticeably their main species include those that contain arachidonic acid (20:4) such as (18:0/20:4) and (16:0/20:4) species, which account for about 35 mol % of total PE-plasmalogens in HDL (208). Similar to PE, PI is also moderately abundant in HDL (0.5-0.8 mol % of total HDL lipids) with 55 % represented by PI (18:0/20:3), and PI (18:0/20:4) species (292). Minor phospholipids in HDL particles include cardiolipin (0.08-0.2 mol %), PS (0.016-0.03 mol %), PG (0.004-0.006 mol %), and phosphatidic acid (0.006-0.009 mol %) (249,260).

Unesterified sterols account for 8.7-13.5 mol % of total HDL lipid, primarily residing in the surface lipid monolayer of HDL, where they partly penetrate the lipid core. This positioning highlights their crucial function in regulating the surface fluidity of HDL. FC is the predominant sterol in HDL, indicating the key role of HDL in cholesterol transport (248). Additionally, HDL contains minor amounts of other sterols, such as oxysterols, estrogens, and phytosterols (294,295).

Neutral lipids including CE, and TG, account for 46.7-54.0 mol % of total HDL lipids (refer to **Table 1.6**). CE are highly hydrophobic lipids that contribute to 35-37 mol % of total HDL lipids. Up to 80% of CE content of HDL are formed in plasma HDL as a result of the trans-esterification between phospholipids and cholesterol catalyzed by LCAT. Most of the CE in HDL is present in the form of cholesteryl linoleate (C18:2) (11). TG accounts for 2.8-3.2 mol % of total HDL lipids with species containing oleic (18:1), palmitic (16:0), and linoleic (18:2) acid moieties represent the majority of TG in HDL (208). Similar to CE, TG are hydrophobic and thus are located in the lipid core of HDL.

The major sphingolipids found in HDL include SM, ceramides, and sphingosine-1-phosphate (S1P) (refer to **Table 1.6**). SM (5.6-6.6 mol % of total HDL lipids) is a structural lipid known to increase HDL surface rigidity (296,297). SM content of HDL largely originates from TGRL, with (d18:1/16:0) and (d18:2/24:0) species forming about 42 mol % of total SM in HDL (208,249). Ceramide is poorly transported by HDL constituting for about 0.022-0.097 mol % total HDL lipids (249). Major molecular species, representing about 65 mol % of total ceramides in HDL, include ceramide (16:0), (24:0) and (24:1) species (208,293). S1P is a phosphorylated sphingoid base and is most known for its role in vascular biology. Although HDL is the major carrier of S1P in plasma (11), the abundance of S1P in HDL is low (~0.05 mol % total HDL lipids) with a majority of S1P (d18:1) and (d18:0) species (249,257). Other lysosphingolipids carried by HDL include lysosphingomyelin and lysosulfatide (249). HDL also contains minor amounts of diacylglycerides, monoacylglycerides, free fatty acids, oxidized

lipids, lipid-soluble vitamins, and antioxidants such as tocopherols (vitamin E), carotenes, and coenzyme Q10 (11).

HDL subpopulations are heterogeneous in their lipid composition (refer to **Table 1.7**). So, taking the example of the content of phospholipids, as percentage of total HDL mass, decreases about 2-fold from HDL_{2b} to HDL_{3c} particles (289). The same tendency is observed with the content of major phospholipid subclasses including PC, PE, PI, LPC, and total fatty acids between HDL_{2b} and HDL_{3c} particles. Similarly, the SM content of HDL decreases gradually from HDL_{2b} to HDL_{3c} in a more pronounced manner than phospholipids, in parallel with a progressive elevation in the PC/SM ratio (289). Lipidomic differences between HDL subpopulations extend also to minor sphingolipids such as S1P, which predominates in HDL₃ compared to HDL₂ (289,298,299). The content of free cholesterol, CE, and TG progressively decrease with increase in HDL density (11).

In conclusion, proteomic and lipidomic studies have revealed the complexity of HDL composition and its potential to provide valuable insights into HDL functionality (11). Hence, HDL's compositional heterogeneity represents a major challenge in the field of CVD, with HDL phospholipidome attracting special attention. Notably, our lab has found strong correlations between multiple phospholipid components of HDL and its atheroprotective functions (10), hinting at the potential for new molecular markers of HDL functionality (i.e. quality) in the context of ASCVD. The next section will explore HDL functionality, its heterogeneity, and the functional relevance of HDL components.

Table 1.6: Major lipid classes of HDL lipidome and their major molecular species.

[Adapted from (11,208,249,281,288,289,291,292,294,300)].

Lipid class	mol % of total HDL lipids	Major subspecies based on their mol % in relative to total content of their lipid class
Glycerophospholipids	37.4-49.3	
Phosphatidylcholine (PC)	32-25	16:0/18:2, 18:0/18:2, 16:0/20:4, 16:0/18:1
PC-plasmalogens	2.2-3.5	18:1/20:4, 18:0/18:2
Lysophosphatidylcholine	1.4-8.1	16:0, 18:0, 18:1, 18:2
Phosphatidylethanolamine	0.7-0.9	18:2/16:0, 18:2/18:0, 18:0/20:4
PE-plasmalogens	0.6-0.9	18:0/20:4, 16:0/20:4
Phosphatidylinositol	0.5-0.8	18:0/20:3, 18:0/20:4
Cardiolipin	0.07-0.2	
Phosphatidylserine	0.016-0.03	16:0/18:0
Phosphatidylglycerol	0.004-0.006	18:1/20:2
Phosphatidic acid	0.006-0.009	20:4/20:0, 20:4/20:2, 18:2/20:1
Neutral lipids	46.7-54.0	
Cholesteryl ester	35-37	Cholesteryl linoleate
Unesterified Sterols	8.7-13.5	Cholesterol
Triacylglycerides	2.8-3.2	18:1/16:0/18:1, 18:2/16:0/18:1
Diacylglycerides	0.17-0.28	16:0/18:1, 18:1 /18:1
Sphingolipids	5.7-6.9	
Sphingomyelin	5.6-6.6	18:1/16:0, 18:2/24:0, 18:1/24:1, 18:2/22:0, 18:1/22:1
Ceramide	0.022-0.097	24:0, 24:1, 23:0, 22:0, 16:0
glycosphingolipids	0.112-0.183	Hexosyl Ceramide, Lactosyl Ceramide
Sphingosine-1-phosphate	0.015-0.046	d18:1
Sphingosine-1-phosphate	0.007	d18:0
Sphingosylphosphorylcholine	0.001	d18:1
Minor lipids		
Free fatty acids	Not determined	16:0, 18:1, 18:1
Isoprostanes-containing Phosphatidylcholines	Very low, nanomolar	36:3, 36:4, 38:4

Table 1.7: Compositional heterogeneity of HDL subfractions. (175, 281, 289,301).

Composition (mol/mol HDL)	HDL2b	HDL2a	HDL3a	HDL3b	HDL3c
Major proteins					
ApoA-I	4-5	4	3-4	3	2-3
ApoA-II	0.8	1.0	1.4	1.0	0.4
Phospholipids	130	140	120	45	25
Phosphatidylcholine	85	100	85	30	20
Phosphatidylinositol	10	10	10	5	2
Phosphatidylethanolamine	2	2	2	1.0	0.5
Lysophosphatidylcholine	1	1	1	0.5	0.5
Sphingomyelin	30	25	20	8	3
Free cholesterol	70	40	25	15	8
Cholesteryl ester	180	160	140	70	40
Triglycerides	30	20	15	10	5

1.3 HDL atheroprotective functions

Currently, it is accepted that HDL functionality (quality) is more important in defining the net atheroprotective capacity of HDL than HDL-C levels (quantity). HDL particles exert multiple atheroprotective activities including their capacity to promote cellular cholesterol efflux, together with anti-inflammatory, antioxidative, cytoprotective, vasodilatory, anti-infectious, and anti-thrombotic activities (illustrated in **Figure 1.15**) (248). Interestingly, our laboratory has revealed recently a new atheroprotective function of HDL, namely, the capacity of HDL to acquire free cholesterol upon TGRL lipolysis (242). Such complex pattern of the biological activities of HDL particles is directly related to their composition and structure (10,11,173). In this section, the most relevant functions of HDL to the pathophysiology of ASCVD will be discussed in details, and the relevance of HDL components to these functions.

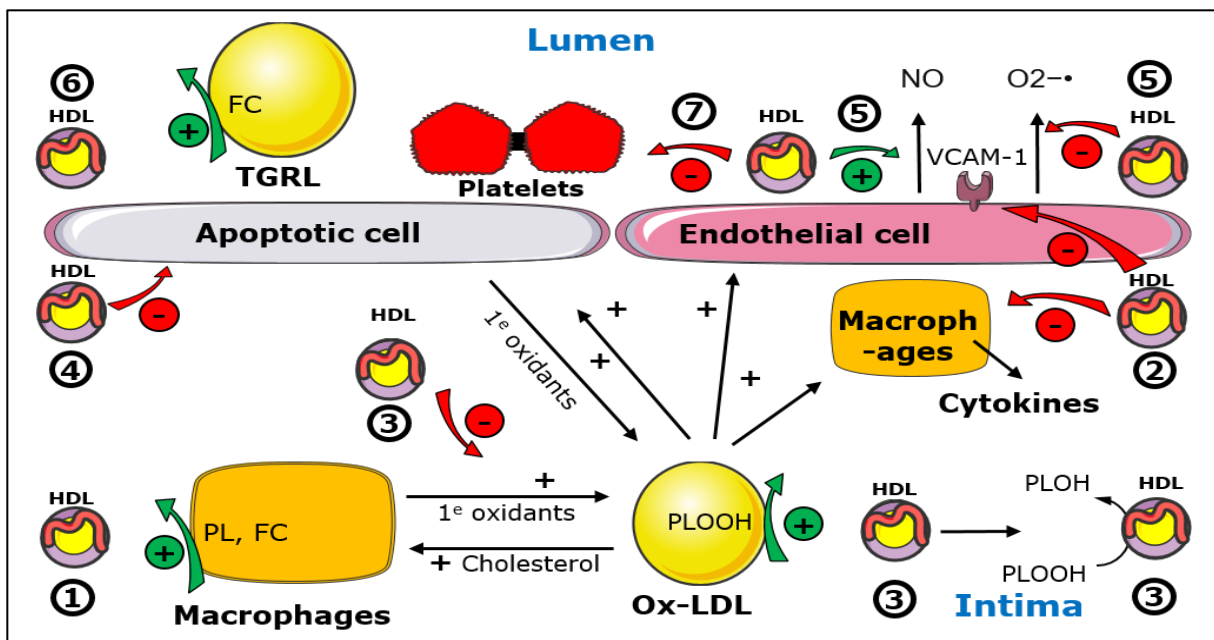


Figure 1.15: HDL functions. Schematic illustration shows the major HDL biological functions including 1) **Cholesterol and phospholipid efflux capacity** from macrophages. 2) **Anti-inflammatory activity** characterized mainly by the inhibition of ox-LDL induced VCAM-1 expression in endothelial cells and cytokine release from macrophages. 3) **Anti-oxidative activity** characterized by the Inhibition of LDL oxidation by one- or two-electron oxidants, as well as the removal and subsequent inactivation of oxidized lipids (PLOOH) from oxidized LDL. 4) **Cytoprotective activity** characterized by the inhibition of oxidized LDL induced endothelial apoptosis. 5) **Vasodilatory activity** characterized by inducing the production of NO and inhibiting the release of superoxide from endothelial cells. 6) **Anti-infectious activity** characterized by binding to LPS. 7) **Anti-thrombotic activity** characterized by inhibiting of platelet aggregation. 1e oxidants, one-electron oxidants; FC, free cholesterol; PL, phospholipid; PLOOH, phospholipid hydroperoxide; PLOH, phospholipid hydroxides; VCAM-1, vascular cell adhesion molecule-1; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NO, nitric oxide; O₂^{-·}, superoxide; LPS, lipopolysaccharide. [Adapted from Kontush and Chapman. 2012 (248)].

1.3.1 Cholesterol Efflux Capacity

The capacity of HDL to transport cholesterol from arterial wall macrophages to the liver for excretion through RCT pathway has been considered the classical atheroprotective function of HDL (250,302). Indeed, clinical studies consistently show that the cholesterol efflux capacity (CEC) of HDL is a stronger predictor of CVD than HDL-C levels (303). HDL mediates the critical initial step in RCT by promoting cholesterol efflux from lipid-loaded macrophages which represents the very early step in atherosclerosis process (8). The CEC of HDL is the most well-known and best studied biological activity of HDL (304).

1.3.1.1 CEC-Mechanisms

The process of cellular cholesterol efflux to HDL is mediated via several mechanisms including unidirectional ATP-dependent cholesterol efflux mediated by ABCA1 and ABCG1 transporters; bidirectional ATP-independent pathway mediated by SR-BI; and receptor-independent passive diffusion through cell membrane (233,304–307). The rate limiting step of this process is the cholesterol desorption from cell membrane, then subsequent collision and incorporation into HDL (213). ABCA1 mediates cholesterol efflux predominantly to lipid-free ApoA-I or lipid-poor pre-beta discoidal HDL (231,308,309), and accounts for about 80% of efflux stimulated by cellular cholesterol loading (310) and regulates more than 50% of total macrophage cholesterol efflux (311). The gene expression of ABCA1 is mainly regulated by nuclear liver X receptors (LXR) - alpha and beta and by retinoid X receptor (RXR) (312). The activity of LXRs is up-regulated by both cellular cholesterol overload and PPAR γ nuclear receptor (231,313). Cellular cholesterol overload increases the production of oxysterols, which activate LXRs to induce cholesterol efflux by increasing the ABCA1 transcription (231). PPAR γ promotes cholesterol efflux by up-regulating LXRs, and it is now well documented that PPAR γ -LXR α -ABCA1 pathway is critical in regulating cellular cholesterol efflux (314,315). ABCG1 is another member of ABC transporters, which was found to promote cholesterol efflux from macrophages to mature spherical HDL₂ and HDL₃ particles (316–318). However, some studies demonstrated that LXR-stimulated cholesterol efflux to HDL from cholesterol-loaded human macrophages involves ABCA1 but not ABCG1 (319). SR-BI mediates bidirectional cellular cholesterol flux between plasma membranes and mature HDL under non-stimulated basal conditions (305). Finally, passive diffusion of cholesterol from plasma membranes to HDL is involved in effluxing cholesterol under non-stimulated basal conditions. However, the

role of SR-BI and passive diffusion is minor upon cholesterol loading of cells as they can't be up-regulated by cellular cholesterol overload (304,310).

1.3.1.2 CEC-functional relevance of HDL proteome

As they predominate on the surface, proteins provide a major contribution to the CEC of HDL particles by mediating their interactions with cellular receptors (320). Among HDL proteins, ApoA-I plays a predominant role in lipid efflux (321). As a result of its unique structure and elevated amphipacity (322), ApoA-I solubilizes and transports cholesterol and phospholipids by adopting a conformation with high affinity for cell membrane receptors in small, cholesterol-poor HDL, which facilitate cellular cholesterol efflux (323). Subsequently, ApoA-I undergoes a conformational change due to cholesterol enrichment, decreasing its affinity for cell membrane receptors, and allowing the dissociation of HDL particles. Other apolipoproteins such as ApoA-II, ApoA-IV, ApoC-I, ApoE, and ApoM are also considered as efficient acceptors of cellular cholesterol (233,324–326). HDL enzymes are also of major relevance for cholesterol efflux and RCT. PON1 is implicated in the CEC of HDL by enhancing the interaction with macrophages through ABCA1 transporter (327) while LCAT also plays a key role in the net transfer of cholesterol from periphery to the liver (231).

1.3.1.3 CEC-functional relevance of HDL lipidome

HDL lipids play an equally crucial role in facilitating HDL-mediated cellular cholesterol efflux, by providing an environment for the acquisition of exogenous lipids, mainly cholesterol. Thus, the properties of HDL surface lipids have a significant influence on the efficiency of lipid efflux to HDL. Among these lipids, phospholipids provide a major contribution to the CEC of HDL. In terms of quantity, increasing phospholipid content of HDL in term of wt%, is associated with a dose-dependent increase in the CEC of HDL via SR-BI (328). Qualitatively, the physical state of phospholipids represents a major structural determinant of the CEC of HDL, with HDL particles enriched by fluid-phase unsaturated phospholipids being more efficient cholesterol acceptors as compared to particles with gel-phase saturated phospholipids (213). This difference is due to the more fluid nature of unsaturated phospholipids, which facilitates the incorporation of exogenous cholesterol into HDL (11). Furthermore, compositional changes during HDL remodeling such as reduced chain length and/or increased level of unsaturation in the surface phospholipids increase the structural flexibility of HDL, a prerequisite for cholesterol efflux (329,330). Conversely, high SM content in HDL reduces both

HDL fluidity of the surrounding liquid-phase lipids and cellular cholesterol efflux (331). Nonetheless, the high affinity of SM for FC can reduce the cholesterol influx from HDL to cells (328), thereby counteracting the impact of reduced HDL fluidity by SM on cellular cholesterol efflux (332).

In the context of HDL's role in preventing cholesterol accumulation within the arterial wall, our laboratory recently demonstrated a new function for HDL that complements its CEC in mediating atheroprotection. We have shown that HDL can acquire FC from TGRL after their lipolysis by LPL *in vitro* (242), which is then transported to the liver, either directly via SR-BI or through apoB-containing lipoproteins and their hepatic receptors (244,245). According to the reverse remnant-cholesterol transport (RRT) hypothesis, proposed by Dr. Anatol Kontush, the removal of cholesterol from TGRL remnants represents a major function of HDL that can reduce cholesterol influx into the arterial wall macrophages by accelerating the clearance of atherogenic TGRL remnants from the circulation (226), which would otherwise accumulate in the arterial wall and promote atherosclerosis (241). Although the relevance of HDL components in this function is still being explored, the number of acceptor HDL particles, and the activities of CETP and LCAT enzymes, along with the content of FC within HDL particles, appear to influence their ability to acquire free cholesterol from TGRL (242,333,334).

1.3.2 Antioxidative activity

The antioxidative activity (AOX) of HDL is mainly expressed as the capacity to inhibit LDL oxidation (335), which represents a key factor in the early development of atherosclerosis (35,37,38). However, HDL protection against oxidation extends to inhibit intracellular oxidative stress in both endothelial cells and macrophages (336–340). The antioxidative activity of HDL is particularly relevant in metabolic conditions that are marked with chronic oxidative stress such as obesity, T2D, and MetS.

1.3.2.1 AOX-Mechanisms

HDL particles potently protect LDL from oxidation induced by one-electron oxidants (free radicals) and two-electron oxidants (hypochlorite). HDL-mediated protection of LDL against free radicals induced oxidation includes the removal of oxidized lipids such phospholipid hydroperoxides (PLOOHs), and CE hydroperoxides (CEOOHs) from LDL, followed by their subsequent inactivation within HDL particles (285). The transfer of oxidized

lipids from LDL to HDL occurs either spontaneously or facilitated by lipid transfer proteins, such as CETP, which accelerates the transfer of both PLOOHs and CEOOHs (341,342). Of note, the capacity of HDL to remove oxidized lipids from LDL is governed by the fluidity of HDL surface monolayer, with less rigid surface efficiently acquires more oxidized lipids (343). Furthermore, multiple studies have indicated that HDL may protect LDL from oxidation in part by serving as a sacrificial target for oxidants (344). This capacity of HDL to scavenge oxidants is influenced by the degree of unsaturation in its phospholipids, with high level of unsaturation offering more sites prone to oxidation (344,345). Importantly, HDL has also been shown to protect macrophages from ox-LDL-induced apoptosis by facilitating the efflux of oxidized sterols, such as 7-ketocholesterol, through ABCG1 (346). Furthermore, HDL reduces cellular oxidative stress by inhibiting the production of reactive oxygen species (ROS), through the interaction with SR-BI in endothelial cells, and both SR-BI and ABCG1 in macrophages (299,340). Following their transfer to HDL, oxidized lipids are inactivated based on their structure via either reduction mainly by the redox-active methionine residues of ApoA-I, or hydrolysis by HDL-hydrolytic enzymes (304).

1.3.2.2 AOX-Functional relevance of HDL proteome

ApoA-I is central to the AOX of HDL, as its methionine (Met) residues 112 and 148 inactivate lipid hydroperoxides (LOOHs) by reducing them into redox-inactive lipid hydroxides (343,347,348). In addition, Histidine residues of ApoA-I may also inhibit the oxidation of LDL as a result of their metal chelating properties (349). Furthermore, ApoA-I facilitates the removal of oxidized lipids from ox-LDL (350). Other apolipoproteins components of HDL including ApoA-II, ApoA-IV, ApoA-V, ApoE, ApoJ, and ApoM are also known to protect LDL from oxidation *in vitro* (257,351,352). Enzymatic components of HDL including PON1, PAF-AH, and LCAT also contribute to the AOX of HDL by promoting hydrolysis of short-chain oxidized phospholipids (250,251). Finally, trypanosome lytic factor (Apolipoprotein L-I, haptoglobin-related protein, and ApoA-I), which is found exclusively in dense HDL have been suggested to provide minor contribution to their LOOHs-inactivating capacity (353,354).

1.3.2.3 AOX-Functional relevance of HDL lipidome

HDL lipids strongly influence the antioxidative activity of HDL mainly via modulating the physical properties of the HDL surface lipids. Indeed, the efficiency of PLOOH transfer from LDL to HDL is mainly determined by the rigidity of the phospholipid monolayer of HDL

(343). Elevated surface fluidity of reconstituted HDL particles was shown to enhance both their antioxidative activity and the transfer rate of PLOOH from oxidized LDL (343). In this context, the fluidity of the phospholipid monolayer of HDL is inversely related with the relative content of SM and free cholesterol to PC, and the relative content of saturated and monounsaturated fatty acids to polyunsaturated ones (343). In addition, the ratio of CE to TG in HDL lipid core impacts the conformation of ApoA-I, thereby modifying the accessibility of Met residues to LOOHs (235,355). Furthermore, the capacity of HDL to scavenge oxidants is influenced by the degree of unsaturation in its phospholipids, with high level of unsaturation offering more sites prone to oxidation (344,345).

1.3.3 Anti-inflammatory activity

HDL particles exert several anti-inflammatory activities (AIA) that may collectively attenuate the chronic inflammatory process of atherosclerosis (356–359). HDL potently inhibits cytokine-induced adhesion molecule expression on endothelial cells, which thereby inhibits the adhesion of monocytes to endothelial cells (360,361). Furthermore, HDL is known to reduce the proliferation of myeloid cells into monocytes, hematopoietic stem cells proliferation, and neutrophil activation (362–364). Indeed, the anti-inflammatory activity of HDL is among the most well-known biological activities that contributes to HDL-mediated atheroprotection (304).

1.3.3.1 AIA-Mechanisms

The multiple effects of HDL against inflammation suggest several mechanisms that may underlie these effects. Among those, cellular lipid efflux appears to provide a mechanistic basis for the capacity of HDL to reduce the expression of endothelial adhesion molecules including VCAM-I, ICAM-I, and E-selectin, to inhibit monocytes and neutrophils activation (362,365), and to reduce myeloid cell proliferation and monocytes production (366). Indeed, the of cellular cholesterol down-regulates macrophage inflammation through attenuating the inflammatory signaling via toll-like receptors (TLRs) (367). Recently, HDL was demonstrated to exhibit early AIA in macrophages via reducing LPS-induced activation of nuclear factor kappa B (NF- κ B), p38 mitogen-activated protein kinase (p38 MAPK), c-Jun N-terminal kinase (JNK) and extracellular-signal-regulated kinase (ERK) signaling, which reflected a TLR-4 downregulation (368). In this study, HDL also exhibited late AIA via reducing LPS-induced phosphorylation of signal transducer and activator of transcription 1 (STAT1) and type 1 interferon (IRF1) signaling. Both the early and late AIA of HDL were dependent on cellular cholesterol efflux to

HDL (368), with ABCA1 appears to be critical for these effects as it diminishes TLR4 trafficking to lipid rafts by reducing membrane cholesterol content (369). In addition, HDL was shown to mediate its AIA in macrophages via inducing the activating transcription factor 3 (ATF3), which in turn downregulates the expression of TLR-induced proinflammatory cytokines (370). In addition, the removal of cholesterol via ABCA1, ABCG1, and SR-B1 equally contribute to the anti-inflammatory effects of HDL in adipocytes (371). ApoA-I interaction with ABCA1 induces also anti-inflammatory effects independently of cholesterol efflux through the activation of Janus kinase 2 (JAK2) and Signal transducer and activator of transcription 3 (STAT3) genes, which reduce cytokine expression in macrophage cells (372). Furthermore, HDL-induced inhibition of endothelial adhesion molecules expression involve inhibition of ox-LDL and TNF α -induced activation of NF- κ B, with subsequent reduction of inflammatory signaling (338,373). Moreover, the capacity of HDL particles to remove and inactivate oxidized lipids from ox-LDL, and/or cells is closely linked to their anti-inflammatory potential, by preventing the local inflammatory response induced by pro-inflammatory oxidized lipids (374,375). It is worth mentioning that some studies have reported pro-inflammatory effects of HDL in macrophages through inducing both TLR-induced protein kinase C (PKC)-NF- κ B/STAT1-IRF1 Signaling (376), and endoplasmic reticulum stress response, mediated by IRE1a (inositol-requiring enzyme 1a)/ASK1 (apoptosis signal-regulating kinase 1)/p38 MAPK signaling (368). Such pro-inflammatory effects were linked to HDL-mediated passive cellular cholesterol depletion.

1.3.3.2 AIA-Functional relevance of HDL proteome

Within HDL proteome, ApoA-I appears to have a major contribution to the anti-inflammatory activity of HDL. Acting through ABCA1, ApoA-I appears to play a central role in mediating cholesterol efflux from membrane lipid rafts, as well as reducing neutrophils and monocytes activation (362,365,377). In addition, ApoA-I is pivotal for the capacity of HDL to inactivate pro-inflammatory oxidized lipids through its reducing actions (343,347,348). Other protein components of HDL particles can also contribute to their AIA. Hence, HDL-associated PAF-AH was shown to resolve inflammation in atherosclerotic plaques through restoring the emigratory process of monocytes derived dendritic cells (378). In addition, PON1 ensures the capacity of HDL to inactivate pro-inflammatory oxidized lipids through its hydrolytic actions (335,379,380). Furthermore, previous shotgun proteomics has identified several complement-regulatory proteins (e.g. C3, C4A, C4B, and C9), and serpins with endopeptidase inhibitor

activity (e.g. serpin peptidase inhibitor, inter- α -trypsin inhibitor H4), to be implicated in the AIA of HDL (351).

1.3.3.3 AIA-Functional relevance of HDL lipidome

HDL phospholipids appear to be the major lipid components that impact the AIA of HDL. For instance, 1-palmitoyl-2-linoleoyl PC (PLPC) containing reconstituted HDL was shown to inhibit pro-inflammatory signaling via NF- κ B, and may contribute to HDL-mediated inhibition of inflammation in cultured endothelial cells (373,381). The impact of phospholipids on the AIA of HDL is determined by both the phospholipid class (i.e. determined by the head group) as well as the phospholipid species within each class (i.e. determined by the fatty acid composition). In terms of phospholipid class, reconstituted HDL particles containing PC and PS demonstrated an enhanced AIA when interacting with activated macrophages compared to particles composed only of PC (382). Expanding on the phospholipid species within the same class, reconstituted HDL particles with different molecular species of PC exhibit distinct abilities to inhibit the expression of VCAM-I in activated endothelial cells. Notably, particles containing species with unsaturated fatty acids exhibit more potent anti-inflammatory effects (383). These differences may be linked to differences in the phase fluidity among different PC species (384). This is in line with the role of reconstituted HDL particles in facilitating cellular lipid efflux, a process that is significantly impacted by lipid phase fluidity. Noticeably, it has been shown that lysosphingolipid content in HDL particles was shown to contribute to their AIA through reducing the aortic expression of MCP-1 (385).

1.3.4 Other biological properties of HDL

HDL protects cells from apoptosis by several mechanisms including promoting cellular efflux of cytotoxic oxidized sterols via ABCG1, decreasing the production of intracellular free radicals, reducing the activation of proapoptotic caspases, maintaining mitochondrial functional integrity (386), and inhibiting the degradation of vascular extracellular matrix by elastases (387). Furthermore, HDL-mediated protection of endothelial cells from apoptosis involves up-regulating the phosphatidylinositol-3-kinase (PI3K), Protein kinase B (Akt), and B-cell lymphoma-extra-large (Bcl-xL) pathways (388). HDL-associated proteins including ApoA-I, ApoE, ApoJ, alpha-1-antitrypsin as well as HDL-associated lysosphingolipids, including S1P, sphingosylphosphorylcholine, and lysosulfatide play an important role in mediating the HDL cytoprotective actions (388–391).

Furthermore, HDL maintains normal function of vascular endothelium via increasing the production of nitric oxide (NO) and prostacyclin (PGI₂) (392,393). NO and PGI₂ are potent vasodilators regulating the inflammatory response in endothelial cells, and they inhibit both platelet activation and proliferation of vascular smooth muscle cells (250,394). Multiple studies have demonstrated that HDL increases the production of NO through increasing the expression and the activity of endothelial nitric oxide synthase (eNOS) (395–398), through SR-BI and S1P receptors, which trigger PI3K/Akt signaling pathways and the phosphorylation of eNOS (395,396). In addition, HDL2 specifically reduce the production endothelial thromboxane A₂, a potent vasoconstrictor and prothrombotic mediator (390). HDL associated lipids play a key role in mediating the vasodilatory effects of HDL particles. Among these, S1P, estradiol, androsterone, and dehydroepiandrosterone appear to mediate vasodilation via stimulation of eNOS enzyme (392,399–402).

Strikingly, HDL also maintain balance in blood coagulation, through exerting potent antithrombotic activity, observed as inhibitory effects on platelets activation and on coagulation factors including tissue factor, factors X, Va, and VIIIa (393,396,403). Moreover, stimulated NO production in endothelial cells and enhanced cholesterol efflux from platelets can contribute to the inhibitory effects of HDL on platelet aggregation (397). HDL-associated phospholipids appear to play a major role in the antithrombotic activity of HDL. Among these, cardiolipin, and the negatively charged PS and PI, may contribute to HDL-mediated effects on coagulation and platelet functions, respectively (291,404).

Many other biological effects have been described with HDL. For instance, HDL has demonstrated anti-infectious activities through binding to lipopolysaccharide (LPS) and facilitating its hepatic clearance, thereby preventing its endotoxic effects (405,406). Furthermore, HDL has emerged as a key player in improving glucose metabolism by enhancing the secretion of insulin, and improving its sensitivity (407). Intriguingly, HDL has also been identified to transport multiple non-coding microRNAs that play a pivotal role in regulating cholesterol homeostasis, including cellular cholesterol efflux (408,409). Among these, miRNA-33 was shown to increase the plasma HDL-C levels, improve RCT and cholesterol efflux from macrophages, and prevent the progression of atherosclerosis *in vivo* (410,411). Interestingly, HDL-mediated transfer of miRNA-223 to endothelial cells was shown to suppress ICAM-I expression, thereby attributing to the AIA of HDL in endothelial cells (412).

In summary, the proteins and lipids components of HDL are the primary determinant of multiple biological functions. The compositional heterogeneity of HDL subpopulations implies a high degree of functional diversity among them (257,413,414). Notably, smaller, denser, protein-rich HDL subpopulations exhibit enhanced biological activities compared to larger, lighter, lipid-rich HDL. For a detailed overview of the functional diversity of HDL subfractions, please refer to **Table 1.8**. In light of this, emerging evidence is demonstrating that alterations in HDL composition are common in cardiometabolic diseases (CMD) and ASCVD. Such changes may result in functional insufficiency or complete dysfunction of HDL, potentially accelerating or contributing to the progression of atherosclerosis. Thus, our focus in the next sections will shift toward understanding how HDL composition and functionality are modified in pathological conditions like CMD and ASCVD, building upon our knowledge of their roles in normal physiological conditions.

Table 1.8: Functional heterogeneity of HDL subfractions. eNOS: endothelial nitric oxide synthase; FC: free cholesterol; PC: phosphatidylcholine; PL: phospholipids; S1P: sphingosine-1-phosphate; SM: sphingomyelin; TFPI: tissue factor pathway inhibitor [adapted from (173,242,415)].

Biologic function	Particles	Feature	Related properties
cholesterol efflux	Small dense HDL	Potent efflux from lipid-loaded macrophages	Low lipid content, high surface fluidity, distinct ApoA-I conformation
		Enhanced efflux via ABCA1	
	Enhanced LCAT activity		
	Large dense HDL	Enhanced efflux via ABCG1 and SR-BI	Elevated PL content
antioxidative	Small dense HDL	Potent protection of LDL from oxidation	Low SM/PC ratio. Increased surface fluidity. Enriched in ApoA-I and hydrolytic enzymes
		Potent inactivation of oxidized lipids	
Anti-inflammatory	Small dense HDL	Inhibition of endothelial cell adhesion molecules expression	Distinct ApoA-I conformation and composition of PL
		Immunomodulatory capacities towards neutrophils	Factor H-related proteins
Cytoprotective	Small dense HDL	Potent protection of endothelial cells from ox-LDL induced apoptosis	Enriched in ApoA-I and S1P
vascular dilatory	Small dense HDL	Potent activation of eNOS and nitric oxide production	Enriched in S1P
	Large dense HDL	Enhanced production of prostacyclin and attenuated production of thromboxane A2	
antithrombotic	Small dense HDL	Potent anticoagulant activity	Enriched in TFPI and S1P
	Large dense HDL	Inhibition of platelet aggregation	Enriched in ApoE
FC acquirement from TGRL	Small dense HDL	Potent capacity to acquire FC upon TGRL lipolysis	Low content of FC

1.4 Altered HDL functionality and dysfunction

In 1975, low plasma HDL-C levels were linked with impaired cholesterol clearance from arterial wall and accelerated atherosclerosis (73), which was interpreted as a reduced cholesterol efflux to HDL as a result of diminished quantity of HDL-C levels. However, both the minimized inverse association between HDL-C levels and cardiovascular risk in patients with CVD and the failure of therapeutic raising of HDL-C levels to improve cardiovascular outcomes (416), have suggested the concept of altered HDL functionality (i.e. diminished HDL quality) in ASCVD. Such concept is supported by the observed reduction in the anti-inflammatory activities of HDL from CAD patient despite high HDL-C levels (417). Emerging evidence is demonstrating that HDL can lose its anti-atherogenic functions under several metabolic conditions and might gain pro-atherogenic properties, thereby contributing to the progression of ASCVD (418).

In CMD such as dyslipidemia, Type 2 diabetes, obesity, and metabolic syndrome, dramatic changes can occur in the composition, metabolism and structure of HDL particles, resulting in reduced atheroprotective function/s (functionally deficient HDL) or completely absent function/s (dysfunctional HDL) with or without gain of dysfunction such as pro-inflammatory properties (251,419). The degree of loss of normal HDL function and gain of dysfunction might differ according to HDL functions. Several studies showed a complete loss of function and gain of dysfunction in the context of HDL anti-inflammatory, vasodilatory and antiapoptotic activities, but a reduction rather than complete loss with the antioxidative activity and cholesterol efflux capacity (418).

1.4.1 Altered HDL composition in cardiometabolic diseases

Altered protein cargo of HDL in CMD is well established (420). For instance, HDL particles from hypercholesterolemic patients showed diminished relative content of apoA-I, while they were enriched with apoE, apoC-I and apoC-II, and importantly Serum amyloid A (SAA), an acute phase inflammatory protein with pro-atherogenic properties (421). Hence, under inflammatory conditions, HDL content of apoA-I can be reduced and replaced by SAA (422,423). As a consequence, SAA can promote the retention of HDL particles within the arterial wall by increasing their binding to extracellular matrix proteoglycans (424). When blocked within the arterial wall, apoA-I in HDL can undergo atherogenic modifications such as myeloperoxidase-induced oxidation, and proteases-induced degradation in patients with CAD

(425,426), and glycosylation in T2D (427). Interestingly, elevated levels of apoC-III were reported in both HDL2 and HDL3 from patients with MetS and CAD (428,429), while HDL3 particles from CAD patients were enriched in apoE and depleted of apoF, PLTP and apoJ (428). In conditions with chronic inflammation and oxidative stress such as T2D, metabolic syndrome, and premature CAD, HDL-associated enzymes such as PON1, PAF-AH, and LCAT can be reduced in quantity and/or become dysfunctional (175,301,417,429,430).

Alterations in HDL lipidome are also common in CMD. For instance, elevated levels of TG were observed in HDL isolated from patients with low HDL-C levels-hypertriglyceridemic status such as in T2D, and MetS, and from patients with MI (288,431,432), whereas such HDL particles were depleted in CE and SM (288,431). Strikingly, LPC levels were depleted in HDL from patients with T2D, MetS, and CHD (288,431,433), whilst elevated in HDL from patients with hypertension (434) and under acute inflammatory conditions (435). In addition, enrichment of HDL in free fatty acids, and isoprostanes, while depletion in PI, and PE, was also observed under acute inflammation (435). Inversely, elevated levels of PE were observed in HDL from patients with hypertension (434). Higher levels of PE were also observed in HDL2 from patients with T2D and CHD, whilst the levels of PI, PC, LPC, and SM were significantly diminished compared to HDL2 from healthy individuals (433). Noticeably, diminished PUFA content of HDL from patients with CAD was also reported (436). Similarly, diminished levels of S1P levels are frequently observed in HDL from patients with type 1 diabetes (T1D), and T2D, and are commonly associated with impairment of multiple HDL functions (419).

1.4.2 Altered HDL metabolism in cardiometabolic diseases

Alterations of HDL proteome and lipidome in CMD mostly originate from altered HDL metabolism, which involve defective HDL formation and/or remodeling. Beside a reduced hepatic production, a deficient lipidation of apoA-I upon interaction with ABCA1, which can reflect apoA-I and/or ABCA1 deficiency, can result in a diminished formation of large HDL, or accelerated catabolism of apoA-I, thereby leading to subnormal HDL-C levels (248). In addition, HDL maturation into large particles can also be altered upon LCAT deficiency, leading to diminished esterification of FC. TG enrichment of HDL can arise from both an increased hepatic production of VLDL, and an increased CETP activity, the latter accelerate CE transfer from HDL to VLDL in exchange for TG, leading to subnormal HDL-C levels (234,437,438). Hence, the presence of TG-enriched HDL is associated with an accelerated lipolysis of its TG content, which in turn enhance the dissociation and renal clearance of apoA-

I, resulting in subnormal levels of ApoA-I, HDL particles, and HDL-C levels (431). Reduced cholesterol levels of HDL can result from defective intravascular hydrolysis of TGRL by LPL, which by diminishing the formation of TGRL surface remnants lead to impaired transfer of FC from TGRL remnants to HDL and subnormal HDL-C levels (248,431). Finally, altered phospholipid content of HDL can result from accelerated remodeling of HDL due to elevated activities of PLTP, endothelial lipase and secretory phospholipase A2 in CMD (248).

In summary, compositional alterations of HDL are common and diverse in CMD. These alterations may contribute to ASCVD progression by impairing the atheroprotective functions of HDL (251). HDL dysfunction primarily involves reduced capacities to prevent LDL oxidation, inflammation and cholesterol accumulation in arterial walls (175). For a detailed overview of the major compositional alterations in CMD and their consequences on HDL functions, please refer to **Figure 1.16** and **Table 1.9** (416,418). Understanding the link between altered HDL composition and function is vital for advancing our understanding of ASCVD and developing therapies to enhance HDL functionality (i.e. quality). The next section will explore therapeutic targeting of defective HDL functionality, with special focus on the reconstituted HDL-based therapeutic approaches.

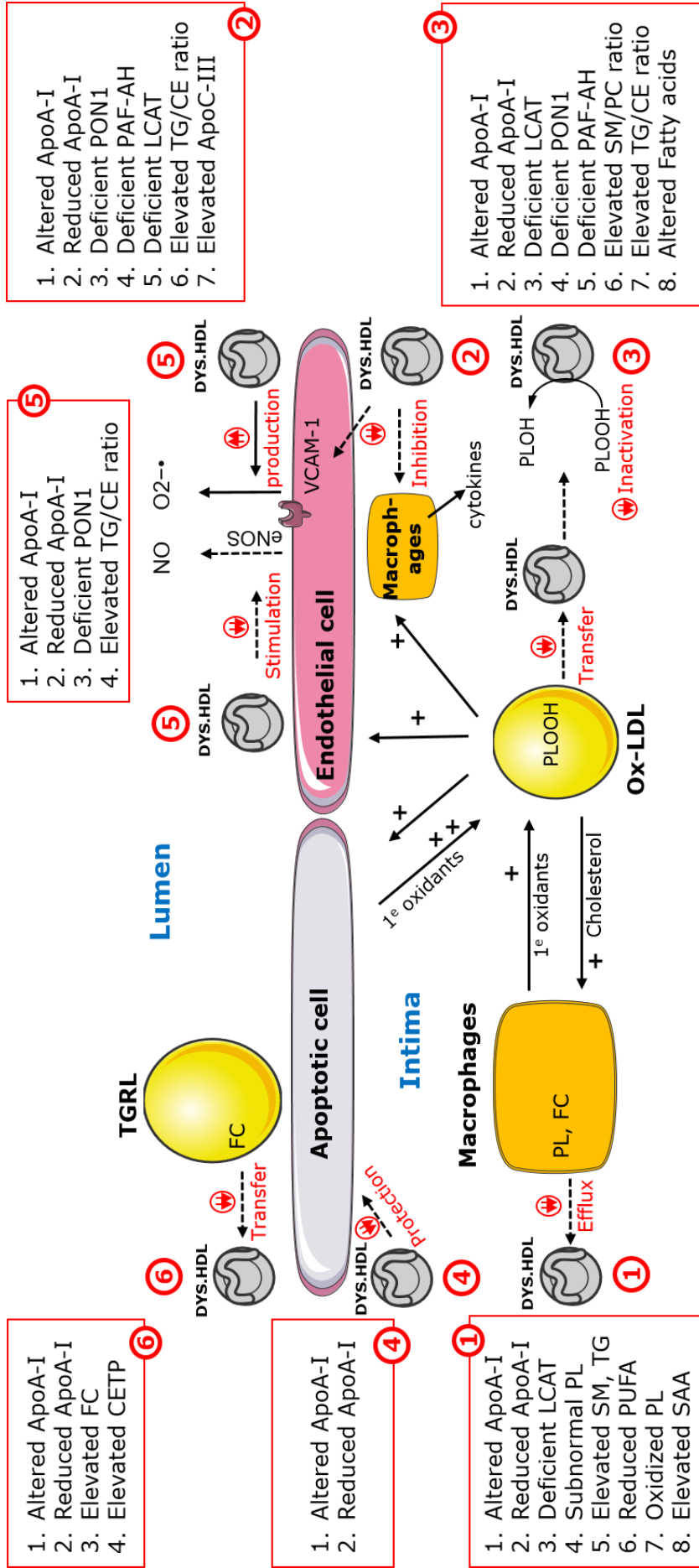


Figure 1.16: Compositional alterations involved in altered HDL functions. Schematic illustration lists altered components of HDL potentially involved in **1**) reduced cholesterol efflux capacity from macrophages, **2**) reduced anti-inflammatory activity in endothelial cells, and macrophages **3**) reduced anti-oxidative activity against LDL oxidation, **4**) reduced protection against TGR1. ApoA-I, apolipoprotein A-I; 1^e oxidants, one-electron oxidants; FC, free cholesterol; PL, phospholipid; SM, sphingomyelin; TG, triglycerides; CE, cholesteryl ester; PC, phosphatidylcholine; PUFA, poly-unsaturated fatty acids; SAA, serum amyloid A; PLOOH, phospholipid hydroperoxide; PLOH, phospholipid hydroxides; VCAM-1, vascular cell adhesion molecule-1; DYS-HDL, dysfunctional high-density lipoprotein;; NO, nitric oxide; O₂⁻, superoxide; CETP, Cholesteryl ester transfer protein; TGR1, triglyceride-rich lipoproteins; LCAT, lecithin-Cholesterol Acyltransferase; NO, endothelial nitric oxide synthase; PON1, paraoxonase 1; ApoC-III, apolipoprotein C-III; PAF-AH, platelet-activating factor acetylhydrolase. [Adapted from Kontush and Chapman. 2012 (248)].

Table 1.9: Altered composition and functions of HDL in cardiometabolic diseases. Data are summarized and reviewed from chapter 10 in (248), except for free cholesterol acquirement from TGRL (226).

Compositional alterations	Functional consequences
CHOLESTEROL EFFLUX CAPACITY	
Low HDL levels with (apoA-I mutations or TG-enriched and PL-depleted HDL)	<ol style="list-style-type: none"> 1. Impaired interaction of HDL with ABCA1 and SR-BI 2. Reduced ability to activate LCAT
Homozygous CETP deficiency (CE-enriched HDL2)	<ol style="list-style-type: none"> 1. Reduced capacity to accept cellular cholesterol
ApoA-I glycation and oxidation	<ol style="list-style-type: none"> 1. Reduced LCAT activation 2. Impaired lipid binding 3. Defective ABCA1 mediated-cholesterol efflux
Replacement of apoA-I by SAA	<ol style="list-style-type: none"> 1. Increased HDL binding to, and CE uptake by, macrophages 2. Diminished cholesterol efflux and ABCA1 expression in macrophages
Enrichment of HDL in apoE	<ol style="list-style-type: none"> 1. Increased HDL catabolism through apoB/apoE receptors
Reduced LCAT activity	<ol style="list-style-type: none"> 1. Reduced cholesterol efflux from, and increased cholesterol influx to, macrophages
Elevated SM/PC ratio and decreased content of PUFAs	<ol style="list-style-type: none"> 1. Increase HDL surface rigidity 2. Reduced LCAT activity
ANTI-OXIDATIVE ACTIVITY	
<p>Reduced content and altered conformation of apoA-I</p> <p>ApoA-I oxidation, and glycation</p> <p>Reduced activity of PON1, PAF-AH, and LCAT</p> <p>Enrichment of HDL in SAA</p>	<ol style="list-style-type: none"> 1. Reduced capacity to inactivate oxidized phospholipids 2. Altered accessibility of Met residues of apoA-I to oxidized phospholipids 3. impaired hydrolysis of short chain oxidized phospholipids

TG-enriched and CE-depleted HDL Increased MUF/PUFA ratio in HDL increased SM and saturated fatty acids contents	Reduced transfer of oxidized phospholipids from LDL to HDL due to reduced HDL surface fluidity
ANTI-INFLAMMATORY ACTIVITY	
Reduced apoA-I content, TG enrichment, PL-depletion	Reduced inhibition of VCAM-1 and ICAM-1
ApoA-I glycation and oxidation	<ol style="list-style-type: none"> 1. Reduced binding to SR-BI 2. Defective capacity to inhibit NFκB activation, ROS production, and the expression of VCAM-1 and ICAM-1
Enrichment of HDL in apoC-III	<ol style="list-style-type: none"> 1. Reduced ability to prevent monocytes adhesion to endothelial cells 2. Increased capacity to activate ERK and NFκB pro-inflammatory pathways
Reduced activity of PON1, PAF-AH, and LCAT and their replacement by SAA	Impaired capacity to inactivate the pro-inflammatory oxidized phospholipids
CYTOPROTECTIVE ACTIVITY	
Reduced apoA-I content ApoA-I oxidation and glycation	<ol style="list-style-type: none"> 1. Reduced binding to SR-BI 2. Increased binding to scavenger receptors (CD36 and SR-A)
VASODILATORY ACTIVITY	
Reduced content and altered conformation of apoA-I, Increased TG/CE ratio	<ol style="list-style-type: none"> 1. Reduced HDL binding to cells 2. Reduced efflux of oxidized lipids that promote vasoconstriction
Reduced PON1 activity	Reduced ability to stimulate eNOS activation, and endothelial NO production
ANTITHROMBOTIC ACTIVITIES	
oxidative modification of HDL components	Increased expression of P-selectin in platelet, and enhanced platelet aggregation via binding to CD36
FREE CHOLESTEROL ACQUIREMENT FROM TGRL	
Reduced content of apoA-I, free cholesterol-enriched HDL, and enhanced CETP activity	Reduced capacity to accept free cholesterol from, and enhanced re-transfer of free cholesterol to, TGRL

1.5 Therapeutic targeting of defective HDL functionality

Therapeutic strategies for improving HDL functionality aim to raise HDL-C and apoA-I levels while normalizing altered HDL composition and metabolism. Major HDL functions-targeting approaches include apoA-I mimetic peptides, and reconstituted HDL (rHDL) formulations, with the goal of reducing atherogenesis and cardiovascular risk.

1.5.1 ApoA-I mimetic peptides

ApoA-I mimetics are amphipathic peptides that structurally resemble native apoA-I in a way that they contain structured analogs of the α -helical domains found in apoA-I. As a result, they display similar biological effects even though their amino-acid sequences are frequently different (248). Examples of ApoA-I mimetics include ATI-5261, 5A, and D-4F peptides which are short-chain peptides, easy to produce compared to full length apoA-I. Although apoA-I mimetics were effective in displaying atheroprotective effects in animal models, recent review of various studies have revealed inconsistencies in the functional properties of these peptides between *in vitro* and *in vivo* settings (439).

1.5.2 Reconstituted HDL

The rHDL is a synthetic discoidal or spherical HDL prepared from purified human or recombinant apoA-I and phospholipids as the essential components, but other proteins and/or lipids can also be used (440,441). Such innovative rHDL that resemble native HDL has experimental, diagnostic and therapeutic application (442,443). The capacity of incorporating specific components into rHDL make it a useful tool for investigating the impact of individual protein or lipid component on the structural and functional aspects of native HDL. In addition, rHDL has also potential therapeutic uses for the management of ASCVD as HDL-replacement therapy (442). Furthermore, rHDL is used in nano-biotechnology as a platform for the delivery of drugs and contrast imaging agents (443).

1.5.2.1 Composition and functional effectiveness of reconstituted HDL

The rHDL particles are usually generated *in vitro* by incubating phospholipid micelles (refer to **Figure 1.17 A&B**) with free apoA-I, which provide the essential amphipathic α -helices that are necessary to initiate the assembly of rHDL (440,443). Given to the fact that PC accounts for about 70% of total phospholipids in native HDL, PC species such as 1-palmitoyl-2-oleoyl

phosphatidylcholine (POPC), 1-palmitoyl-2-arachidonoyl phosphatidylcholine (PAPC), or PLPC are usually used as principle phospholipid to generate rHDL (444). Other phospholipids such as PS, and PE as well as cholesterol can also be incorporated with variable proportions in the presence of PC (445). The conformation of rHDL is largely dependent on its composition, as well as the preparation method (including the incubation time) and it includes discoidal rHDL particles with a size range of 7-17nm, similar to native HDL (446) and spherical rHDL (refer to **Figure 1.17 C&D**) (447). Consistent with its pivotal roles in native HDL, ApoA-I plays a central role in the anti-atherogenic effects of rHDL. While the phospholipid component (i.e. PC) in rHDL appears to have a regulatory role (248), the presence of bioactive phospholipids like S1P and PS has been found to significantly influence the atheroprotective properties of rHDL. This suggests that the ability of rHDL to convey atheroprotection is, to some extent, influenced by the phospholipid composition of the particle (382,448,449). Interestingly, rHDL formulations have been shown to effectively exert all major atheroprotective functions attributed to native HDL (248).

1.5.2.2 Therapeutic applications of reconstituted HDL

The rHDL holds promise for ASCVD management, due to its surprisingly acute and rapid effects in elevating plasma HDL levels and in reducing the size of the atherosclerotic plaque following intravenous infusion (450). Weekly infusions of ETC-216, the first rHDL to reach clinical trials and composed of PC and apoA-I Milano (apoA-I variant with potent anti-atherogenic properties), for 5 weeks in patients with a history ACS reduced the atherosclerotic plaque volume in relative to baseline (451). However, ETC-216 was associated with dose-dependent increase in neutrophils. Seeking for more safe and cost-effective formulations, CSL-111, which consists of human apoA-I complexed with soybean PC was developed. In individuals with CAD, weekly infusions of CSL-111 for 4 weeks reduced the atherosclerotic plaque volume and improved the plaque characterization index and coronary score (452). However, CSL-111 infusions were mildly hepatotoxic, possibly due to the residual cholate utilized for rHDL production (442). As a result, CSL-111 was reformulated as CSL-112, which lacked the hepatotoxic cholate. CSL-112 was able to raise plasma apoA-I levels immediately after infusion, enhance capacity of plasma to efflux cellular cholesterol and it displayed good safety profile (453). Another rHDL formulation, CER-001, contains human recombinant apoA-I with SM and PG. While CER-001 didn't impact coronary atherosclerotic burden in statin-

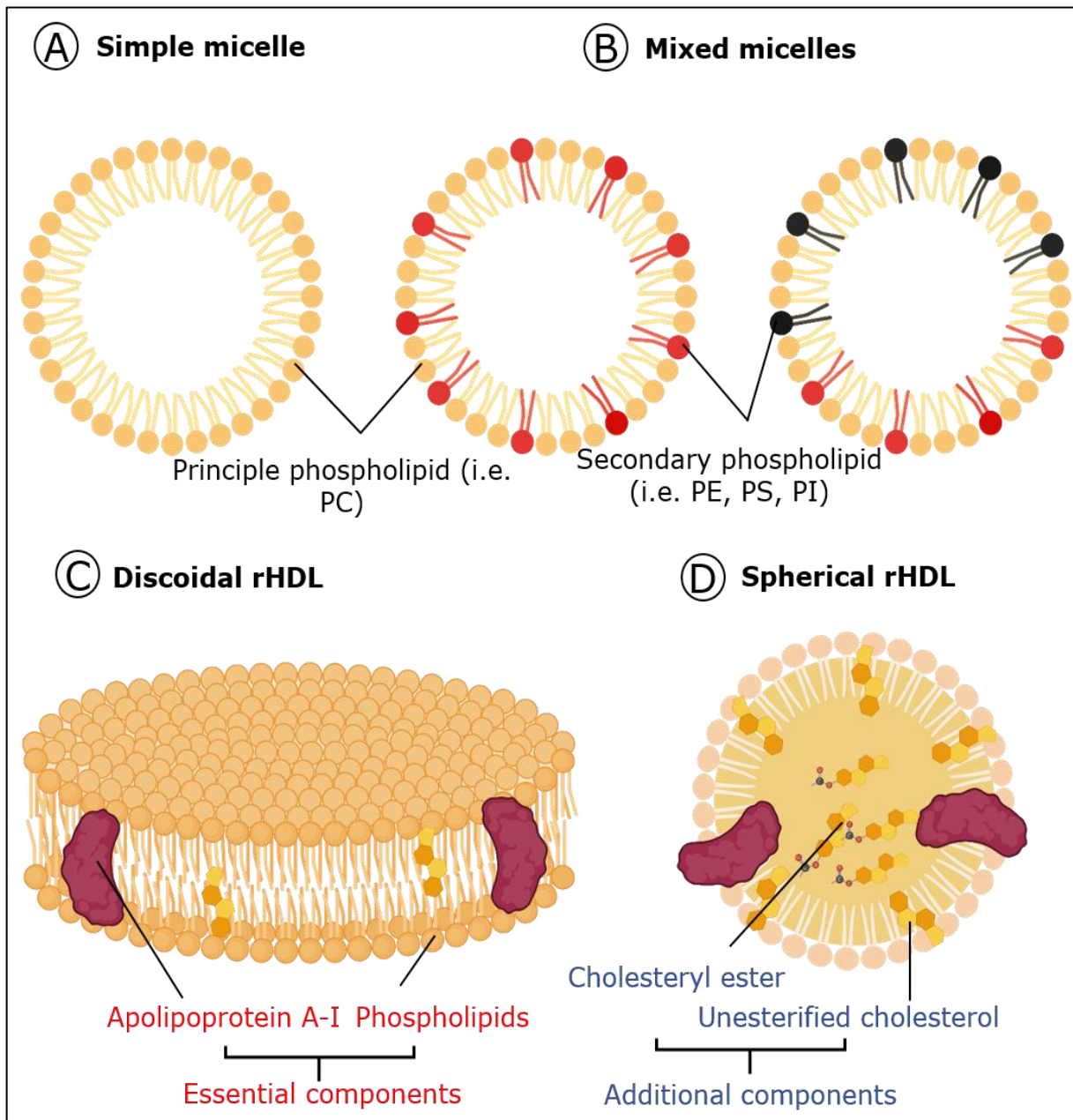


Figure 1.17: reconstituted HDL. Schematic illustration shows different phospholipid micelles used to generate rHDL particles in the presence of apolipoprotein A-I. **A)** Simple micelle contains only PC, the principle phospholipid moiety that is usually used to generate rHDL, **B)** Mixed micelles composed of more than one class of phospholipid, with PC as the principle and predominant phospholipid, which usually used to study the impact of bioactive phospholipids such as PE, PS, and PI on HDL structural and functional properties, **C)** Discoidal rHDL particle generated from mixing phospholipid micelles with apolipoprotein A-I with or without unesterified cholesterol. **D)** Spherical rHDL particles generated by mixing discoidal rHDL particles contain unesterified cholesterol with LCAT enzyme that promotes the formation of cholesteryl ester formation, which in turn migrates into the lipid core of the particle. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; rHDL, reconstituted HDL. [Adapted from Ryan RO. Et al 2010 (443)].

treated ACS patients (454,455), it effectively reduced carotid wall area in patients with homozygous familial hypercholesterolemia (456).

The rapid regression of atherosclerotic plaque volume induced by rHDL (i.e. ETC-216) is most likely related to the accelerated cholesterol efflux from arterial wall macrophages (451). Other anti-atherogenic activities of rHDL, notably potent anti-inflammatory and antioxidative properties may also contribute to plaque regression in humans. Furthermore, potent vasodilatory and anti-thrombotic effects of rHDL (i.e. CSL-111) have been demonstrated in hypercholesterolemic and type 2 diabetes patients, respectively (397,457). In animal models, studies have consistently shown that various rHDL formulations inhibited the expression of endothelial cell adhesion molecules (i.e. VCAM-1 and ICAM-1), as well as the LPS-induced release of pro-inflammatory cytokines, and ROS production, along with atherosclerotic plaque stabilization (458–462). Furthermore, rHDL (i.e. apoA-I plus POPC) was shown to display cytoprotective and anti-infectious effects in animal models (463,464).

1.5.2.3 Experimental applications of reconstituted HDL

In parallel with its therapeutic applications, rHDL serves as a valuable tool to decipher the role of individual HDL components on its structural and functional properties. For instance, rHDL enables the study of lipids like PE, PS, and SM and their impact on the kinetic, thermodynamic, and physical (i.e. size, fluidity, and stability) properties of rHDL (445,465). rHDL can also be used to characterize the underlying mechanisms of the atheroprotective functions of native HDL such as their anti-inflammatory effects in various cellular and animal models (368). Finally, using rHDL help to identify potential lipid biomarkers, such as PS, EPA-PC, and specific fatty acids, related to HDL functionality (466,467). Given the prevalence of PC in native HDL and the established effectiveness of rHDL formulations consisting only of PC and apoA-1, PC-only rHDL is typically used as a standard control in these research studies.

By using both the new knowledge offered by the lipidomic studies of ASCVD patients (15,16), and the possibility offered by rHDL reconstitution, it is now possible to elucidate the role of new biomarkers and therapeutic targets. Thus, the next section explores the implications of plasma lipidomics in ASCVD, with a particular focus on PE species.

1.6 Molecular lipid biomarkers in ASCVD

[This section will be formulated and published as a review article]

Lipids play a key role in the formation and subsequent disruption of atherosclerotic plaques (15). While plasma levels of major lipids such as total cholesterol, LDL-C, TG, and oxidized phospholipids, help in predicting the CVD risk, the bulk of CVD cannot be fully explained by traditional lipid-related risk factors alone (468). In addition, it is increasingly becoming evident that many other lipid types play a significant role in atherogenesis (15). Progress in mass spectrometry-based analytical techniques have identified a large variety of lipid molecular species directly associated to the development of obesity, diabetes mellitus, hypertension, and ASCVD (469–471). Given that the composition of lipid species profoundly influences their biological effects, identifying the detailed composition-including individual fatty acids - of the lipid species associated to CVD could provide mechanistic insights into their potential implication in disease pathogenesis (472). Thus, an in-depth knowledge about the lipid molecular species that hold potential mechanistic links to ASCVD may help to identify better biomarkers and novel therapeutic potentials (472,473).

The primary application of lipidomic techniques was to identify specific molecular lipid species that potentially predict the risk for CVD events. Among the various lipid classes investigated, plasma phospholipids have emerged as a source of several novel CVD biomarkers. The detailed findings of the lipidomic studies reviewed in this study are outlined in **Table 1.10**. In the context of plasma PC and related lipids, Meikle et al. revealed that plasma levels of twenty PC and LPC molecular species were negatively associated with unstable CAD compared to stable CAD, which in turn was negatively associated to plasma levels of twenty-two species of PC, PC-plasmalogens, alkyl-PC and LPC species compared to healthy individuals (15). This effect was confirmed by Sigruener et al. who has identified four polyunsaturated PC and LPC species that appeared to confer protective effects against cardiovascular and total mortality in patients with CAD (474). While Paapstel et al. found that low serum levels of six species of PC, alkyl-PC, and LPC were associated with atherosclerosis (475). Altogether, these findings collectively underscore the potential protective roles of several PC, alkyl-PC, PC-plasmalogens, and LPC species in ASCVD. However, it's important to note that several other PC, alkyl-PC and LPC species have been associated with increased ASCVD risk. For instance, Meikle et al. reported positive associations between four PC species including PC (32:1), PC (35:2), PC (38:3), and PC (38:4) and two LPC species including LPC (20:3) and LPC (22:1) and stable

CAD (15). Similarly, Stegemann et al. found that elevated plasma levels of PC (32:1), PC (38:2), and PC (38:3) species were significantly linked to CVD risk in a large population-based study (16). Moreover, Sigruener et al. and Ganna et al. reported positive associations between numerous PC, alkyl-PC, and LPC species [particularly LPC (18:1), LPC (18:2) species] and both total and CAD mortality, as well as incident CVD (474,476). In another study conducted by Alshehry et al. on patients with T2D, seventeen species of PC, alkyl-PC, PC-plasmalogens, and LPC, with the exception of PC (O) (36:5), were significantly associated with future cardiovascular events and death (refer to **Table 1.10**). In addition, PC (O) (36:1), PC (28:0), LPC (18:2), LPC (20:0), and PC (35:4) species were reported to improve CVD risk prediction (477), while eight PC and LPC species were reported to be enriched in atherosclerotic plaque (478). Ménégaut et al. reported a specific enrichment of 2-arachidonoyl-LPC species in carotid atheroma plaques from diabetic patients compared to non-diabetic patients (479). Strikingly, such enrichment of 2-arachidonoyl-LPC species in carotid plaques was confirmed in a recent MASCADI (Arachidonic Acid Metabolism in Carotid Stenosis Plaque in Diabetic Patients) clinical study of T2D elderly patients with advanced carotid atherosclerosis, and it was correlated with plaque inflammation, suggesting the potential role of this specific LPC species in diabetic atherosclerosis (480). Collectively, this body of evidence suggests that plasma PC and related lipids (alkyl-PC, PC-plasmalogens, and LPC) may play dual roles in both protection and progression of ASCVD, pointing out the importance of specific molecular species.

PE has gained prominence as a crucial phospholipid class in the context of CVD. Up to date, several plasma lipidomic studies have consistently demonstrated a positive association between the majority of plasma PE species and CVD risk and mortality. Meikle et al. reported the majority of detected plasma PE (i.e. 16 species out of 20), and four species of PE-plasmalogens, and LPE to be positively associated with stable CAD (refer to **Table 1.10**) (15). Notably, the positive association observed with PE (34:1), PE (34:2), PE (36:2), PE (36:4), PE (36:5), and PE (38:4) by Meikle et al. was further confirmed by larger population-based studies such as the Bruneck and LURIC studies. In these large-scale lipidomic studies, these specific PE species maintained significant association with incident CVD, even when controlling for multiple comparison (16), and they were found to be associated with CAD and/or overall mortality, underscoring the significance of these specific PE species as valuable biomarkers for ASCVD (474). This was confirmed by Alshehry et al. who identified the potential of plasma alkyl-PE (O) (36:5) in improving the prediction of CVD events in individuals with T2D (477). Additionally, Stegemann et al. reported the enrichment of PE (38:3) species in atherosclerotic

plaques (478), shedding light on the potential role of PE species in the pathogenesis of atherosclerosis. In contrast, it's noteworthy that several alkyl-PE species (ten species), PE-plasmalogens (seven species), and LPE (18:1) exhibited a negative association with unstable CAD (versus stable CAD), hinting at their potential role in stabilizing atherosclerotic plaques (15). In conclusion, plasma PE species and related lipids have emerged as novel biomarkers in the context of ASCVD, with certain species showing consistent positive associations with incident CVD risk, CAD, and overall mortality, hence, warranting further research.

The implication of other phospholipid classes including PI, PS, and PG in ASCVD was also demonstrated by lipidomic studies. For instance, Meikle et al. reported negative association between the plasma levels of eight PI species and unstable CAD, while the plasma levels of five PI species, four of them contain ARA, including PI (36:4), PI (38:4), PI (40:4), PI (40:5), were positively associated with stable CAD compared to control individuals (15). In this study, the plasma levels of PG (16:0/18:1) showed positive associations with stable CAD. In the context of PS, Stegemann et al., reported the enrichment of PS (38:5) and PS (38:2) in atherosclerotic plaques (478). In summary, despite the limited number of lipidomic studies, this existing data enhance our understanding about the implication of individual phospholipid species in ASCVD, underscoring the importance of the precise composition of these species in influencing their association with ASCVD.

Beside phospholipids, lipidomic studies have revealed interesting insights into the involvement of several sphingolipid species such as SM and Ceramides in ASCVD. Hence, three SM species including SM (18:0), SM (18:1), and SM (18:2) were positively associated with the unstable CAD (15). SM (18:2) was also negatively associated with stable CAD (15), suggesting a potential role for this sphingolipid species in increasing plaque vulnerability. 5 SM species including SM (34:2), SM (42:2), SM (16:0), SM (16:1), and SM (24:1) have also been associated with both incident CVD, and increased CAD and/or all mortality (16,474), or, for 4 other SM species (d32:2/ d33:1/ d33:2/ d38:2) with only CAD incidence by Ganna et al. (476). Among these species, SM (d33:1) was specifically detected in atherosclerotic plaques (478). Such existing data point out the potential deleterious involvement of several SM species, particularly SM (18:2) and SM (d33:1) in ASCVD. However, some SM species including SM (22:0), SM (22:1), SM (23:0), SM (23:1), and SM (24:0) were reported to have protective effects against CAD and/or total mortality (474). In the context of plasma ceramides (Cer), Meikle et al. reported 5 species of Cer including Cer (16:0), Cer (18:0), Cer (20:0), Cer (22:0),

and Cer (24:0) to be positively associated with stable CAD, as well as 2 species of di-Hexosylceramide (Hex2cer), a glycosylated ceramides, including Hex2cer (22:0) and Hex2cer (24:0) (15). Strikingly, the plasma levels these two of Hex2cer species were also reported in another study to be associated with both future CV events and death in individuals with T2D, while several other glycosylated ceramides including 6 species of HexCer, 4 species of Hex2Cer, and 3 species of Hex3Cer (refer to **Table 1.10**), were positively associated with future CV events and death (477). Collectively, such lipidomic studies strongly support the notion that specific sphingolipid species in plasma could serve as biomarkers for ASCVD pathogenesis, progression, and associated mortality.

Given the well-established role of plasma cholesterol and TG in the development of ASCVD, advancements in plasma lipidomics have brought to light specific molecular species within these lipid classes that hold significant importance. To delve into these findings further, Meikle et al. study has identified seven species of esterified cholesterol, known as cholesteryl ester (CE), that displayed a positive association with stable CAD (refer to **Table 1.10**) (15). Of these, CE (14:0), and CE (16:1) were also significantly associated with increased CVD risk, with CE (16:1) being consistently linked to incident CVD, and improved prediction and classification of CVD risk (16). Furthermore, these two species were identified alongside with seven other CE species to be enriched in atherosclerotic plaques, shedding light on their involvement in the pathogenesis and progression of ASCVD (478). In particular, polyunsaturated CE with long-chain fatty acids including CE (22:3), CE (22:4), CE (22:5), and CE (22:6) showed the greatest relative enrichment in plaques and formed part of a lipid signature for vulnerable and stable plaque. In addition, Alshehry et al. reported the significant association of plasma CE (16:0) and CE (18:0) in patients with T2D, with future CV events and death (477). Notably, CE (18:0) was found to significantly enhance the prediction of CVD risk. Together, these lipidomic studies underscore the critical roles of specific CE molecular species in ASCVD development, highlighting the potential significance of short CE molecular species with saturated fatty acids such as Myristic acid (C14:0), Palmitic acid (C16:0), and Stearic acid (C18:0).

Plasma TG showed to have “dual” effects on ASCVD depending on their molecular species. Twenty-one species of TG being negatively associated with unstable CAD, while sixteen species were positively linked to stable CAD (refer to **Table 1.10**) (15). In this study, the majority of TG species were predominantly composed of Myristic acid (C14:0), Palmitic

acid (C16:0), Palmitoleic acid (C16:1), Stearic acid (C18:0), Oleic acid (C18:1), and Linoleic acid (C18:2) fatty acids. The positive association of 10 species of TG, and particularly TG (54:2) with increased CVD risk was confirmed by Stegemann et al. in the population based-Bruneck study (16). While in patients with T2D, TG (56:6) showed positive association with future CV events and death (477). Beyond TG species, the significance of several mono-, and di-, acyl glycerides (MG, and DG) species in CVD was also reported. Meikle et al. reported the positive association of 10 DG species, mostly contain Palmitic acid (C16:0), Oleic acid (C18:1), and Arachidonic acid (C20:4), with stable CAD (15). MG (18:2), in turn, was reported to be consistently associated with CAD incidence, independently of the main cardiovascular risk factors, with causal involvement in the development of CAD (476).

In conclusion, these studies highlight that specific molecular lipid species could hold diagnostic and prognostic significance in identifying individuals at increased risk of CAD events and mortality. **Table 1.11** summarizes the most promising molecular lipid species as potential biomarkers for CVD. Notably, within the various lipid species, the majority of PE species consistently demonstrated a positive and replicated association with ASCVD risk and related mortality. Thus, the next section will focus on this particular class of phospholipids in the context of ASCVD.

Table 1.10: A summary of major findings of lipidomics in CVD. ARA: arachidonic acid; CE: cholesteryl ester; DG: diglyceride; HexCer: Hexosylceramide; MG: monoglyceride; LPE: Lyso-PE; LPI: Lyso-PI; LPC: Lyso-PC; PC (O): alkyl-PC; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PC (P): plasmalogen-PC; PE (O): alkyl-PE PI: Phosphatidylinositol; PS: phosphatidylserine; SM: sphingomyelin; TG: triglyceride.

Disease/ sample/ N°	Plasma lipid species	Reference
CAD/ plasma/ 220	<ol style="list-style-type: none"> 1. CE : (14:0), (16:1), (16:2), (17:1), (18:3) 2. DG : (14:0/16:0), DG (14:0/18:0), DG (14:0/18:2) 3. TG : 21 species, predominantly composed of different combinations of (14:0), (14:1), (16:0), (16:1), (17:0), (18:0), (18:1), (18:2) 4. SM : (14:1) 5. PC : (28:0), (30:0), (34:3), (34:4), (34:5), (36:4), (36:6), (38:6), (38:7) 6. Odd-PC : (33:3), (35:5) 7. Alkyl-PC : (32:2), (34:2), (34:4), (35:4) 8. Plasmalogen-PC: (34:3) 9. LPC : (14:0), (16:1), (17:1), (18:0), (18:1), (18:2), (18:3), (20:2), (20:3), (20:5), (22:0), (22:1) 10. PI : (36:0), (36:1), (36:3), (36:4), (38:2), (38:3), (38:4), (40:4) 11. alkyl-PE (O): (34:1), (34:2), (36:2), (36:3), (36:4), (36:5), (36:6), (38:4), (38:5), (40:5) 12. PE-p: (16:0/18:2), (16:0/20:4), (16:0/22:5), (17:0/20:4), (18:0/18:1), (18:0/18:2), (18:0/22:5) 13. LPE : (18:1) <p>were negatively associated with unstable CAD</p> <ol style="list-style-type: none"> 1. Cer: (18:0) 2. Dihexosylceramide: (16:0) 3. SM : (18:0), (18:1), (18:2) <p>were positively associated with unstable CAD</p> <ol style="list-style-type: none"> 1. SM : (18:2) 2. PC 32:3, 34:2, 36:2, 36:4 3. Odd-PC: (33:2), (35:2) 4. Alkyl-PC: (34:2), (36:0), (36:3) 5. LPC: 14:0, 15:0, 16:0, 17:0, 17:1, 18:0, 18:2, 22:1 6. Plasmalogen-PC: (32:0), (34:1), (34:3), (36:1), (36:2), (40:5) 7. PE-P: (18:0/18:2) <p>Were negatively associated with stable CAD</p> <ol style="list-style-type: none"> 1. Cer: (16:0), (18:0), (20:0), (22:0), (24:1) 2. Dihexosylceramide: (22:0), (24:0) 	Meikle et al. (15)

	<p>3. CE: (14:0), (16:1), (18:1), (20:3), (20:4), (22:4), (24:6)</p> <p>4. DG: (16:0/20:3), (16:0/20:4), (16:0/22:5), (16:1/18:1), (18:0/18:1), (18:1/18:1), (18:1/18:2), (18:1/18:3), (18:1/20:3), (18:1/20:4)</p> <p>5. TG: 16 species</p> <p>6. PC: (32:1), (38:3), (38:4)</p> <p>7. LPC: (20:3), (22:1)</p> <p>8. PI: (36:4), (38:3), (38:4), (40:4), PI (40:5)</p> <p>9. PE: (32:1), (34:1), (34:2), (34:3), (35:1), (36:1), (36:4), (36:5), (38:3), (38:4), (38:5), (38:6), (40:4), (40:5), (40:6), (40:7)</p> <p>10. PE-p: (16:0/20:4), (20:0/20:4)</p> <p>11. PG: (16:0/18:1)</p> <p>12. LPE: (20:4), (22:6)</p> <p>were positively associated with stable CAD</p>	
Incident CVD/ plasma/ 685	<p>1. TAG (50:1), TAG (50:2), TAG (50:3), TAG (52:2), TAG (52:3), TAG (52:5), TAG (54:2), TAG (56:1), TAG (56:5), TAG (56:6), PE(34:1), PE(34:2), PE(36:2), PE(36:4), PE(36:5), PE(38:3), PE(38:4), PE(38:5), PE (O) (38:6), SM (34:2), SM (42:2), PC (32:1), PC (38:2), PC (38:3), CE(14:0), CE(16:0), CE(16:1), CE(22:2) were significantly associated with CVD risk after multiple comparison</p> <p>2. TAG(54:2) and CE(16:1), and PE(36:5) were consistently linked to incident CVD, and improved risk classification and CVD prediction</p>	<p>Bruneck study</p> <p>Stegeman n et al.</p> <p>(16)</p>
CAD and total mortality/ plasma/ 3316	<p>1. PC (32:0), PC (38:0), PC (30:1), PC (34:1), PC (36:1), PC (O) (32:0), PC (O) (34:0), PC (O) (32:1), PC (O) (34:1), PC (O) (38:5), PE (30:1), PE (32:1), PE (34:1), PE (36:1), PE (40:1), PE (34:2), PE (36:2), PE (38:2), PE (36:3), PE (36:4), SM (16:0), SM (16:1), SM (24:1) were positively associated with total and/or CAD mortality</p> <p>2. PE (36:5), PE (38:4), PE (38:7), PE (O) (32:0), PE (O) (34:1), and PE (O) (36:2) were positively associated with total mortality</p> <p>3. PC (36:4), PC (36:5), PC (40:6), PC (40:7), LPC (16:0), LPC (18:0), LPC (18:2), SM (22:0), SM (23:0), SM (24:0), SM (22:1), and SM (23:1) were associated with protective effects.</p>	<p>LURIC study</p> <p>Siguener et al.</p> <p>(474)</p>

Incident CAD events /plasma/1028	<ol style="list-style-type: none"> MG (18:2), LPC (18:1), LPC (18:2), LPC (20:0), LPC (22:5), PC (34:1), LPC (18:3), LPC (20:4), PC (O-36:5), PC (31:1), PC (36:1), PC (32:2), PC (32:1), PE (35:2), PE (34:1), PE (39:1), PE (35:1), SM (d18:2/14:0), SM (d38:2), SM (d18:2/15:0), SM (d33:1) were associated with CAD incidence MG (18:2), LPC (18:1), LPC (18:2), and SM (28:1) were consistently associated with CAD 	Ganna et al. (476)
Incident CAD events in T2D /plasma/3779	<ol style="list-style-type: none"> Cer: HexCer (d18:1/16:0), (d18:1/18:0), (d18:1/20:0), (d18:1/22:0), (d18:1/24:0), (d18:1/24:1)/ Hex2Cer (d18:1/16:0), (d18:1/22:0), (d18:1/24:0), (d18:1/24:1)/ Hex3Cer (d18:1/20:0), (d18:1/22:0), (d18:1/24:0), (d18:1/24:1) PC: (34:5), (35:4), (40:6) ; PC (P): (32:1), (34:1) ; Alkyl-PC (O): (32:0), (32:1), (34:1), (36:1), (36:2), ; LPC (O): (18:0), (18:1), (22:0), (22:1), (24:0), (24:1), (24:2) CE(16:0), CE(24:1), SM(34:1), TG(56:6) <p>were significantly associated with both future CV events and death</p> <ol style="list-style-type: none"> PC (O) (36:1), CE(18:0), PE(O) (36:4), PC(28:0), LPC(20:0), and PC(35:4), LPC(18:2) improved the prediction of cardiovascular events 	Alshehry et al. (477)
Atherosclerosis/plaque/26	<ol style="list-style-type: none"> LPS (20:0), PS(38:5), PS(38:2), LPC (14:0), LPC (O) (16:0), LPC (O) (18:0), LPC (18:2), LPC (22:5), LPC (22:4), PC(O)(16:0/22:5),PC(18:0/20:3), CE(10:0), CE(14:0), CE(16:1), CE(18:1), CE(18:3), CE(22:6), CE(22:5), CE(22:4), CE(22:3), PE (38:3), SM(d18:1/14:0), SM(d18:1/15:0), SM(d18:0/15:0) were enriched in atherosclerotic plaque 	Stegemann et al. (478)
Atherosclerosis/serum/124	<ol style="list-style-type: none"> Low serum levels of PC (28:1), PC (30:0), PC (32:2), PC (O) (30:0), PC (O) (34:2) and LPC (18:2) were observed in patients with atherosclerosis compared to healthy control 	Paapstel et al. (475)

Table 1.11: The implication of specific molecular lipid species in CVD.

Lipid species	Implication in CVD	Reference
PC (32:1), and PC (38:3)	Positive association with the presence of stable CAD and incident CVD	(15, 16)
PC (28:0), and PC (35:4)	Improve CVD risk prediction	(477)
LPC (18:2)	Positive association with CVD incidents and CVD mortality. Improves CVD risk prediction	(474, 476, 477)
2- arachidonoyl- LPC	Positive association with atherosclerotic plaque inflammation in diabetic patients	(479, 480)
PE (36:5)	Consistent positive association with stable CAD and incident CVD. Improves the prediction and classification of CVD risk	(15, 16)
SM (18:2)	Positively associated with unstable CAD while negatively associated with stable CAD. Potential role in increasing plaque vulnerability	(15)
SM (d33:1)	Positive association with CAD incidence, and increased abundance in atherosclerotic plaque	(476, 478)
Hex2cer (22:0), Hex2cer (24:0)	Positive association with the presence of stable CAD, future CVD events and death in diabetic patients	(15, 477)
CE (16:1)	Positive association with stable CAD, and incident CVD. Increased abundance in atherosclerotic plaque	(15, 16, 478)
TG (54:2)	Positive association with stable CAD, and incident CVD	(15, 16)

1.7 Phosphatidylethanolamines

PE are the second most abundant glycerophospholipids in eukaryotic cells, comprising 15-25% of total phospholipids in mammalian cells. PE are functionally associated with a wide range of cellular processes, and disturbances in their metabolism have been implicated in the development of several chronic diseases (481).

1.7.1 Phosphatidylethanolamine-Structure, synthesis, and Functions

PE is a cone-shaped phospholipid containing a small polar head group in proportion to its fatty-acid chains as shown in **Figure 1.18B** (481,482). Typically, PE is composed of a glycerol backbone containing two fatty acid chains linked via ester linkage (at the sn-1 and sn-2 positions (di-acyl form) and an ethanolamine ($\text{HOCH}_2\text{CH}_2\text{NH}_2$)-linked phosphate head group at the sn-3 position as shown in **Figure 1.18A and C**. As for all phospholipids, PE subspecies arise from differences in their fatty acid composition. Saturated fatty acids are predominantly found at the sn-1 position, while the sn-2 position is characterized by an abundance of unsaturated fatty acyl chains (481). In addition to the diacyl form (constitutes the vast majority of PE, about 95-100%) (483), PE tends to exist in another two forms, namely alkyl-acyl or PE-plasmanyl (PE (O)), wherein the fatty acid at the sn-1 is linked via an ether linkage (illustrated in Figure 1.14.d) and alkenyl-acyl form, in which the fatty acid at the sn-1 is linked via a vinyl-ether linkage (illustrated in Figure 1.14.e), the latter known as PE-plasmalogen (PE (P)). Lyso-PE is a well-known metabolite of PE and it is formed mainly by phospholipase A2 - mediated hydrolysis of the fatty acid at sn-2 position of PE (illustrated in Figure 1.14.e) (484).

In mammalian cells, PE are generated by four independent pathways: 1) Cytidine diphosphate (CDP)-ethanolamine pathway or Kennedy pathway (485); 2) Acylation of Lyso-PE; 3) Head group base-exchange reactions, with the three of them occur in the endoplasmic reticulum (ER) membrane and 4) phosphatidylserine decarboxylase (PSD) pathway which occurs in the mitochondria (481,484). The four biosynthetic pathways of PE are outlined in **Figure 1.19**. PSD and CDP-ethanolamine pathways are the two predominant pathways utilized for the PE biosynthesis (486), while the acylation of LPE and head group base exchange have minor contribution to the overall cellular production of PE (487,488). In mammalian cells (i.e. macrophages and chinese hamster ovary (CHO) cells), it was reported that the CDP-ethanolamine pathway preferentially produces a species of PE with mono- or di-unsaturated fatty acids on the sn-2 position (e.g. PE (16:0/18:2) and PE (18:1-18:2)), whereas the

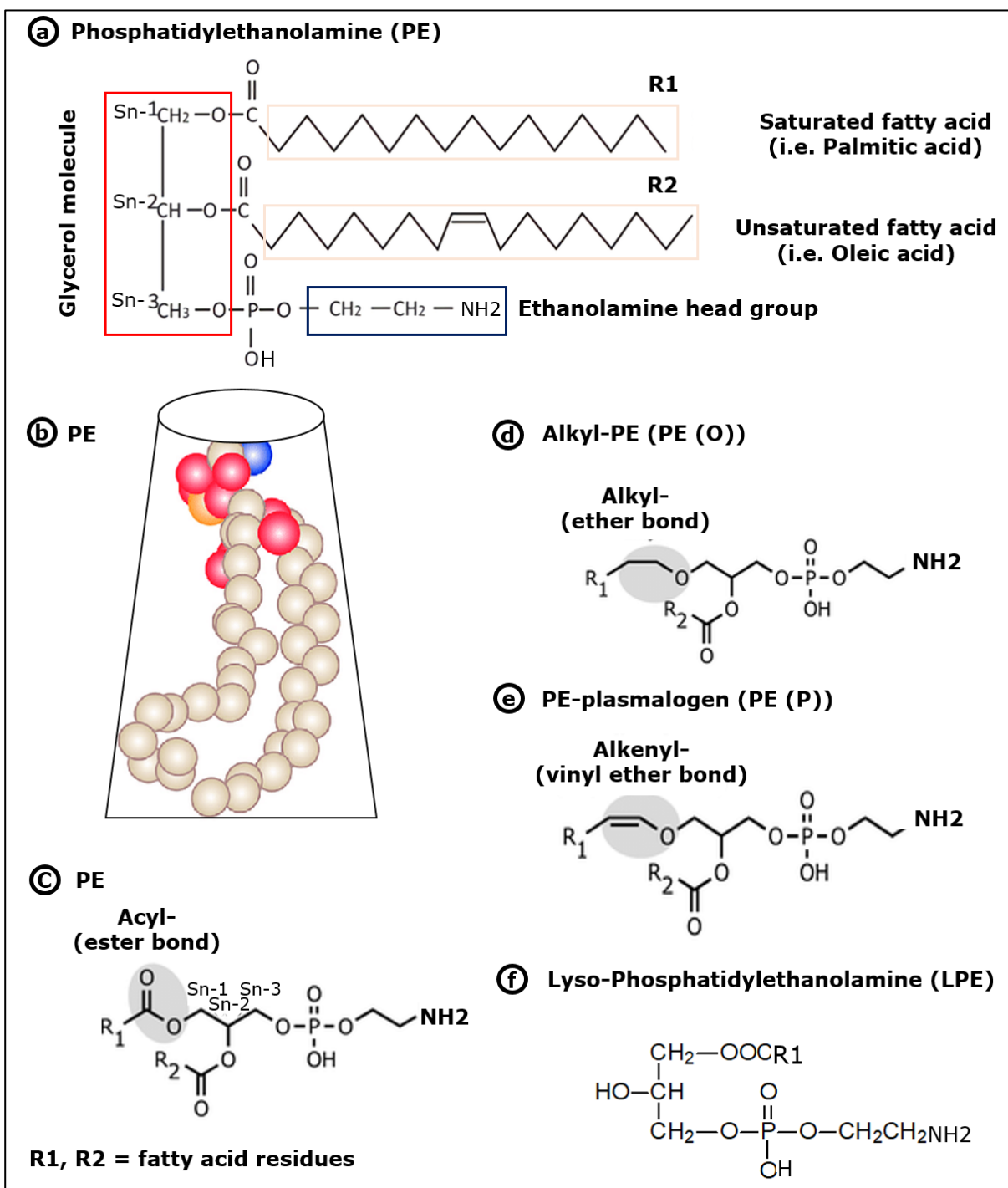


Figure 1.18: The structure of phosphatidylethanolamine and related lipids. A. General Phosphatidylethanolamine (PE) structure. In general, unsaturated fatty acids are mostly found in position sn-2 while saturated fatty acids most abundant in position sn-1. **B.** Diagram of PE structure shows the small polar head group of PE in proportion to its fatty-acid chains resulting in a cone-shaped structure, tan spheres: carbon atoms; red: oxygen; orange: phosphate; and blue: nitrogen while the hydrogen atoms are not presented. In animal tissues, PE tend to exist in **C.** diacyl (PE), **D.** alkyl-acyl (PE (O)), and **E.** alkenyl-acyl (PE-plasmalogen) forms **F.** General structure of Lyso-PE, formed mainly by hydrolysis of phosphatidylethanolamine by the enzyme phospholipase A2. [Adapted from Calzada et al. 2016 (481) and Otoki Y, et al. 2021 (482)].

mitochondrial PSD pathway generates species with mainly PUFA on the sn-2 position (e.g. PE (18:0/20:4), PE (18:0-20:5), and PE (18:0/22:6)). Importantly, it should be noted that ether PE (i.e. PE (O) and PE (P)) are generated via acyl-dihydroxyacetone (DHAP) pathway which starts in the peroxisome organelles and complete in the ER (489), (this pathway is not covered here, as it falls beyond the scope of this thesis).

Once made, PE influences all cell membrane functions such as membrane protein topology, membranes fusion, cell division, glucose metabolism, oxidative phosphorylation, mitochondrial protein biogenesis, and lysosomal autophagy (481). In addition, PE is used as a precursor for the production of PC via methylation reactions catalyzed by PE methyltransferase (PEMT) enzyme (Figure 1.19) (490,491), maintaining an appropriate PC/PE molar ratio, which is particularly important for liver cells to maintain normal liver functioning, regeneration, and production of bile acids (492,493). Importantly, it was shown that PE play an important role in lipoproteins metabolism, namely in the assembly and secretion of VLDL particles (494). PE was reported to be efficiently removed from VLDL, after its secretion, in circulation (300,495) possibly via PLTP-mediated transfer to other lipoproteins like HDL and LDL.

Disturbances in PE metabolism have been implicated in several chronic diseases. Several studies have demonstrated that PE can contribute to pathogenesis of Alzheimer's and Parkinson's diseases by being either increased or decreased, respectively (481,496,497). In addition, altered relative abundance of PE in the liver (i.e. increased PE production by Kennedy pathway or reduced biosynthesis of PC or reduced PC/PE ratio) was observed in patients with nonalcoholic fatty liver disease (NAFLD) (498,499), a condition that is often associated with obesity, insulin resistance, metabolic syndrome and ASCVD (500,501). Our understanding of the role of PE in CMD is just in its early stages and there is a growing interest of defining the mechanistic role of PE in these disease conditions. Thus, in the following section, the implication of plasma PE in the context of ASCVD will be discussed.

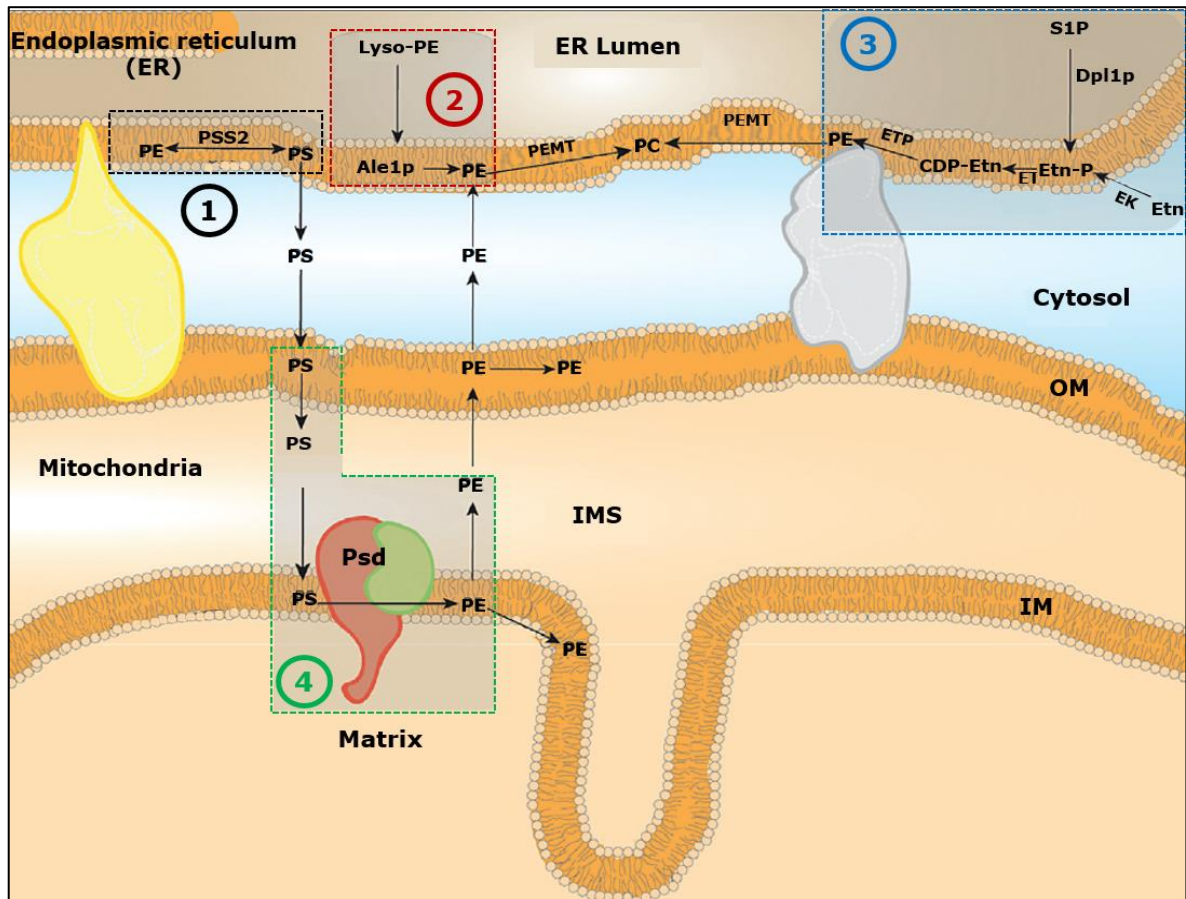


Figure 1.19: Phosphatidylethanolamine biosynthesis pathways. There are three distinct PE biosynthetic pathways in the endoplasmic reticulum (ER). **1. Base Exchange pathway.** In this pathway, the serine residue of phosphatidylserine (PS) is exchanged for ethanolamine under the action of phosphatidylserine synthase-2 (PSS2), resulting in the formation of PE. **2. Acylation of Lyso-PE.** In this pathway, dietary Lyso-PE is first translocated across the plasma membrane and then acylated into PE by the acyl-CoA-dependent acyltransferase (Ale1p) in the ER. **3. CDP-ethanolamine pathway** involves first the production of phosphoethanolamine (Etn-P) by the phosphorylation of ethanolamine by ethanolamine kinase (EK) or through degradation of sphingosine-1-phosphate by dihydro- sphingosine-1-lyase (Dpl1p). The produced Etn-P is then converted to CDP-ethanolamine (CDP-Etn) under the action of CTP: phosphoethanolamine cytidyltransferase (ET) enzyme, which is produced by PCYT2 gene. Finally, CDP-Etn undergoes a condensation reaction with diacylglycerol (DAG) under the action of 1, 2-diacylglycerol ethanolamine phosphotransferase (ETP) to form the final product, PE. In contrast to the three previously mentioned pathways, **4. Phosphatidylserine decarboxylase (PSD) pathway**, which is the second major route for PE production, resides in mitochondria. In this pathway, PS decarboxylation to PE is performed by Phosphatidylserine decarboxylase (Psd), which is embedded in the inner mitochondrial membrane. Biosynthesis of PE by PSD pathway necessitates lipid trafficking steps within organelles to transport PS from the ER (site of PS synthesis) to mitochondria (site of PE synthesis). PE generated by any of these pathways can be converted to phosphatidylcholine (PC) through the action of PE methyltransferase (PEMT). [Adapted from Calzada et al. 2016 (481) and van der Veen et al. 2017 (484)]. OM: outer membrane; IMS: inter-membrane space; IM: inner-membrane.

1.7.2 Plasma Phosphatidylethanolamines-In the context of CVD

To date, several lipidomic studies have reported the implication of PE in the context of ASCVD. Meikle et al. (15) reported that lower plasma levels of total PE (O) and total PE (P) were significantly ($p < 0.05$) associated with unstable CAD, while higher levels of total PE, and total Lyso-PE were significantly ($p < 0.05$) associated with stable CAD (versus healthy individuals). At the level of individual lipid species, 10 out of 12 species of alkyl-PE (PE (O)), and 3 out of 12 species of PE-plasmalogens (PE (P)) were negatively ($p < 0.01$) associated with unstable CAD, while 16 out of 20 species of PE, and 2 out of 6 species of Lyso-PE were positively ($p < 0.01$) associated with stable CAD as shown in **Figure 1.20**. In addition, among PE (P) species, only 2 individual species of PE (P) containing ARA (C20:4) were associated with stable CAD. The negative associations of PE (O) and PE (P) with unstable CAD appear to relate to the atherosclerotic plaque stability rather than plaque burden, whilst the positive associations of PE and LPE with stable CAD suggest their potential implication in the onset and pathogenesis of the disease. Furthermore, in a prospective population-based Bruneck Study, Stegemann and her colleagues identified several molecular PE species as a promising biomarkers to predict CVD risk (16). In this study, the sum of plasma PE was significantly ($p < 0.01$) associated with incident CVD (end point: MI, Stroke, and sudden cardiac death). Among individual species, 8 out of 15 species of PE including PE (34:1), PE (34:2), PE (36:2), PE (36:4), PE (36:5), PE (38:3), PE (38:4), PE (38:5) and 1 out of 3 species of PE (O) (i.e. PE (O) (38:5)) maintained significant association ($p < 0.01$) with incident CVD after adjustment for age, sex, and statin therapy. Interestingly, using 3 different selection algorithms, PE (36:5) was identified as one of only three lipid species, alongside TG (54:2) and CE (16:1), consistently linked to incident CVD. In a standard Cox regression model with progressive adjustment, PE (36:5) maintained significant association with incident CVD as illustrated in **Figure 1.21**. Furthermore, adding the plasma levels of PE (36:5) on top of the traditional CVD risk factors resulted in improved prediction and classification for CVD risk (16). PE (36:5), among 13 PE species, was also positively associated with total and/or CAD mortality (474). These studies strongly question the potential implication of plasma PE species, particularly PE (36:5), in the pathogenesis and progression of ASCVD.

Notably, plasma PE species are not equally distributed within plasma lipoproteins with 60% in HDL, 19% in VLDL, and 21% in LDL (208). While HDL is well-recognized for its anti-atherogenic functions, and PE (36:5) has consistently shown a deleterious association with

ASCVD, there is currently only circumstantial evidence establishing a link between PE (36:5) and HDL functions. Given the pivotal role of the fatty acid composition in determining the biological roles of phospholipids, the next section will discuss the potential biological forms of PE (36:5) and their respective fatty acid compositions.

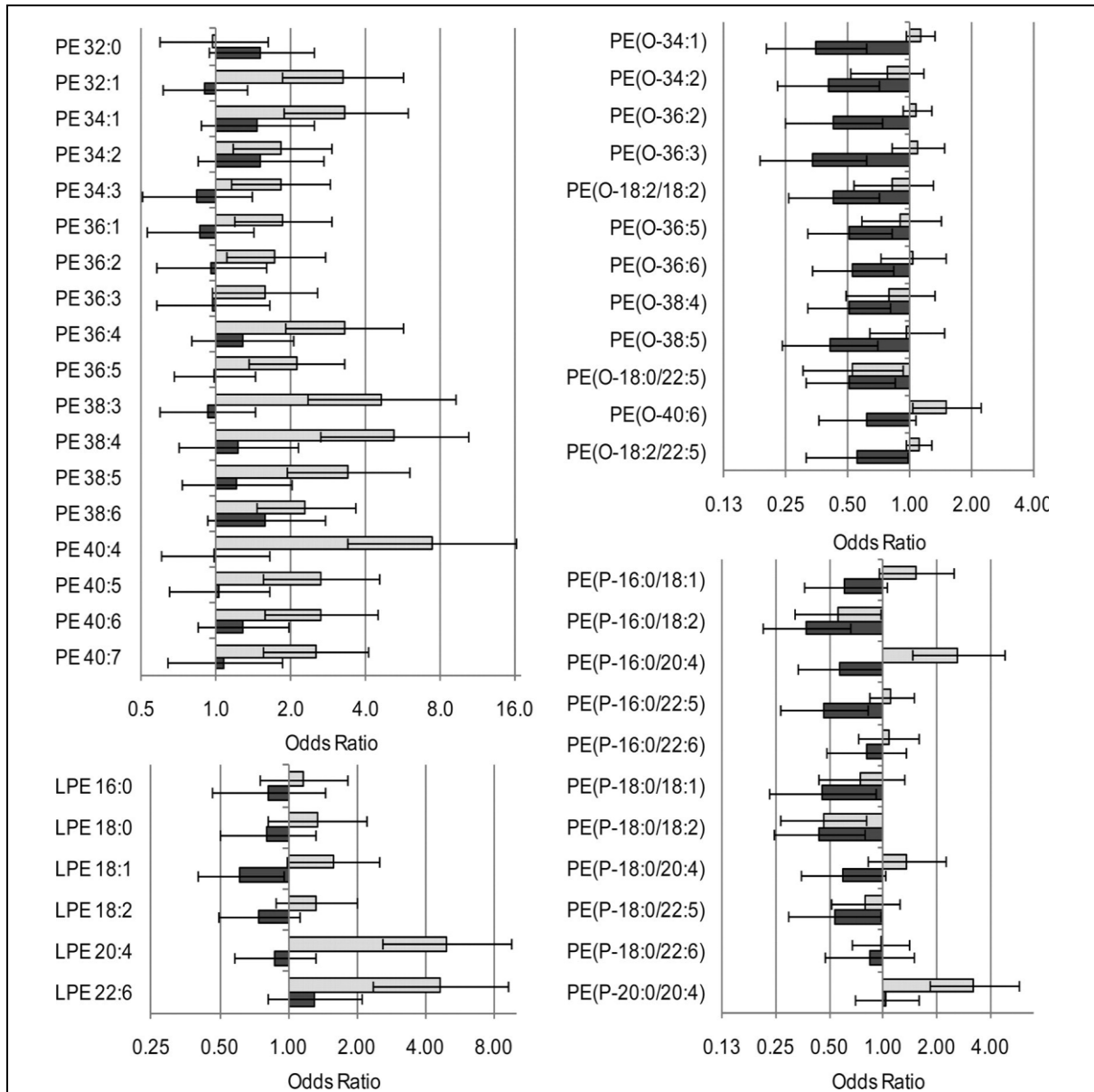


Figure 1.20: Logistic regression analysis of PE and Lyso-PE species against unstable CAD (vs stable CAD) and stable CAD (vs control). Logistic regression was performed for each lipid species against unstable CAD relative to stable CAD (dark bars), adjusting for age, sex, body mass index (BMI), systolic blood pressure (SBP), and statin use, and against stable CAD relative to control (light bars), adjusting for age, sex, BMI, SBP, and fasting blood glucose. Shown are the IQR odds ratios and 95% confidence intervals for the individual lipid species [from Meikle et al. 2011 (15)].

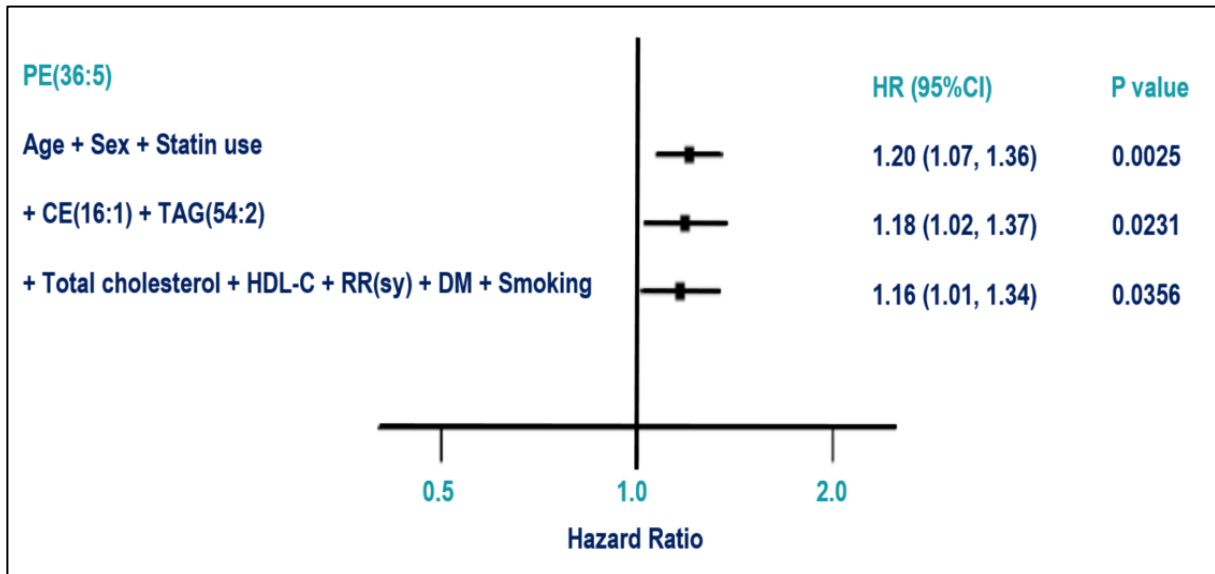


Figure 1.21: Association of PE (36:5) with incident CVD. Multivariable effect estimates and confidence intervals (CIs) for PE (36:5). The composite CVD end point considers myocardial infarction, stroke, and sudden cardiac death (Bruneck Study, n=90). HR and 95% CI were derived from standard Cox regression models with progressive adjustment and calculated for a 1-SD-higher lipid level. CE: cholesteryl ester; DM: diabetes mellitus; HDL-C: high-density lipoprotein cholesterol; HR: hazard ratio; PE: phosphatidylethanolamine; RR (sy): systolic blood pressure; and TAG: triacylglycerol [Adapted from Stegeman et al. 2014 (16)].

1.7.3 Phosphatidylethanolamine (36:5)-Biological forms

While plasma PE (36:5) has been identified as a lipid signature to predict CVD risk (16), no study determined the precise component composition (e.g. fatty acid molecular species) that comprise PE (36:5) (473). PE (36:5) is a GPL consisting of ethanolamine head group and 2 fatty acids, together contain 36 carbons and 5 double bonds; thus it represents a composite of numerous combinatorial molecular species (17). Based on the total number of carbon atoms and double bonds in the PE (36:5), six molecular species could possibly exist in human plasma including PE (14:1/22:4), PE (16:0/20:5), PE (16:1/20:4), PE (18:1/18:4), and two species of PE (18:2/18:3) with either α -linolenic acid (C18:3n3) or γ -linolenic acid (C18:3n6) (illustrated in **Figure 1.22**). Based on the blood concentrations of various PE (36:5) species quantified in normolipidemic healthy individuals (17), PE (16:0/20:5), and PE (16:1/20:4) are considered the major molecular species of PE (36:5). PE (16:0/20:5) consists of palmitic acid (C16:0) chain at the sn-1 position and EPA (C20:5n3) at the sn-2 position, referred to latter as EPA-PE, while PE (16:1/20:4) consists of palmitoleic acid (C16:1) chain at the sn-1 position and ARA (C20:4n6) at the sn-2 position, referred to latter as ARA-PE. In this regard, PE is well-known as a reservoir of PUFA (481,484).

EPA is a key anti-inflammatory/anti-aggregatory, whilst ARA acts as a precursor for several pro-inflammatory/pro-aggregatory mediators. Thus, low plasma EPA:ARA ratio is considered a marker of chronic inflammation, which is central to the development of ASCVD (502). Kondo et al. showed that patients with stable CAD have a significantly lower EPA:ARA ratio than healthy volunteers (503). Indeed, a low EPA: ARA ratio was linked to a higher risk of AS plaque progression and rupture (504–506), and was suggested as a predictive marker for early onset of ACS (507,508). Taken together, these findings collectively suggest a higher prevalence of plasma ARA over EPA in ASCVD, and implies the potential involvement of ARA as a component of PE (36:5) in CVD. Given that PE is a reservoir of PUFA (481,484), thus we speculate the predominance of ARA-PE molecular species of PE (36:5) over the EPA-PE species in these pathological conditions. The following section will discuss briefly the effects of ARA and EPA in ASCVD.

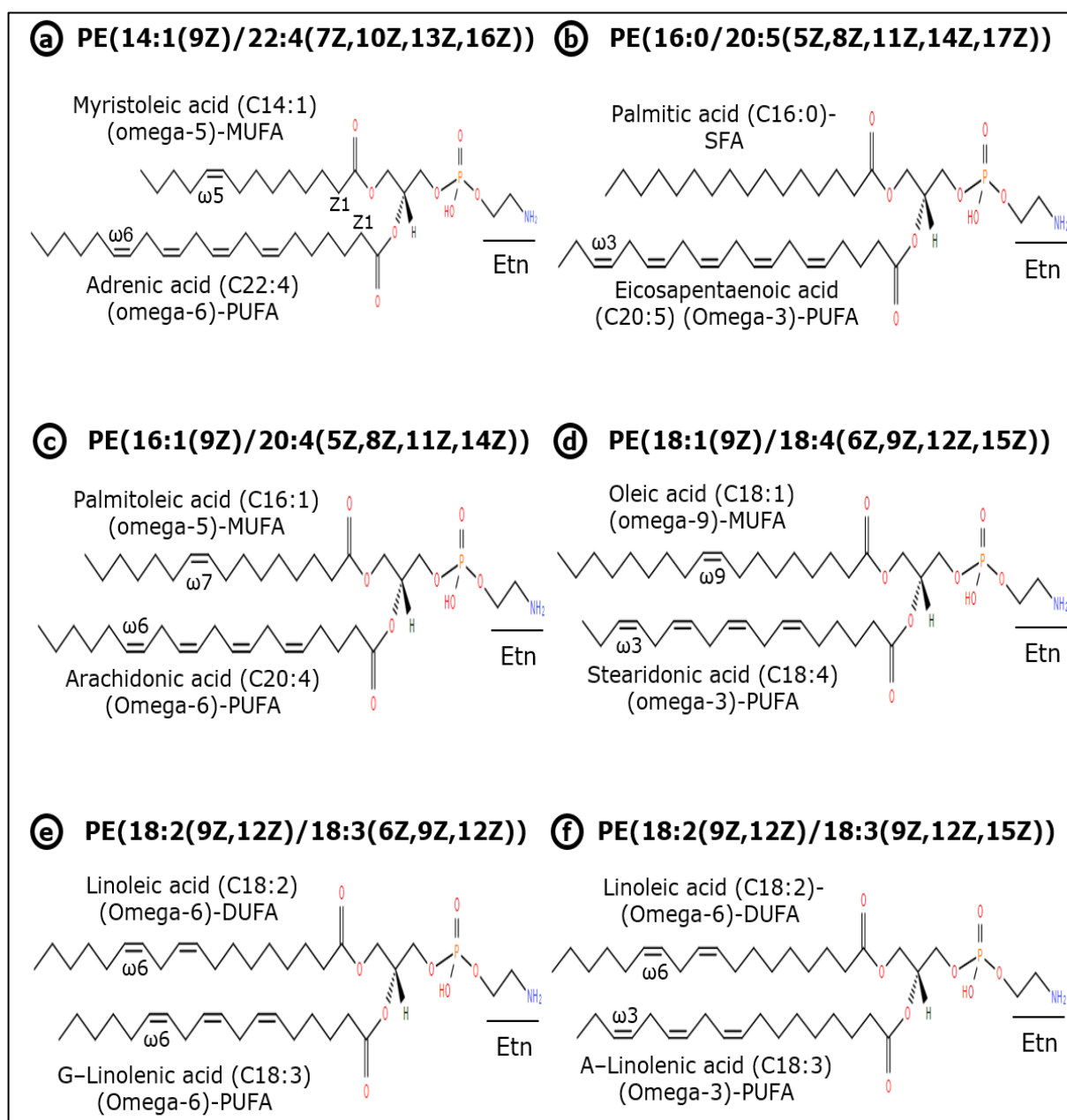


Figure 1.22: Putative molecular species of PE (36:5) and their fatty acids composition.

Average molecular weight of PE (36:5) is 737.986 (Dalton). According to the total number of carbon atoms double bonds in PE (36:5), six molecular species are possible. **A.** PE (14:1/22:4) (1-Myristoleoyl-2-adrenoyl-sn-glycero-3-phosphoethanolamine). **B.** PE (16:0/20:5) (1-Palmitoyl-2-eicosapentaenoyl-sn-glycero-3-phosphoethanolamine). **C.** PE (16:1/20:4) (1-Palmitoleoyl-2-arachidonoyl-sn-glycero-3-phosphoethanolamine). **D.** PE (18:1/18:4) 1-oleoyl-2-stearidonoyl-Sn-glycero-3- phosphoethanolamine). **E.** PE (18:2/18:3) (1-Linoleoyl-2-a-linolenoyl-sn-glycero-3-phosphoethanolamine). **F.** PE (18:2/18:3) (1-linoleoyl-2-gamma-linolenoyl-sn-glycero-3-phosphoethanolamine). DUFA: di-unsaturated fatty acid; Etn: ethanolamine; MUFA: monounsaturated fatty acid; PE: phosphatidylethanolamine; SFA: saturated fatty acid; PUFA: polyunsaturated fatty acid. Z indicate Cis double bond, while the number indicate the position of the double bond. The ω indicates omega. G indicate gamma. A indicates alpha. [Adapted from Wishart et al. 2022 (17)].

1.7.4 Arachidonic acid and Eicosapentaenoic acid in ASCVD

Both omega-3 PUFAs (i.e. α -linolenic acid (ALA), EPA, DHA, and docosapentaenoic acid (DPA)), and omega-6 PUFA (i.e. linoleic acid (LA) and ARA) are closely linked to inflammation, thus holding a pivotal role in the emergence, progression, and management of ASCVD (509,510). The key link between PUFA and inflammation is their role of serving as precursors for bioactive lipid mediators including prostanoids (i.e. prostaglandins (PGs) thromboxanes (TXs)), leukotrienes (LTs), and resolvins which are collectively involved in modulating the intensity, duration, and resolution of inflammation (511–514). Omega-6-derived mediators predominantly exert pro-inflammatory effects, whereas omega-3-derivatives mostly have anti-inflammatory effects. The metabolic pathways for production of prostanoids, LTs, and resolvins from PUFA are illustrated in **Figure 1.23**. Given their relevance to the objectives of this thesis, only ARA and EPA will be discussed in details.

1.7.4.1 Arachidonic acid in atherosclerosis

In atherosclerosis, ARA-derivatives are mainly implicated in inflammation, platelet aggregation, vascular tone and permeability (515). Following various cellular signals such as inflammation and TLR4 activation, the cytosolic Ca^{2+} dependent phospholipase A2 (cPLA2) enzyme mediate the generation of ARA from cell membrane phospholipids to control the inflammatory response (509,516). The released free ARA is metabolized mainly by the lipoxygenases (LOXs), and cyclooxygenases (COXs) enzymes into bioactive lipid metabolites (figure 1.19) (514). COXs induce the production of TXA₂, PGI₂ and PGs (i.e. PGD₂, PGE₂, PGF₂ α , and PGJ₂), while the LOXs promote the production of hydroxyicosatetraenoic acids (HETEs), LTs, and lipoxins (LXs) (511,514). ARA derived lipid metabolites are predominantly pro-inflammatory mediators, except for the LXs, which have pro-resolving, anti-inflammatory properties (509). In atherosclerosis, continuous recruitment and activation of monocytes, as well as generation and accumulation of foam cells are striking features of progressive atherogenesis as they contribute to chronic local inflammation (38,39). In this regard, ARA metabolites (i.e. PGs, TXs, LTs, and HETEs) are implicated in the inflammatory process of atherosclerosis as they contribute to monocytes recruitment and maintain the inflammatory state (509,514). Indeed, ARA was shown to enhance monocytes binding to endothelial cells, and increase the expression of adhesion molecules like VCAM-1, ICAM-1, E-selection, and pro-inflammatory cytokines including IL-6, and TNF α (517). Among the most important ARA-

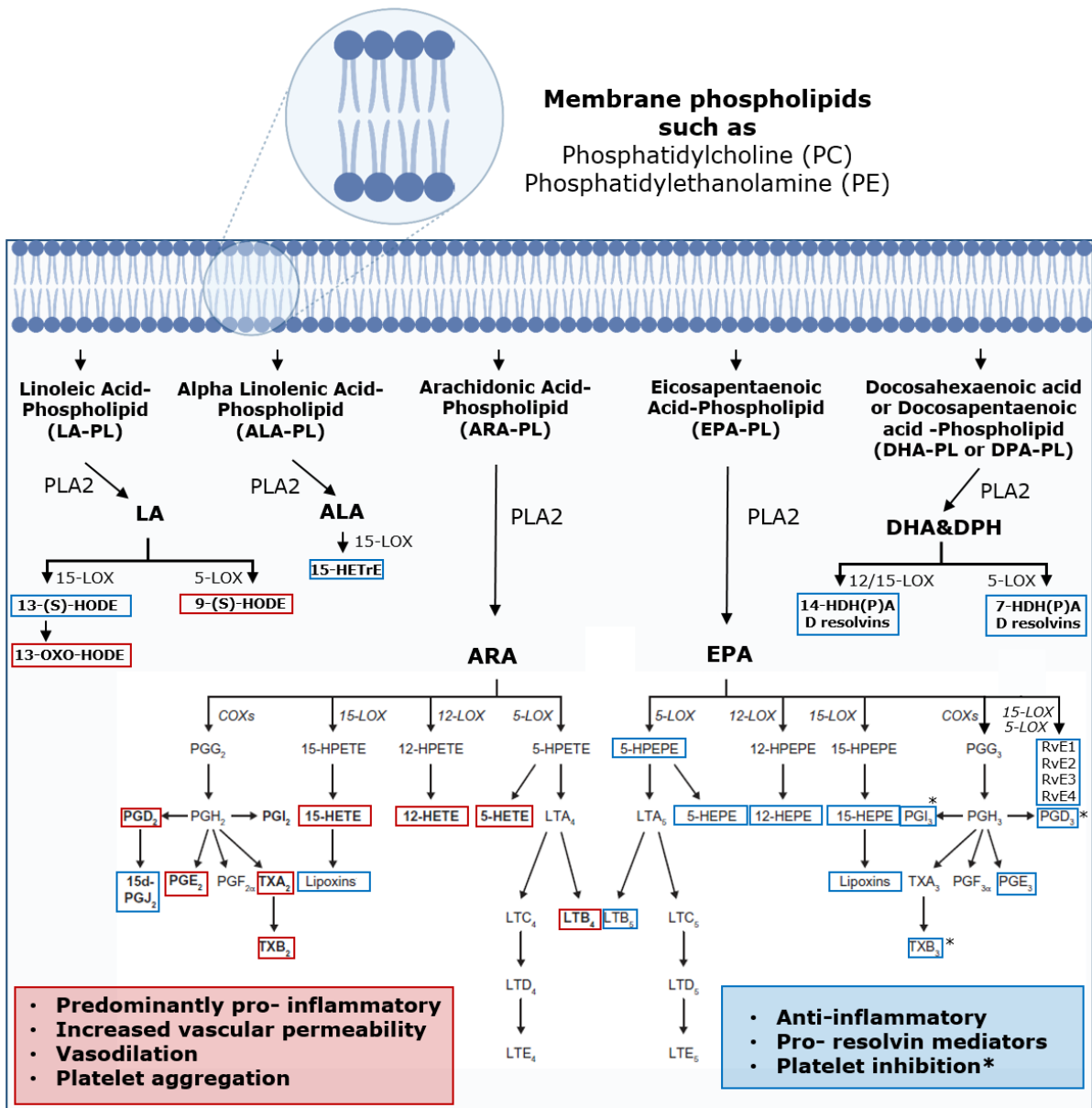


Figure 1.23: Metabolism and metabolic products of PUFA. Phospholipase A2 (PLA2) mediates the release of fatty acids from membrane phospholipids (PL), including omega-6 PUFA (i.e. linoleic acid (LA, C18:2), and arachidonic acid (ARA, C20:4)), and omega-3 PUFA (i.e. alpha linolenic acid (ALA, C18:3), eicosapentaenoic acid (EPA, C20:5), docosahexaenoic acid (DHA, C22:6), docosapentaenoic acid (DPA, C22:5)). These fatty acids can be metabolized further by lipoxygenase (LOX), or cyclooxygenase (COX) enzymes resulting in the production of a wide range of pro- and anti-inflammatory metabolites. Pro-inflammatory metabolites are marked by red box while anti-inflammatory ones are marked in blue. HETrE: hydroxyeicosatrienoic acid; HETE: hydroxyeicosatetraenoic acid; HPETE: hydroperoxyeicosatetraenoic acid; HEPE: hydroxyeicosapentaenoic acid; HPEPE: hydroperoxyeicosapentaenoic acid; HDHA: hydroxydocosahexaenoic acid; HDPA: hydroxydocosapentaenoic acid; HODE: hydroxyoctadecadienoic acid; PG: prostaglandin; TX: Thromboxane; LT: leukotriene; RV: resolvin. [Adapted from (Coras et al. 2020 (511), Larsson et al. 2004 (512), Ahluwalia et al. 2022 (513), and Calder, 2003 (514)].

derived pro-inflammatory/pro-aggregatory mediators are PGE₂, LTB₄, TXA₂ (514,518). PGE₂ was shown to increase the production of interleukin 6 (IL-6), while LTB₄ was shown also to increase IL-1 β , and to act as a chemotactic agent for monocytes (502,514).

It is noteworthy that ARA is not entirely pro-inflammatory, and it can contribute to inflammation resolution under certain conditions such as late-phase inflammation, through the production of LXs, following a switch in enzymatic ARA processing from 5-LOX to 15-LOX enzyme (509). Notably, ARA-derived LXA₄ was shown to impair the production of various pro-inflammatory cytokines, stop neutrophil chemotaxis, attenuate pro-inflammatory M1 macrophages, and foster anti-inflammatory M2 macrophages (519,520). However, when the atherogenic inflammatory stimuli such as the accumulation of oxidized LDL persist within the vascular wall, inflammation resolution by lipoxins may fail, leading to unresolved persistent inflammation established by the ARA-derived PGs, LTs, and TXs (509).

1.7.4.2 Eicosapentaenoic acid in atherosclerosis

EPA has been shown to slow the development and progression of atherosclerosis by exerting pleiotropic beneficial effects at multiple steps in the atherogenic process as illustrated in **Figure 1.24** (521–523). EPA was reported to reverse endothelial dysfunction in cultured human umbilical vein endothelial cells (HUVECs), via increasing the production of NO, synergistically with statins, while attenuating both oxidized LDL-induced ROS production (524) and palmitic acid-induced expression of adhesion molecules and cytokines, as well as apoptosis (525). Thus, EPA was shown to inhibit monocytes rolling and adhesion to lipopolysaccharide (LPS)-and TNF α -activated endothelial cells (517), and to reduce LPS-induced VCAM-1 expression (526). Moreover, the incorporation of EPA in the plasma membrane of THP-1 macrophages increased ABCA1-mediated cellular cholesterol efflux to ApoA-I, suggesting a beneficial effect of EPA in reducing cellular cholesterol accumulation and thereby foam cell formation (527). Finally, EPA was also shown to attenuate both copper-induced LDL oxidation, and glucose-induced lipid peroxidation in multilamellar lipid vesicles (528). The actions of EPA could be explained by its role as a precursor for 3-series prostanoids (e.g. PGI₃, PGE₃, TXB₃), and 5-series leukotrienes (e.g. LTB₅), which exhibit anti-inflammatory and/or anti-aggregatory effects, as shown in **Figure 1.23** (511,512,514,521). In addition, EPA serves a precursor for several pro-resolving mediators (i.e. LXs, resolvins, and protectins), which reduce monocytes recruitment, and cytokines production, thereby helping to correct and resolve the impaired resolution of inflammation seen in atherosclerosis (521,529).

Furthermore, EPA can compete with ARA to form less inflammatory mediators via competing to incorporate into membrane phospholipids as well as via direct inhibition of COX-2 and 5-LOX, resulting in a switch to produce EPA-derived prostanoids and leukotrienes (529).

Interestingly, several clinical studies confirmed the potential beneficial effects of EPA in atherosclerosis. Hence, EPA was suggested to reduce the macrophage infiltration in atherosclerotic plaques of patients who received omega-3 fish oil for a median of 42 days before undergoing carotid endarterectomy (530). In addition, when patients with very high TG levels received Icosapent ethyl, a pure ethyl ester of EPA, the plasma levels of ox-LDL levels were significantly reduced, which might result in better clearance of LDL and contribute to less foam cell formation (531).

At the level of AS plaques, EPA was shown to help stabilizing atherosclerotic plaques and thereby prevent their rupture. In ACS patients, EPA supplementation (1.8 g/day for 8 months) significantly increased fibrous cap thickness compared with baseline and untreated controls (532). The addition of highly purified EPA to statin therapy also increased fibrous-cap thickness, while decreased plaque lipid content in ACS patients, suggesting better plaque stabilizing effects than the statin alone (533). Furthermore, in patients with T2D (534) and hypertriglyceridemia (535), EPA was also shown to decrease the cIMT. Interestingly, the EPA content of carotid plaque phospholipids was inversely correlated with the median score of seven plaque features including lipid core, foam cells, haemorrhage, overall inflammation, fibrous cap inflammation, macrophages in plaque, macrophages in cap (536). Altogether, these studies provide evidence that EPA have a range of overlapping benefits across the spectrum of ASCVD disease, resulting in improved vascular and coronary health.

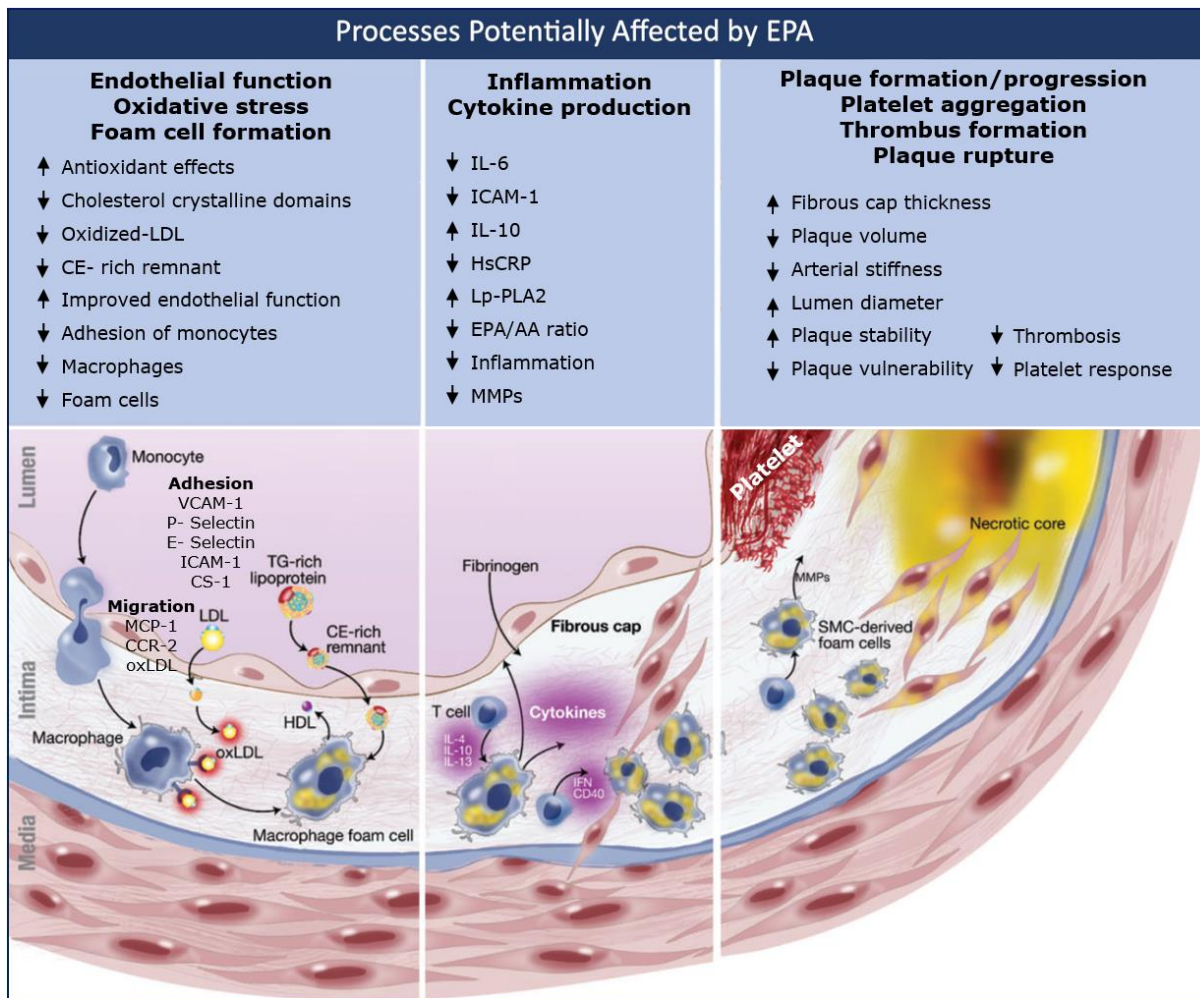


Figure 1.24: The cellular and molecular beneficial effects of EPA on atherosclerosis. Atherosclerosis is a multistep process starting from endothelial dysfunction and advancing to plaque development, progression, and rupture. EPA has been reported to have beneficial effects on multiple atherosclerosis processes including endothelial function, oxidative stress, foam-cell formation, inflammation/ cytokines, plaque formation/progression, platelet aggregation, thrombus formation, and plaque rupture. CCR: C-C chemokine receptor; CS: connecting segment; CE: Cholesteryl ester; EPA: eicosapentaenoic acid; EPA/AA: eicosapentaenoic acid/arachidonic acid ratio; HDL: high-density lipoprotein; HsCRP: high-sensitivity C-reactive protein; ICAM: intercellular adhesion molecule; IFN: interferon; IL: interleukin; LDL: low-density lipoprotein; Lp-PLA2: lipoprotein-associated phospholipase A2; MCP: monocyte chemotactic protein; MMP: matrix metalloproteinase; oxLDL: oxidized LDL; SMC: smooth muscle cell; Th: T helper; VCAM: vascular cell adhesion molecule. [Adapted from borrow et al. 2015 (521), Nelson et.al. 2021 (522), and Budoff et al. 2018 (523)].

HYPOTHESIS AND OBJECTIVES

2.1 HYPOTHESIS

2.2 OBJECTIVES AND EXPERIMENTAL APPROACH

2

"The greatest discoveries have come from people who have looked at a standard situation and seen it differently." - Albert Szent-György

Chapter 2. Working hypothesis and objectives

2.1 Hypothesis

It has been widely accepted that low HDL-C is a strong and independent risk factor for ASCVD. However, the failure of HDL-C raising therapies to reduce the ASCVD risk have illuminated a crucial insight: the "quantity" of HDL-C may not be the key therapeutic target. Instead, the spotlight turns to the concept of HDL functionality "quality" as a more accurate predictor of ASCVD risk. HDL particles are highly heterogeneous in their structure (i.e. size and shape) and composition (i.e. mainly lipids and proteins), with such heterogeneity closely linked to their atheroprotective functions. Within HDL lipidome, phospholipids have emerged in several studies as pivotal determinants of HDL functions. In CMD and ASCVD, the HDL phospholipidome often undergoes alterations, accompanied by a reduction or complete loss of HDL's anti-atherogenic function/s. This suggests a profound link between specific phospholipid species within HDL, its altered functionality, and an elevated risk of ASCVD. Consequently, understanding the impact of such phospholipid species on HDL functionality could help to identify novel biomarkers for evaluating and enhancing HDL function as a therapeutic strategy to ameliorate ASCVD outcomes.

Available plasma lipidomic data reveals a significant positive correlation between most PE species and the initiation and progression of ASCVD. Among these, PE (36:5) emerges as a signature lipid species, displaying the strongest predictive value for ASCVD. These observations highlighted the strong interest to unravel the mechanistic implications of these lipid species in ASCVD pathogenesis, and to define the precise molecular species that drive the strong association of PE (36:5) with incident CVD. Of the six putative molecular species of PE (36:5), two species including PE (16:0/20:5) or EPA-containing PE (36:5), and PE (16:1/20:4) or ARA-containing PE (36:5) are considered the major species of PE (36:5) exists in human plasma, with ARA-PE (36:5) dominating conditions linked to chronic inflammation, such as CMD and atherosclerosis.

With the majority of plasma PE cargo is carried by HDL, and previous indications that total PE content in HDL potentially influences both the structural properties of HDL, and the capacity of human serum to promote cellular cholesterol efflux, a pivotal hypothesis arises.

Although plasma HDL is well-recognized for its anti-atherogenic functions, and the plasma PE, notably PE (36:5), have consistently shown a deleterious association with ASCVD in lipidomic studies, there is currently no direct evidence establishing a link between PE, and PE (36:5) in particular, and HDL functions. Here we directly tested the hypothesis of that the positive deleterious association between plasma PE and PE (36:5) and CVD reflects deleterious link between PE and HDL functionality "i.e. quality". In specific, we propose that the presence of PE and PE (36:5) within HDL particles might affect their anti-atherogenic functions, and their role in atheroprotection and potentially accelerating atherogenesis, and elevating ASCVD risk (**Figure 2.1, H-1**). In light of the well-known deleterious effects of ARA in ASCVD, our hypothesis suggests that the presence of ARA in PE within HDL could add more deleterious effects on HDL functions (**Figure 2.1, H-2**), whilst the incorporation of EPA in PE in HDL is expected to promote beneficial effects, building upon the well-known protective effects of EPA in ASCVD (**Figure 2.1, H-3**).

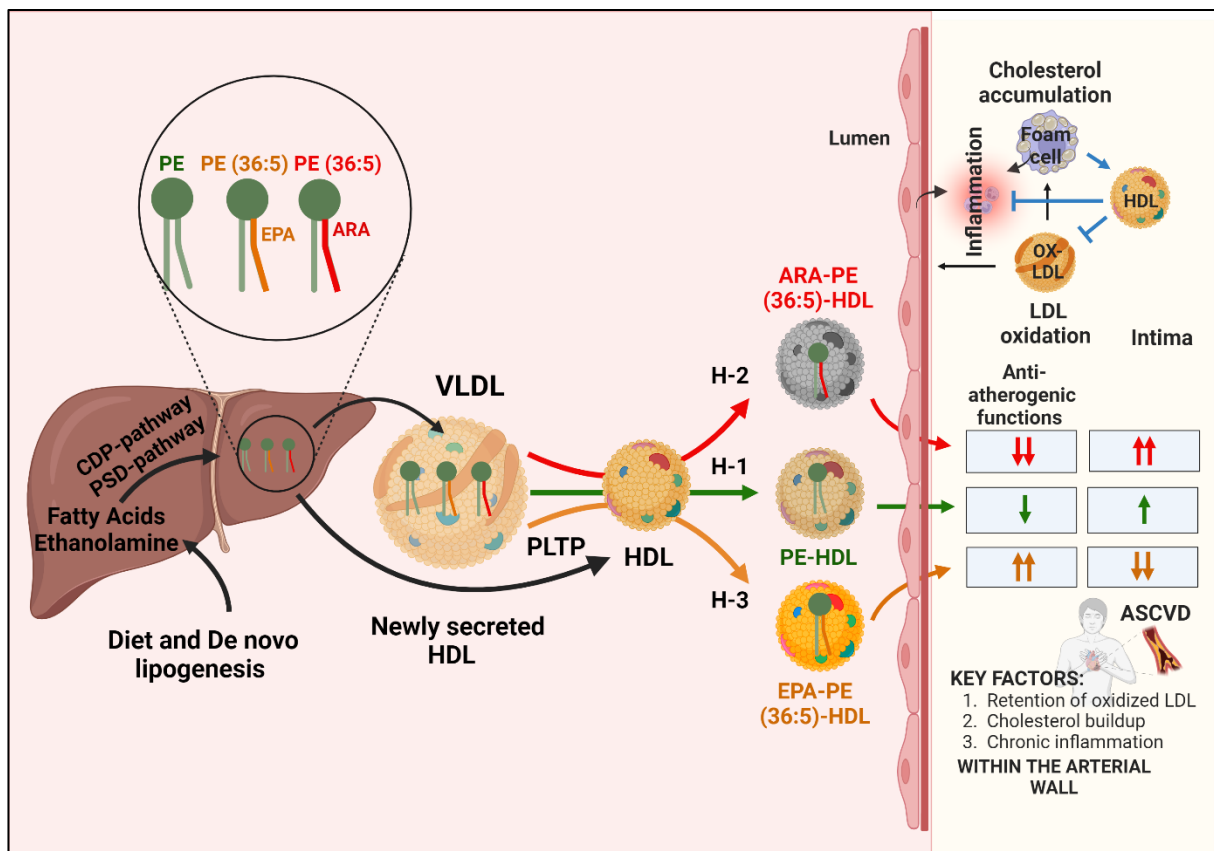


Figure 2.1: Proposed mechanistic insights into how plasma PE and PE (36:5) may promote atherosclerosis. ARA: arachidonic acid; CDP: Cytidine diphosphate; EPA: eicosapentaenoic acid; H: hypothesis; HDL: high density lipoprotein; VLDL: very low density lipoprotein; PE: phosphatidylethanolamine; PLTP: phospholipid transfer protein; PSD: Phosphatidylserine decarboxylase; Ox-LDL: oxidized low density lipoprotein.

2.2 Objectives and experimental Approaches

In the pursuit of a deeper understanding of the potential mechanisms that underlie the strong positive association of plasma PE species with ASCVD, this doctoral dissertation sets forth a series of objectives that could collectively contribute to unraveling this association. The overall objective of this study is to elucidate the impact of PE, and particularly the PE (36:5) molecular species, on the atheroprotective functions of HDL. To address these objectives, a multifaceted experimental approach encompassing both clinical and preclinical methodologies has been employed.

Objectives and approaches

Objective one: Association between PE and Atherosclerosis: The first objective of this study is to explore the potential association between both total PE and PE (36:5) contents within HDL particles and the development of atherosclerosis. By investigating the relationships of these lipid components of HDL with established clinical markers of atherosclerosis, a comprehensive assessment of their potential role in atherosclerosis development will be accomplished.

Approach: Human study: this clinical approach (**Figure 2.2A**) involves the isolation of HDL2 particles from women diagnosed with metabolic syndrome, followed by quantifying the content of total PE and PE (36:5) within these isolated particles. Then, the associations between the content of these lipid species in HDL and atherosclerosis markers such as cIMT, the presence of AS plaque in carotid arteries, and CAC scores will be evaluated.

Objective two: HDL's Efficacy to reverse atherosclerosis and ARA-PE (36:5): The second objective seeks to evaluate whether HDL's capacity to mitigate the development of atherosclerosis is adversely affected by the presence of ARA-PE (36:5) molecular species. Through the employment of a relevant murine model, the influence of this specific molecular species on the development of atherosclerotic plaque will be elucidated.

Approach: *In vivo* atherostudy: this approach (**Figure 2.2B**) involves employing TgCETP x *Ldlr*^{-/-} female mice model subjected to a high-cholesterol diet to induce atherosclerosis. Then, mice will be treated with ARA-PE (36:5)-containing rHDL, and the impact of these particles on the size of aortic AS plaque will be evaluated.

Objective three: Structural and Functional Modifications of HDL: The third objective aims to delve into the potential modifications induced by the presence of PE and particularly ARA-PE (36:5) and EPA-PE (36:5), on the structural and functional properties of HDL. By assessing the impact of these lipid species on rHDL particles, a deep understanding of their impact on HDL's quality (i.e. structure and function) will be achieved.

Approach: *In vitro* study: This approach (**Figure 2.2C**) involves generation of rHDL particles containing various PE species, including Soy-PE, ARA-PE, and EPA-PE, and then evaluating the main structural and functional properties of these particles.

By achieving these approaches, this dissertation strives to establish a direct link between PE, particularly PE (36:5), and HDL functions. These efforts aim to provide mechanistic insights to explain the positive association of PE species and ASCVD and to decipher novel biomarkers of HDL functionality (i.e. quality), which could have promising implications for the development of future therapeutic approaches in the context of ASCVD.

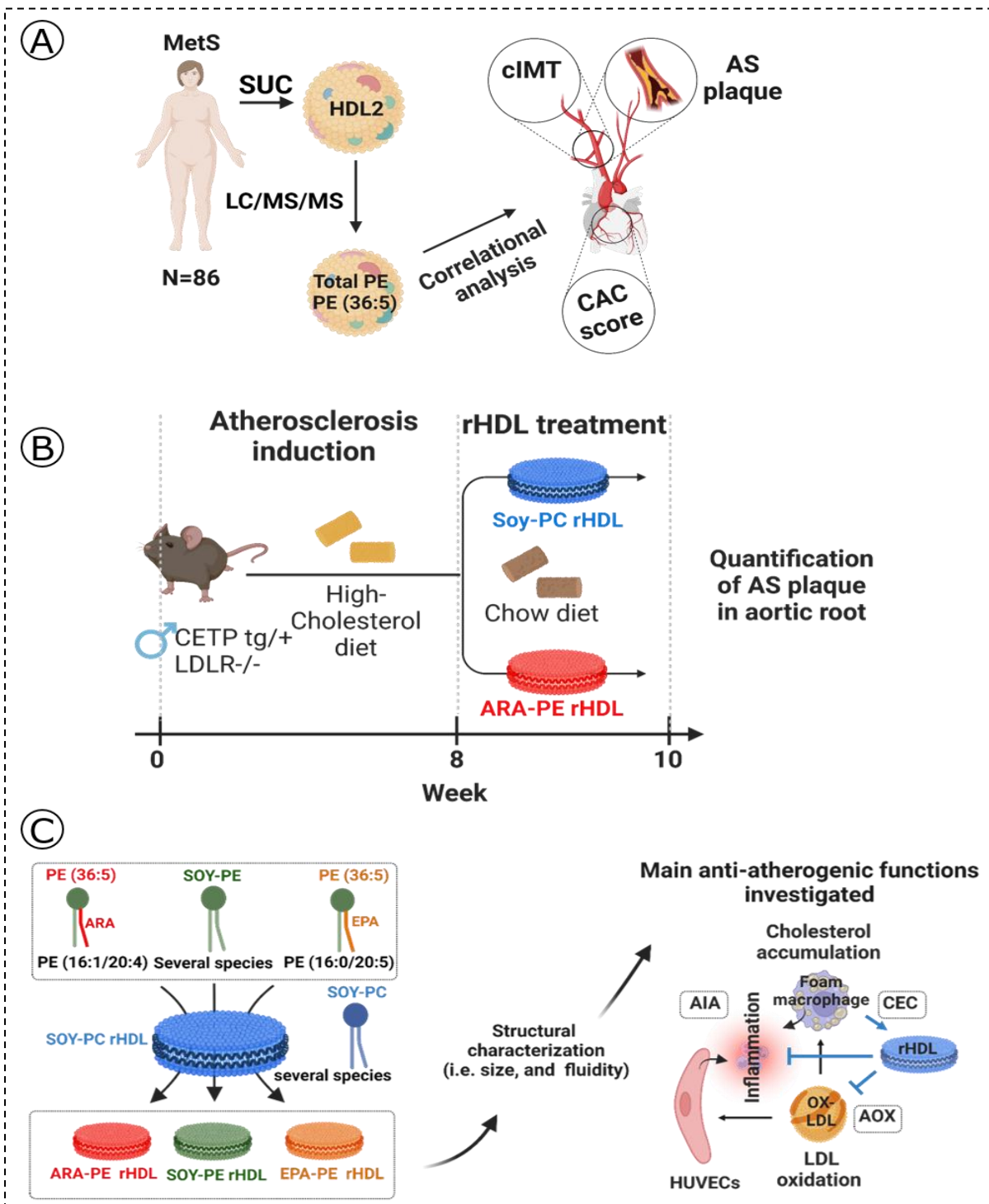


Figure 2.2: Experimental strategies. Our study involved three methodological approaches. **A.** Human study. **B.** *In vivo* atherosclerosis study and **C.** *In vitro* study. AIA: anti-inflammatory activities; AOX: anti-oxidative activities; AS: Atherosclerotic; ARA: arachidonic acid; CAC score: coronary artery calcium score; CETP: Cholesteryl ester transfer protein; cIMT: carotid intima-media thickness; CEC: cholesterol efflux capacity; HUVECs: human umbilical vein endothelial cells; MetS: Metabolic syndrome; SUC: sequential ultracentrifugation; LC/MS/MS: Liquid chromatography-tandem mass spectrometry; Ox-LDL: oxidized low density lipoprotein; LDLr: LDL receptor; PC: phosphatidylcholine; PE: phosphatidylethanolamine. In both *in vivo* and *in vitro* studies, SOY-PC rHDL which contains only Soy-PC was used as a control rHDL.

RESEARCH WORK

REGULATION OF HDL DYSFUNCTIONALITY BY
PHOSPHATIDYLETHANOLAMINE LINKS POLY-
UNSATURATED FATTY ACIDS WITH
ATHEROSCLEROTIC CARDIOVASCULAR
DISEASES

3

*"No amount of experimentation can ever prove me right; a single experiment can
prove me wrong." - Albert Einstein*

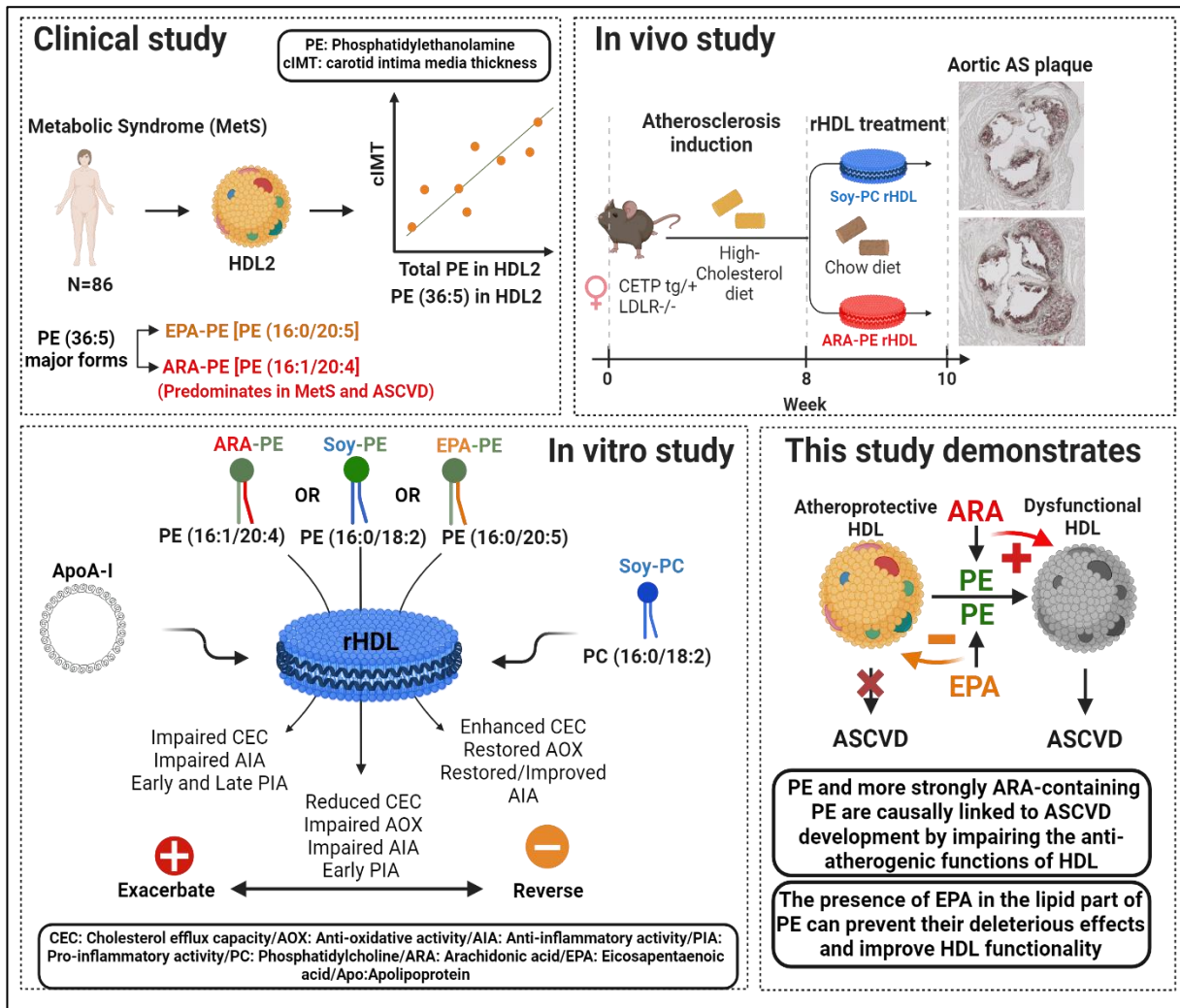
Chapter 3. Research work

Regulation of HDL dysfunctionality by phosphatidylethanolamine links poly-unsaturated fatty acids with atherosclerotic cardiovascular diseases

Malik Taradeh, Lise Hardy, Veronica Dahik, Marie Lhomme, Hua Wang, Canelle Reydellet, Clément Materne, Pukar KC, Jean-Paul Pais-De-Barros, Sophie Galier, Eric Frisdal, Hervé Durand, Maharajah Ponnaiah, Petra El Khoury, Elise F. Villard, Thierry Huby, Philippe Lesnik, Antonio Gallo, Philippe Giral, Eric Bruckert, Maryse Guerin, Anatol Kontush, Isabelle Guillas and Wilfried Le Goff

Submitted for publication

Graphical abstract



REGULATION OF HDL DYSFUNCTIONALITY BY PHOSPHATIDYLETHANOLAMINE LINKS POLY-UNSATURATED FATTY ACIDS WITH ATHEROSCLEROTIC CARDIOVASCULAR DISEASES

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Abstract.

Aims. Low plasma high-density lipoprotein (HDL)-cholesterol levels are associated with elevated risk of atherosclerotic cardiovascular disease (ASCVD), an effect potentially reflecting the antiatherogenic functions of HDL. These functions, including cholesterol efflux capacity (CEC), anti-inflammatory (AIA) and anti-oxidative (AOX) activities, are largely influenced by the HDL phospholipidome. Patients with cardiovascular diseases frequently exhibit alterations of phospholipidome and functions of HDL. Several studies reported positive associations of plasma levels of phosphatidylethanolamines (PE) species, notably PE (36:5), with ASCVD although the mechanisms are unknown. Plasma PE (36:5) exists as eicosapentaenoic (EPA)-PE and arachidonic acid (ARA)-PE, with the latter predominating in ASCVD. The objective of this study was to determine whether the association of PE (36:5) with ASCVD might result from an impairment of the antiatherogenic functions of HDL.

Methods and Results. We observed that total PE and PE (36:5) contents of large HDL isolated from 86 women with metabolic syndrome were positively associated with carotid intima-media thickness in multivariate regression analysis adjusted for traditional risk factors. Retro-orbital injection of reconstituted HDL (rHDL) containing ARA-PE led to an increase of the size of atherosclerotic plaques in TgCETP x Ldlr^{-/-} mice fed a high-cholesterol diet in comparison to the injection of control rHDL containing phosphatidylcholine (PC) alone. *In vitro* studies showed that PE rHDL exhibited a reduced CEC and an impaired AIA in human THP-1 macrophages, and a diminished AOX against copper-induced LDL oxidation compared to control rHDL. Strikingly, ARA-PE rHDL exhibited more profound alterations of the HDL function, while EPA-PE rHDL counteracted the dysfunction of PE and ARA-PE rHDL and could potentiate the functionality of rHDL.

Conclusion. Our findings uncover a causal link between PE species, especially, ARA-PE, with HDL dysfunction and atherosclerosis, which can be potentially reversed by EPA. This study supports the therapeutic usefulness of EPA in the restoration of the HDL functions and the reduction of ASCVD.

Keywords

High-density lipoproteins, phosphatidylethanolamine, arachidonic acid, eicosapentaenoic acid, atherosclerosis

Running title

Modulation of HDL functions by phosphatidylethanolamine

Translational Perspective.

This study provides new and potentially important information on ongoing therapies actually in development in patients with a high risk of cardiovascular diseases whose therapeutic efficacy is highly debated. By bringing to light the role of polyunsaturated fatty acid in phosphatidylethanolamine species for HDL functions, this study proposes that omega-3 eicosapentaenoic acid (EPA) might counteract the dysfunctionality of HDL observed in patients with atherosclerotic cardiovascular diseases and provides potential clues on the cardioprotective effect of EPA reported in the REDUCE-IT clinical trial. Those findings support the usefulness of the consumption of pure EPA, and possibly infusion of EPA-PE containing reconstituted HDL, in patients with atherosclerotic cardiovascular diseases.

Abbreviations.

Apo: apolipoprotein
ARA: arachidonic acid
ASCVD: atherosclerotic cardiovascular disease
CAD: coronary artery disease
CETP: cholesteryl ester transfer protein
cIMT: carotid intima-media thickness
CMD: cardiometabolic diseases
DPH: diphenylhexatriene
EDTA: ethylenediaminetetraacetic acid
EPA: eicosapentaenoic acid
ER: endoplasmic reticulum
FPLC: fast protein liquid chromatography
HDL-C: high-density lipoprotein
IL-1 β : interleukin-1 beta
LA: linoleic acid
LPC: lysophosphatidylcholine
LPDP: lipoprotein-deficient plasma
LPS: lipopolysaccharide
Met: methionine
MetS: metabolic syndrome
PBS: phosphate buffer saline
PC: phosphatidylcholine
PE: phosphatidylethanolamine
PI: phosphatidylinositol
PLOOH: phospholipid hydroperoxide
PUFA: polyunsaturated fatty acid
rHDL: reconstituted HDL
Soy-PC: L- α phosphatidylcholine
Soy-PE: L- α phosphatidylethanolamine
TMA-DPH: trimethylamine-diphenylhexatriene

Introduction.

Atherosclerotic cardiovascular diseases (ASCVD) are the leading cause of morbidity and mortality in Western countries. It is well established that low circulating concentrations of cholesterol associated with high-density lipoproteins (HDL-C) are predictive of ASCVD incidence¹, making HDL a promising target to reduce ASCVD². However, therapeutic strategies raising HDL-C have failed to reduce ASCVD mortality³. Multiple biological activities are displayed by HDL, including macrophage cholesterol efflux, anti-oxidative, anti-inflammatory, and cytoprotective activities². Among them, HDL capacity to promote cholesterol efflux from macrophages is proposed to underlie the association between HDL-C and ASCVD. Indeed, the capacity of HDL to promote macrophage cholesterol efflux is inversely associated with atherosclerosis development⁴, incident CV events⁵, and mortality in patients with myocardial infarction⁶ independently of HDL-C levels. Those studies paved the way to the notion that HDL functionality could represent a better indicator of ASCVD risk than HDL-C levels⁷.

Structure (size and shape) and composition (lipid and protein content) of HDL particles are highly heterogeneous, with such a heterogeneity being intimately linked to their biological functions^{2,8}. Besides apolipoprotein (apo) A-I which constitutes around 70% of total proteins, proteomic and lipidomic studies identified hundreds of individual protein and lipid species in HDL^{9,10}. Phospholipids account for 35-50% of total HDL lipidome, with phosphatidylcholines (PC, 33–45wt%) being the major phospholipid class^{10,11} followed by moderately abundant classes such as lysophosphatidylcholine (LPC, 0.5–5wt%), phosphatidylethanolamine (PE, 0.5–1.5wt%), and phosphatidylinositol (PI, 0.5–1.5wt%)¹⁰. Phospholipids were reported previously to modulate HDL functionality based on their class, abundance, and biophysical properties^{12–14}. Alterations of both phospholipidome and biological functions of HDL are detected in ASCVD patients, suggesting that the presence and/or the abundance of specific phospholipid species within HDL can be associated with altered HDL functionality and increased CVD risk^{15–17}.

Lipidomic studies have revealed a positive association between the plasma levels of several PE species and ASCVD^{16,18}. Among them, PE (36:5) displayed the strongest association with ASCVD¹⁸, with

earlier lipidomic analyses of plasma from control individuals and patients with CVD highlighting the deleterious role of PE (36:5). Thus, plasma concentrations of PE (36:5) were significantly higher in patients with stable coronary artery disease (CAD)¹⁶. Even more strikingly, the prospective population-based Bruneck Study identified PE (36:5) among only three lipid species displaying the strongest predictive value for CVD¹⁸. Moreover, inclusion of PE (36:5) on top of traditional risk factors resulted in improved risk prediction and classification in this cohort¹⁸. However, the molecular mechanisms through which PE (36:5) and total PE promote ASCVD are unknown¹⁹.

In plasma, about 60% of total PE cargo is carried by HDL particles¹¹. In this regard, PE was reported to influence structural properties of HDL²⁰, and its content in HDL was negatively correlated with the capacity of human serum to promote cellular cholesterol efflux²¹. Two major PE (36:5) subspecies are found in human plasma²², namely PE (16:0/20:5), and PE (16:1/20:4), with the latter being speculated to predominate in cardiometabolic diseases based on the reduced eicosapentaenoic acid (EPA, 20:5) to arachidonic acid (ARA, 20:4) ratio observed in patients with ASCVD and in obese women with metabolic dysfunction^{23,24}. EPA is an omega (ω)-3 polyunsaturated fatty acid (PUFA) that exerts several beneficial effects against ASCVD²³. In this context, the cardiovascular risk was reported to be lower in patients receiving a highly purified EPA ethyl ester than in those on placebo²⁵. Conversely, ARA, an ω -6 PUFA, is widely known for its deleterious pro-inflammatory roles in ASCVD²⁶. Several studies reported the capacity of PUFA to modulate HDL functionality. Hence, ω -3 fatty acids, such as EPA and linolenic acid, improve, while ω -6 fatty acids, such as linoleic acid (LA), attenuate the atheroprotective functions of reconstituted HDL²⁷⁻²⁹. However, knowledge about the effects of EPA- and ARA-containing PE (36:5) on HDL functionality remains unknown.

In this study, we addressed the hypothesis that PE, and particularly ARA-containing PE (36:5), can promote ASCVD by altering the atheroprotective functions of HDL. We demonstrated that total PE and PE (36:5) contents in HDL are associated with atherosclerosis development in women with metabolic syndrome (MetS). Injection of reconstituted HDL (rHDL) in *TgCETP x Ldlr^{-/-}* mice confirmed

the proatherogenic role of ARA containing-PE (36:5). Finally, our *in vitro* studies uncovered that both total PE and ARA-containing PE (36:5) attenuated the functions of rHDL, which were restored, or even improved, with EPA-containing PE (36:5). Taken together, our study suggests that the presence of PE and PE (16:1/C20:4) in HDL may represent novel biomarkers for HDL dysfunctionality and may underlie the deleterious role of total PE and PE (36:5) in ASCVD. By contrast, the presence of PE (16:0/C20:5) in HDL reverses this deleterious effect, providing new mechanistic clues in the understanding of the protective role of EPA in ASCVD.

Materials and Methods.

Patients with metabolic syndrome.

Assessment of the impact of the PE and PE (36:5) content of large HDL (HDL2) on the development of atherosclerosis was performed in a group of 86 women with metabolic syndrome (MetS) for whom carotid intima-media thickness (cIMT) was measured using ultrasound as previously described³⁰. Mean cIMT was defined for each individual as the average of the right and the left common cIMT. Major clinical and biochemical parameters of women with MetS are presented in **Supplemental Table 1**. Patients were classified as displaying MetS on the basis of modified Adult Treatment Panel III criteria as described in our early cohort³⁰. The study was performed in accordance with ethical principles outlined in the declaration of Helsinki. Written informed consent was obtained from all patients.

Isolation and characterization of plasma HDL.

HDL2 were isolated from human plasma by sequential ultracentrifugation. Briefly, the density of plasma or medium was increased to 1.063 g/ml by addition of dry solid potassium bromide. Apolipoprotein (apo) B-containing lipoproteins ($d < 1.063$ g/ml) were removed after a first ultracentrifugation at 45,000 rpm for 24 h at 15°C using a Beckman 50.4 rotor in a Beckman XL70 ultracentrifuge. Then, the density of plasma was increased to 1.21 g/ml and HDL fractions ($d < 1.21$ g/ml) were isolated after a second ultracentrifugation under the same settings of the first one. Purified HDL fractions were dialyzed using Spectrapor membrane tubing against phosphate buffer saline (PBS) at pH 7.4 before analysis for their lipid and protein content and used in functional assays.

Preparation of reconstituted HDL.

Isolation of human ApoA-I. Human ApoA-I was isolated from human plasma and purified by fast protein liquid chromatography (FPLC). Total HDL was isolated by sequential ultracentrifugation. The first ultracentrifugation step in Beckman 70 Ti rotor at 45,000 rpm for 24 hours at 15°C at a density of 1.063 g/ml allowed to remove all apo B-containing lipoproteins. Then total HDL was recovered after the second ultracentrifugation step at a density of 1.21 g/ml under the same conditions. Total HDL were

extensively dialyzed against ammonium buffer (5 mM, pH 7.4) and lyophilized, resuspended, and delipidated completely with methanol/ether (1:4) at -20°C. Precipitated proteins were dried under speed vacuum and ApoA-I was purified by ion-exchange chromatography. After protein solubilization in buffer A (20 mM Tris/6 M urea, pH 8.5) at a concentration > 12mg/ml, ApoA-I was loaded on XK26-40 chromatography column and separated at a flow rate of 3 ml/min in Tris-urea buffer by biologic DuoFlow systems (BioRad, France). The purity of individual ApoA-I-containing fractions detected at 280 nm was assessed on 20% denaturing acrylamide gel revealed with Coomassie blue. Pure fractions were pooled and dialyzed against ammonium buffer (20 mM, pH 7.4) and lyophilized.

Preparation of reconstituted HDL. Reconstituted HDL (rHDL) particles were prepared by sodium cholate dialysis as previously described³¹. Briefly, human ApoA-I was mixed with L- α phosphatidylcholine (Soy-PC) without or with either L- α phosphatidylethanolamine (Soy-PE; Avanti Polar Lipids, AL, USA), PE (16:1/20:4), or PE (16:0/20:5; synthesized by ICBMS, Lyon, France), at a molar ratio of 1:90 (ApoA-I: Soy-PC) for Soy-PC rHDL and 1:70:20 (ApoA-I:Soy-PC:PE) for either Soy-PE, PE (16:1/20:4), or PE (16:0/20:5) rHDLs (Supplemental Table 2). The required amounts of phospholipids in chloroform were mixed and dried under nitrogen gas. To form micelles, sodium cholate (30 mg/ml) was added to the dried lipids at a molar ratio of 1:1 (sodium cholate: phospholipid) in Tris-buffered saline (TBS, pH 7.4) and vortexed every 15 minutes until the solution became clear. Then ApoA-I was added to the mixture and incubated for 2h at 4°C. Finally, rHDL particles were dialyzed against TBS for 5 days and against PBS for 3 days and stored at -80°C. All the rHDL concentrations were based on their ApoA-I content, which was measured using Indiko™ Plus clinical chemistry analyzer (Thermo Scientific, US) according to the manufacturer's instructions. Quality control of rHDL and ApoA-I was performed using non-denaturing TBE 4–20% gradient polyacrylamide gel electrophoresis and staining with Coomassie Brilliant Blue (Supplemental Figure 1A and B)²⁷.

Lipidomic analysis.

LC/MS/MS was used to characterize the phospholipidome of rHDL particles as previously described³². All internal standards were purchased from Avanti Polar Lipids (Alabaster, USA). LC/MS grade or UPLC grade solvents were obtained from Sigma-Aldrich (St Louis, USA) and used without further purification. Plasma lipids were extracted using a modified Folch method^{12,33}. Briefly, HDL samples containing 2 µg phospholipid supplemented with a mixture of internal standards were mixed with 1600 µL acidified methanol: 0.1N HCl (1:1 v/v) and 800 µL chloroform. The lower organic phase was dried, lipids were reconstituted into 40 µL of LC/MS-compatible solvent and injected into LC/MS/MS system. Lipids were quantified by LC-ESI/MS/MS using a Prominence UFLC (Shimadzu, Tokyo, Japan) and QTrap 4000 mass spectrometer (AB Sciex, Framingham, USA) equipped with a turbo spray ion source (450°C) combined with an LC20AD HPLC system, a SIL-20AC autosampler (Shimadzu, Kyoto, Japan) and the Analyst 1.5 data acquisition system (AB Sciex, Framingham, MA, USA). Quantification of phospholipids, sphingolipids and neutral lipids was performed in positive ion mode. Sample (4 µL) was injected onto a Kinetex HILIC 2.6 µm (2.1x150 mm) column (Phenomenex, USA). Mobile phases consisted of water and acetonitrile containing ammonium acetate and acetic acid. Lipid species were detected using scheduled multiple reaction monitoring (sMRM). N₂ was used as a nebulization and collision gas. Lipids were quantified using 37 calibration curves specific for 9 lipid subclasses [phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylserine (PS), sphingomyelin (SM), triglycerides (TG), ceramides (Cer) and phosphatidylglycerol (PG)] and up to 12 fatty acid moieties. Highly abundant lipid species which displayed non-linear responses in non-diluted extracts were quantified using a 20-fold diluted sample. An in-house developed R script was employed to correct for isotopic contribution to MRM signals as adapted from³⁴.

Atherosclerosis development and injection of reconstituted HDL in mice.

Human transgenic cholesteryl ester transfer protein (CETP) and *Ldlr*^{-/-} mice (TgCETP x *Ldlr*^{-/-}) were generated by breeding human CETP transgenic mice [Tg (CETP) 5203Tall/J; Jackson Laboratory] with *Ldlr*^{-/-} (*Ldlrtm1Her*/J; Jackson Laboratories) mice. Generated TgCETP x *Ldlr*^{-/-} mice were always

hemizygous for the human *CETP* transgene. Mice were housed in a conventional animal facility and fed ad libitum a normal chow diet. For the study of atherosclerosis, female *TgCETP* x *Ldlr*^{-/-} mice who were 9-10 weeks of age (average weight of 22 g) were fed a high-cholesterol diet (HCD) (1.25% cholesterol and 16% cocoa butter, SAFE diet N°CD002510, France) for 8 weeks before the injection of rHDL. Mice under isoflurane anesthesia (2% isoflurane/0.2 L O₂/min) were next retro-orbitally injected with either ARA-PE rHDL (6 mice) or control Soy-PC rHDL (5 mice) at a dose of 15 mg ApoA-I/kg of body weight three times per week for a period of two weeks upon a normal chow diet.

Injection of rHDL was validated through the quantification of plasma levels of human ApoA-I at various time points after a single injection of Soy-PC rHDL (15 mg ApoA-I/kg of body weight) or buffered saline in female *TgCETP* x *Ldlr*^{-/-} mice (Supplemental Figure 2A), while the HCD was validated through the quantification of plasma cholesterol levels after 8 weeks of HCD (Supplemental Figure 2B). The effectiveness of control Soy-PC rHDL toward atherosclerosis regression was validated in an independent study by injecting female *TgCETP* x *Ldlr*^{-/-} mice with either 15 mg ApoA-I/kg of Soy-PC rHDL or buffered saline three times per week for a period of 4 weeks upon a normal chow diet (Supplemental Figure 2C and 2D). Quantification of plasma protein and lipid levels was performed as previously described¹², in blood samples collected in ethylenediaminetetraacetic acid (EDTA)-coated tubes (Microvette, Sarstedt) by retro-orbital bleeding under isoflurane anaesthesia (2% isoflurane/0.2 L O₂/min). Plasma samples were stored frozen at -80°C prior use. Mice were sacrificed by cervical dislocation and tissues were collected, snap-frozen and stored at -80°C or fixed in 10% formalin for further analysis. All procedures were approved and accredited (No. 02458.02) by the French Ministry of Agriculture and were in accordance with the guidelines of the Charles Darwin Ethics Committee on animal experimentation

Quantification of atherosclerotic plaques.

The animals were sacrificed under isoflurane anesthesia and perfused through the heart with sterile PBS. The hearts were collected and fixed in 10% formalin solution as previously described³⁵ for 24

hours at 4°C prior to be incubated in 20% sucrose-PBS solution for additional 24 hours. Hearts were then dissected at the level of the aortic root, embedded in Tissue-Tek optimum cutting temperature (OCT) medium (Sakura Finetek Europe, The Netherlands) and snap frozen in liquid nitrogen. A total of 60 cryosections (10 µm thickness) were cut through the proximal aorta using Leica CM1900 Cryostat (Leica Biosystems). Sections were fixed in 10% formalin for 5 minutes and stained with filtered Oil red-O for 10 minutes. The extent of the atherosclerotic lipid lesions in the aortic root was quantified using ImageJ software (National Institutes of Health) on the images captured by a Zeiss AxioImager M2 microscope and plaque area measured with the AxioVision Zeiss software.

Human macrophages.

Human macrophages. Human THP-1 monocytes obtained from American Type Culture Collection were maintained at 37°C in 5% CO₂ in RPMI 1640 media containing 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L glutamine, and 100 U/ml penicillin/streptomycin. Cells were differentiated into macrophage-like cells with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) for 48-72h.

Cholesterol efflux capacity of reconstituted HDL.

Analysis of the capacity of reconstituted HDL to promote cholesterol efflux from human THP-1 macrophages was performed as previously described³⁶. Cellular cholesterol efflux to Soy-PC, Soy-PE, ARA-PE, and EPA-PE rHDL (at 5, 10, 20, and 50 µg ApoA-I/ml) was assayed in a serum-free medium for a 4-hour chase period.

Measurement of the antioxidative activity of reconstituted HDL.

The antioxidative activity of rHDL (final concentration of 50µg ApoA-I/ml) was evaluated towards reference LDL (final concentration of 0.2 mg cholesterol/ml) isolated from a pool of plasma obtained from healthy subjects from the Etablissement Français du Sang (EFS). Human LDL was isolated from normolipidemic human plasma by isopycnic nondenaturing density gradient ultracentrifugation in a Beckman SW41 Ti rotor at 40,000 rpm for 44 hours at 15°C in optima Beckman

XPN-100 ultracentrifuge as described earlier¹⁷. After ultracentrifugation, the gradient was fractionated in predefined volumes, and five LDL subfractions (LDL1-LDL5) were isolated and pooled together. Isolated LDL was dialyzed against phosphate-buffered saline (PBS, pH 7.4) in the dark at 4°C, stored at 4°C and utilized within one week for functional assays. The chemical composition of isolated LDL was determined by enzymatic colorimetric tests using commercially available assays (Diasys, Germany)¹⁷. The rHDL particles were added to LDL directly before oxidation. LDL oxidation was induced by copper sulfate (CuSO₄, final concentration of 0.05 μM). The extent of LDL oxidation in the presence or absence of rHDLs was assessed using 2', 7'-dichlorofluorescein diacetate (DCFH) fluorescent probe by Fluorescence Microplate Reader (Gemini, Molecular Devices, USA) as previously described³⁷. The oxidability of rHDL particles alone was also measured, in the absence of LDL, in the incubations containing DCFH, CuSO₄ and rHDL at the concentrations indicated above.

Anti-inflammatory activity of reconstituted HDL in THP-1 macrophages.

Human THP-1 macrophages plated at a density of 1.0×10^6 cells/well into 24-well plates were treated with different rHDLs (20 μg ApoA-I/ml) in a serum-free RPMI media for a 4-hour (early) or a 16-hour (late) period. Subsequently, rHDL-containing media were removed and cells were washed twice with PBS before the induction of inflammation with lipopolysaccharide (LPS; 100 ng/ml) for additional 4 hours when indicated. At the end of the LPS treatment, the supernatants were collected and the levels of secreted Interleukin-1 beta (IL-1β) in the culture media were measured by a MILLIPLEX Magnetic Bead Panel (Millipore) and the MAGPIX device (Luminex) system according to the manufacturer's instructions. The mRNA levels of chemokines and cytokines were quantified by qPCR analysis as described below.

RNA extraction, retrotranscription and real-time quantitative PCR.

Total RNAs from cell cultures were extracted using the NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer's instructions. Reverse transcription and real-time qPCR using a LightCycler LC480 (Roche) were performed as previously described³⁶. Human mRNA levels were

normalized to the mean expression of three housekeeping genes, including human non-POU domain-containing octamer-binding housekeeping gene (NONO), human α -tubulin (TUBA) and human heat shock protein 90kDa alpha (cytosolic), class B member 1 (HSP90AB1). Data were expressed as a fold change in mRNA expression relative to control values. The primers used in this study are listed in **Supplemental Table 3**.

Phospholipid transfer assay from LDL to HDL.

The transfer of phospholipids from LDL to rHDL was evaluated using human LDL labeled with Dil (1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindocarbocyanine perchlorate) fluorescent probe as described previously³⁸ with slight modifications. Briefly, LDL was labeled with Dil probe (5 μ M) at a Dil: LDL-phospholipid mass ratio of 1:13 via gentle stirring overnight at 37°C in the presence of lipoprotein-deficient plasma (LPDP) as a source of lipid transfer proteins at a LPDP: LDL volume ratio of 1:100. The Dil-labelled LDL was separated from unbound Dil by filtration through PD-10 Sephadex G-25 desalting column (GE HealthCare, US) and Dil-LDL filtrate was collected. Dil-LDL (7.5 mg phospholipid/dL) was mixed with rHDL (4 mg ApoA-I/dL), or reference ApoB-deficient plasma (1:30 v/v) obtained from a healthy normolipidemic donor, 50 μ l of Tris buffer (0.4 M, PH 8) and PBS to a final volume of 200 μ l, and the mixtures were incubated at 37°C for 1 hour to achieve phospholipid transfer. The reaction was terminated by placing the mixture on ice, and Dil-rHDL-containing supernatants were isolated from each sample using ApoB-depleting precipitant (i.e. phosphotungstic acid, MgCl₂, NaOH; pH 6.2) as previously described³⁸. The fluorescence intensity of Dil-HDL was measured at an excitation wavelength of 550 nm and emission wavelength of 564 nm using Fluorescence Microplate Reader (Gemini, Molecular Devices, USA). The phospholipid transfer from LDL to rHDL was calculated via normalizing the fluorescence intensity in rHDL-containing supernatant to that of a reference normolipidemic HDL (employed as ApoB-deficient plasma) and presented as a percentage.

Lipid surface and core rigidity of reconstituted HDL.

The rigidity of the lipid surface and core of rHDLs was evaluated using trimethylamine-diphenylhexatriene (TMA-DPH), and diphenylhexatriene (DPH; Cayman Chemical, USA) fluorescent probes, respectively³⁹. Reconstituted HDL particles (3.3 mg ApoA-I/dL) were incubated with DPH (final concentration of 0.80 μ M, dissolved in tetrahydrofuran) or TMA-DPH (final concentration of 0.32 μ M, dissolved in dimethylformamide) at 37C° for 1 hour to achieve the incorporation of the fluorescent probes into the lipoproteins. Anisotropy of the probe fluorescence was measured by FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices, USA) using a standard polarizer set as previously described³⁹.

Quantification of cell surface expression of TLR4.

Cell surface expression of TLR-4 was investigated using R-Phycoerythrin (PE) fluorescent labeled-mouse anti-human TLR-4 (CD284) antibody (Clone TF901, 564215; BD Biosciences, US). THP-1 macrophages treated with 20 μ g ApoA-I/ml of rHDL for a period of 4 hours were detached with EDTA-trypsin and stained with 100 μ l of eFluor™ 520 Fixable Viability dye (Invitrogen, US) diluted in PBS-FBS 5% (1:1000 v/v) for 30 minutes. After 5 minutes of centrifugation at 400 g, supernatant was removed and cells were stained with 100 μ l of PE fluorescent- labeled mouse anti-human TLR-4 (CD284) antibody diluted in PBS-FBS 5% (1:50 v/v) with FcR blocking reagent (Miltenyi Biotec) for 30 minutes. Cells were fixed using FOXP3 fixation diluent (Invitrogen, Thermo Fisher Scientific, US) before flow cytometry analysis on a LSR II FORTESSA SORP (BD Biosciences).

Western blotting analysis.

Total protein from THP-1 macrophages plated at a density of 4.0×10^6 cells/well into 6-well plates treated with different rHDLs (20 μ g ApoA-I/ml) for a period of 4 hours, followed by 30 minutes stimulation with LPS, was extracted and analyzed by Western blotting as previously described¹⁴. Twenty micrograms of protein were separated by electrophoresis (2.5 hours, 120 Volt) on a 10% bis-

Tris polyacrylamide gel and transferred onto a nitrocellulose membrane (overnight at 40 Volt; Cytiva Life Sciences, USA). Subsequently, the membrane was blocked, incubated with primary antibodies at 1:1000 overnight and revealed with IR dye 680RD and 800CW secondary antibodies at 1:10000 for 3 hours (Li-COR, USA). Quantification of Western blots was performed using Li-Cor scanner (Odyssey system, Li-COR Biosciences, Germany). Phospho-SAPK/ JNK (Thr183/Tyr185) antibody (#9251), SAPK/JNK antibody (#9252), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb (#4370), p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb (#4695), phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP® Rabbit mAb (#4511), and p38 mitogen-activated protein kinase (MAPK) antibody (#9212), were purchased from Cell Signaling Technology (Massachusetts, USA).

Quantification of eicosanoid production by LC/MS-MS.

The impact of rHDLs on the long-term eicosanoids production was investigated in THP-1 macrophages plated at a density of 4.0×10^6 cells/well into 6-well plates and treated with different rHDLs (20 µg ApoA-I/ml) for a period of 16 hours. At the end of the treatment, cells were detached with EDTA-trypsin and centrifuged, supernatant was aspirated and cell pellets were stored at -80°C.

Cell pellets (100 µL) were spiked with an internal standard mixture (5µl) containing 0.5, 0.5, 2, 2 and 0.5 ng of TxB2-d4, PgE2-d4, 13(S)HODE-d4, 9(S) HODE-d4 and 15(S)HETE-d8, respectively. Lipids were extracted with 400 µl of cold methanol containing Butylated Hydroxytoluene (BHT; 50 mg/L) for 10 min at 0°C. After a centrifugation for 5 min at 4°C, and 15 000g, supernatants were collected and the pellets were reextracted as previously described. Pooled supernatants were evaporated to dryness under vacuum. Dried extracts were finally solubilized with 50 µL of methanol/water (50/50 v/v).

High performance liquid chromatography (HPLC) was performed on Vanquish UPLC System equipped with an autosampler, a binary pump and a column oven (ThermoScientific, France). Lipids were separated on a Luna C18 3µm – 250 x 2 mm column (Phenomenex, France) maintained at 45°C. Mobile phases consisted of acetic acid 0.1 % in water; A) and acetonitrile (B). The flow rate was maintained constant at 0.3 ml/min. Before each new injection, the column was equilibrated with 25 % of the phase

B for 5 min. For lipid species separation, the gradient was then ramped to 45 % B in 3 min, maintained at 45% B for 2 min, ramped to 60 % B in 5 min, then to 98 % B over the subsequent 6 min and finally maintained at 98 % B for the next 4 min. The LC system was coupled to an Altis Plus triple quadrupole (ThermoScientific, France) equipped with an H-ESI electrospray ionization source. Source vaporizer and ion transfer tube temperatures were set at 350°C and 325°C respectively. The sheath gas, auxiliary gas and sweep gas nitrogen pressures were set at 50, 10 and 1, respectively (arbitrary units).

Analysis was conducted in negative selected reaction monitoring mode (SRM) using the following transitions: PgE2 351.1 → 271.1 / PgE2-d4 355.1 → 275.1 (Collision energy (Coll) 16 V); TxB2 369.4 → 169.0 / TxB2-d4 373.3 → 173.1 (Coll 5 V); 13(S)HODE 295.3 → 195.0 / 13(S)HODE-d4 299.3 → 195.0 / 9(S)HODE 295.3 → 171.1 / 9(S)HODE-d4 299.3 → 172.0 (Coll 18 V); 15(S)HETE 319.2 → 219.0 / 15(S)HETE-d8 327.3 → 226.1 (Coll 12V); 11(S)HETE 319.2 → 167.2 (Coll 15V); 5(S)HETE 319.2 → 115.2 (Coll 14V).

Calibration curves were obtained using authentic standards, with the exception of 11(S) HETE and 15(S) HETE for which 5(S) HETE curve with 15(S) HETE-d8 as an internal standard was used. All standards were extracted using the same method as the samples. Linear regression was applied for the calculations.

Statistical analyses.

Data are presented as mean ± S.E.M or median [range]. Experiments were performed in triplicate and values shown represent at least three independent experiments. The normal distribution of the data was evaluated using Shapiro-Wilk and Kolmogorov-Smirnov tests. For normally distributed data, comparisons were performed by a unpaired two-tailed Student's t-test without assuming same standard deviation (SD) for both populations, unless indicated in the graph legends. For not normally distributed data, Mann Whitney test was used. Statistical analyses were performed by Prism software from GraphPad (San Diego, CA, USA). Association between the abundance of PE (36:5) in large HDL₂

with cIMT was evaluated by univariate and multivariate linear regression using the R statistical software-version 3.3.2 (R Foundation for Statistical Computing).

Results.**Total PE and PE (36:5) contents of large HDL were associated with atherosclerosis in women with MetS.**

Earlier lipidomic studies have reported that plasma levels of PE species, especially PE (36:5), are strongly and positively associated with ASCVD^{16,18}. To test if these effects result from the presence of PE and PE (36:5) in HDL, the association of their contents in large HDL₂ particles, the primary contributors to PE cargo among plasma HDL subparticles⁴⁰, with carotid intima-media thickness (cIMT) was investigated in a cohort of 86 women with MetS. Unadjusted linear regression analysis showed that both total PE and PE (36:5) contents of large HDL were positively associated with cIMT ($\beta=0.285$, $p=0.0087$, Figure 1A; $\beta=0.359$, $p=0.0008$, Figure 1B, respectively). This association remained significant with total PE ($\beta=0.308$, $p=0.0053$, Figure 1A) and PE (36:5) ($\beta=0.268$, $p=0.0078$, Figure 1B) after adjustment for ASCVD risk factors including age, diabetes mellitus, hypertension, smoking, plasma total cholesterol and triglycerides levels. This result indicates that total PE and PE (36:5) contents of large HDL were intimately associated with atherosclerosis in women with MetS, suggesting a potentially deleterious role of PE in the atheroprotective function of HDL.

Lipidomic characterization and fatty acid profiling of reconstituted HDL.

In order to investigate the impact of PE in general and PE (36:5) in particular on the functions of HDL, we generated four types of rHDL: Soy-PC rHDL (as a control), Soy-PE rHDL, ARA-PE rHDL, and EPA-PE rHDL. Soy-PC and Soy-PE were used as natural mixtures of several PC and PE species, while ARA-PE (16:1/20:4) and EPA-PE (16:0/20:5) were used as the two major subspecies of PE (36:5) detected in human plasma²². Soy-PC and Soy-PE displayed similar fatty acid composition, with linoleic acid (C18:2) and palmitic acid (C16:0) representing about 65wt% and 15wt% of total fatty acids, respectively (Supplemental Figure 3A), but differed in their head groups (choline vs ethanolamine) which allowed us to study the biological effects of the PE head group. Soy-PE, ARA-PE and EPA-PE had

the same head group but different fatty acid composition, which allowed us to study the biological effects of their fatty acid moieties (Supplemental Figure 3A).

To determine the precise phospholipid composition of the rHDLs, lipidomic analysis was performed. We observed that Soy-PC rHDL was composed mostly by 99.98% of PC alone, while Soy-PE rHDL was composed of PC and PE at the PC: PE molar ratio of 7.4:2.6. The phospholipidome of ARA-PE rHDL and EPA-PE rHDL was composed of PC with either ARA-PE at the PC: ARA-PE molar ratio of 7.2:2.8 or EPA-PE at the PC: EPA-PE molar ratio of 7.0:3.0, respectively (Supplemental Figure 3B). Such phospholipid composition is typical for rHDL prepared *in vitro*³¹ while the used ApoA-I: Phospholipids of 1:90 (Supplemental Table 2) is close to that measured in human plasma HDL^{14,40}.

ARA-PE impaired the capacity of HDL to reverse atherosclerosis in mice.

Although PE (36:5) exists in human plasma mainly as ARA-PE and EPA-PE, previous studies revealed a higher prevalence of ARA-PE in obese women with metabolic dysfunction and in patients with ASCVD^{23,24}, suggesting that ARA-PE predominates in HDL from these patients. In order to test the impact of ARA-PE HDL on atherosclerosis in mice, female TgCETP x *Ldlr*^{-/-} mice fed a high cholesterol diet were injected with either control (Soy-PC rHDL) or ARA-PE rHDL as described in Figure 2A. Analysis of the atherosclerotic plaques in the aortic root (Figure 2B) revealed an accelerated atherosclerosis in mice injected with ARA-PE rHDL than in those injected with control Soy-PC rHDL (51.2% vs 41.0% of total aortic area, p=0.0005, respectively). This data indicates that the presence of ARA-PE in HDL not only abolished the capacity of rHDLs to reduce atherosclerosis in mice but rather rendered them proatherogenic.

ARA-PE impaired the capacity of HDL to promote macrophage cholesterol efflux while EPA-PE increases it.

To test if the effects observed in both humans and mice resulted from alterations of the biological activities of HDL by ARA-PE, we conducted *in vitro* studies investigating the impact of PE and PE (36:5), in the both ARA and EPA forms, on the major cardioprotective functions of HDL. First, we

explored the impact of the composition of rHDL particles (Figure 3A) on their macrophage cholesterol efflux capacity (CEC), which is the function of HDL proposed to underlie the association between low HDL-C levels and ASCVD^{2,41}. As shown in **Figure 3**, control Soy-PC rHDL exhibited a dose-dependent increase in CEC from human THP-1 macrophages, confirming the robust biological activity of our rHDL preparation. The CEC of Soy-PE rHDL was only reduced relative to Soy-PC rHDL at the highest concentration of rHDL at 50 µg/mL (-16%, $p=0.002$). Moreover, we observed a marked reduction of the CEC of ARA-PE rHDL in comparison to the both control Soy-PC and Soy-PE rHDLs at concentration of 20 µg/mL (-15% and -32%, respectively, $p<0.05$) and 50 µg/mL (-19% and -19%, respectively, $p<0.05$). Interestingly, such alterations of the CEC were not detected in EPA-PE rHDL; this latter being even greatly enhanced by an average of 87% ($p<0.05$) as compared to the other rHDL particles. The quantification of mRNA levels by qPCR as well as of the ABCA1 surface expression by flow cytometry in human THP-1 macrophages following incubation with the rHDLs (Supplemental Figure 4A-G) suggested that the opposite effects of ARA-PE and EPA-PE on the CEC of rHDL were not due to a modification of the expression of the major lipid transporters involved in cholesterol efflux pathways. Taken together, these findings indicate that the presence of ARA-PE impaired the CEC of rHDL in human macrophages while that of EPA-PE improved it.

EPA and ARA counteract the abolishment of the AOX of rHDL mediated by PE.

We next investigated the capacity of the PE-containing rHDLs to prevent LDL oxidation, which is a characteristic feature in the pathogenesis of atherosclerosis². The antioxidative activity (AOX) of rHDLs was evaluated as their capacity to prevent copper-induced LDL oxidation using a DCFH fluorescent probe. The extent of LDL oxidation was evaluated using a biphasic oxidation kinetics (Supplemental Figure 5), including the amount of oxidized DCFH formed at the end of the experiment and the oxidation rate in the propagation phase which are proportional to the level of LDL oxidation, and the duration of the lag phase which is inversely related to LDL oxidation. In accordance with oxidation kinetics in Figure 4A, we observed that control Soy-PC rHDL efficiently reduced both the

amount of oxidized DCFH (-33%, $p=0.002$, Figure 4B), and the oxidation rate in the propagation phase (-58%, $p<0.0001$, Figure 4C), and prolonged the lag phase 2.7-fold ($p<0.0001$, Figure 4D), indicating an efficient AOX of control Soy-PC rHDL. Strikingly, this AOX was lacking with Soy-PE rHDL but conserved when either ARA-PE or EPA-PE rHDLs were used (Figure 4B-D), suggesting that ARA and EPA may restore the AOX of PE-rHDL.

Several studies reported that HDL may protect LDL from oxidation in part by acting as a sacrificial target for oxidation⁴². Thus, we investigated the oxidability of rHDLs by copper ions in the absence of LDL. Both ARA-PE and EPA-PE rHDLs were more susceptible to oxidation than Soy-PC or Soy-PE rHDLs (+72%, $p=0.015$ and 200%, $p=0.011$, respectively, Figure 4E). Overall, our findings demonstrate that PE abolished the AOX of rHDL but this effect could be reversed by EPA and ARA, likely by increasing scavenging capacity of rHDL for oxidants.

EPA and ARA restored the PE-mediated alteration of the phospholipid transfer from LDL to rHDL by decreasing its lipid rigidity.

Because the first step in the AOX of HDL is thought to involve the transfer of oxidized phospholipids from LDL to HDL, a process controlled by the rigidity of lipids at the surface of HDL⁴³, we investigated the capacity of rHDLs to acquire phospholipids from LDL labelled with a Dil fluorescent probe. As shown in Figure 5A, the transfer of the fluorescent phospholipids from Dil-labelled LDL to control Soy-PC rHDL was similar to that to a reference normolipidemic HDL (employed as ApoB-depleted plasma), highlighting the efficient capacity of rHDL to accept phospholipids. However, the transfer of fluorescent phospholipids from Dil-labelled LDL to Soy-PE rHDL was significantly reduced (-26%, $p<0.05$). By contrast, the transfer was increased to EPA-PE and ARA-PE rHDLs when compared to both control Soy-PC rHDL and Soy-PE rHDL (EPA-PE: +42%, $p<0.05$ and +91%, $p<0.0001$; ARA-PE: +27%, $p<0.001$ and +72%, $p=0.0002$, respectively). In an attempt to obtain mechanistic clues regarding the impact of PE and PE(36:5) on the rigidity of HDL, we next explored the surface and core fluidity of rHDL, using TMA-DPH and DPH fluorescent probes³⁹, respectively. Fluorescence anisotropy values for Soy-PE

rHDL with TMA-DPH (Figure 5B) and DPH (Figure 5C) probes were not different from those for control Soy-PC rHDL. By contrast, the anisotropy values with the TMA-DPH probe were decreased for both EPA-PE (-46%, $p < 0.05$) and ARA-PE (-43%, $p < 0.05$) rHDL consistent with a decreased lipid surface rigidity. A drop of the anisotropy value with the DPH probe was only detected for the EPA-PE rHDL (-96%, $p < 0.05$), indicative of a decreased lipid core rigidity.

Taken together, these findings led us to propose that the loss of the AOX in PE-containing rHDL could result from an alteration of the phospholipid transfer from LDL. On the contrary, such a transfer of oxidized phospholipids was enhanced when PE were enriched in EPA and ARA through a mechanism involving an increase of the fluidity of rHDL. It was noteworthy that EPA exhibited the strongest fluidizing effect by enhancing both surface and core fluidity of rHDL.

EPA but not ARA rescued the anti-inflammatory activity of PE-containing rHDL in macrophages upon LPS treatment.

Because HDL exert potent anti-inflammatory activities (AIA) allowing a reduction of inflammatory activation of arterial macrophages², we investigated the influence of a short (ST, 4h) and long term (LT, 16h) treatment with rHDLs on the production of pro-inflammatory IL-1 β cytokine whose inhibition was reported to reduce cardiovascular events in patients with CVD⁴⁴. In human THP-1 macrophages, the expression and secretion of IL-1 β triggered by LPS were significantly reduced following both ST (-37%, $p < 0.0001$ and -27%, $p = 0.014$; Figure 6A) and LT (-30%, $p = 0.004$ and -50%, $p = 0.02$; Figure 6B) pre-incubation with control Soy-PC rHDL. The inhibitory effect of the ST and LT pre-incubation with rHDL was no longer observed with either Soy-PE or ARA-PE rHDLs, whereas it was preserved with EPA-PE rHDL. A more pronounced inhibitory effect on IL-1 β secretion was even detected following the ST pre-incubation with EPA-PE rHDL (-63%, $p < 0.01$; Figure 6A). It is of note that rHDLs were without effect on the cell surface expression of the LPS binding receptor TLR4 (Supplemental Figure 4H).

To test if the anti-inflammatory effect of rHDLs involved modulation of intracellular inflammatory signaling pathways in macrophages, we investigated their impact on the LPS-induced activation of ERK, p38 MAPK and JNK signaling pathways which were previously reported to be downregulated in macrophages by HDL⁴⁵. Control Soy-PC rHDL significantly reduced the LPS-induced phosphorylation of ERK, p38 MAPK and JNK in human THP-1 macrophages (-19%, $p < 0.05$; -30%, $p < 0.001$ and -36%, $p < 0.001$, respectively, Figure 6C-E). Such inhibitory effects were abolished with Soy-PE rHDL and ARA-PE rHDL, with the exception of JNK phosphorylation which was reduced with ARA-PE rHDL to the same extent as with control Soy-PC rHDL. By contrast, the reduction of the LPS-induced phosphorylation of ERK, p38 and JNK was retained in EPA-PE rHDL (-32%, $p < 0.01$; -34%, $p < 0.01$ and 32%, $p < 0.01$, respectively).

These findings demonstrate that PE and ARA-PE abrogated the anti-inflammatory property of rHDL in LPS-treated human macrophages by acting on inflammatory signaling pathways and that the presence of EPA was able to restore it.

Composition of PE-containing rHDL in ARA and EPA played an important role in eicosanoid production and IL-1 β secretion in human macrophages.

We finally addressed the hypothesis that ARA carried by HDL could promote inflammation in macrophages by providing a source of ARA which could be oxidized to produce pro-inflammatory eicosanoids. As shown in Figure 7A, 16h-treatment of human THP-1 macrophages with ARA-PE rHDL was accompanied by a marked increase of the production of eicosanoids, including hydroxyeicosatetraenoic acids (5S-, 11S-, and 15S-HETEs), prostaglandin (PGE₂) and thromboxane (TXB₂), in comparison to control Soy-PC and Soy-PE rHDLs. Substitution of ARA by EPA in PE-rHDL abolished this effect and led to a marked decrease of 5S-HETE compared to both control Soy-PC and Soy-PE rHDLs. Interestingly, the impact of PUFAs (ARA and EPA) in PE-rHDLs on eicosanoids production was associated with a concomitant effect on the secretion of pro-inflammatory IL-1 β cytokine by human THP-1 macrophages (Figure 7B-C). Strikingly, whereas treatment with ARA-PE rHDL led to a 5-

fold increase of IL-1 β secretion in comparison to control Soy-PC and Soy-PE rHDLs, the secretion of IL-1 β was almost undetectable in macrophages incubated with EPA-PE rHDL.

These results provide evidences that the ARA content in PE-containing rHDLs contributed to the production of eicosanoids and the secretion of the pro-inflammatory IL-1 β cytokine by human macrophages. By contrast, the EPA content in PE-containing rHDL exhibited a potent inhibitory action toward IL-1 β secretion from human macrophages.

Discussion.

Plasma lipidomic studies showed a robust positive association between several PE species, notably PE (36:5), with ASCVD^{16,18} although the underlying mechanisms are unknown. In this study, we demonstrated that the contents of total PE and PE (36:5) in large HDL are linked to atherosclerosis development in women with MetS. This atherogenic effect was confirmed in mice injected with rHDL containing ARA-PE, the predominant PE (36:5) form in patients with cardiometabolic diseases^{23,24}. Our *in vitro* investigations revealed that the presence of PE in rHDL impaired their biological activities, an effect worsened with ARA-PE, suggesting a deleterious effects of both PE head group and lipid chain in anti-atherogenic functions of HDL with potential implications in ASCVD pathogenesis. On the contrary, EPA-PE counteracted the PE-mediated HDL dysfunctionality and enhanced the anti-atherogenic functions of HDL, providing mechanistic insights in the protective role of EPA in patients with ASCVD.

In agreement with our findings in MetS patients, Giraud et al. reported increased levels of ARA-containing PE species in HDL from patients with rheumatoid arthritis; their abundance was associated with inflammation and CVD risk⁴⁶. Taken together with the present study, these data suggest that the association of plasma PE (36:5) with ASCVD may result in part from the presence of ARA-PE, i.e. PE (16:1/20:4), in HDL, rather than EPA-PE, i.e. PE (16:0/20:5), the other form of PE (36:5) detected in human plasma²². This hypothesis is reinforced by the numerous studies reporting the positive link of ARA (C20:4), a key omega-6 PUFA, with ASCVD²³, in contrast to the multiple anti-atherogenic properties of EPA (C20:5), an omega-3 PUFA⁴⁷. Although our findings undoubtedly warrant further validation in larger cohorts of patients with ASCVD composed of both women and men, the atherogenic property of ARA-PE HDL was validated in our *in vivo* experiments in a mice model of atherosclerosis injected with rHDL. Indeed, we found that ARA-PE HDL lacked a capacity to reduce atherosclerosis compared to control rHDL, underlying the deleterious role of ARA-PE on HDL-mediated atheroprotection.

The present study demonstrated that PE and ARA-PE impaired several biological activities of HDL particles proposed to contribute to their atheroprotective action. Thus, the presence of Soy-PE (a mixture of PE species) led to an attenuation of the CEC of HDL from human macrophages in comparison to Soy-PC HDL with a similar fatty acid composition. This result is coherent with a previous study showing an inverse correlation between the total PE content of HDL and the capacity of human serum to promote cellular cholesterol efflux²¹. Because mRNA levels of transporters and receptors controlling cholesterol efflux from macrophages, such as ABCA1, the major cholesterol transporter in human macrophages^{36,40}, were not altered by PE-containing HDL, these data support an extracellular rather than an intracellular influence of PE on the CEC of HDL. This hypothesis is supported by the work of Demel et al. who described a lower affinity of PE compared to PC toward cholesterol⁴⁸, suggesting a reduced membrane cholesterol transfer into PE HDL. Moreover, PE can alter the dynamic structure and interaction of HDL with cell membrane receptors like ABCA1^{20,49}. Taken together, these mechanisms may contribute to the capacity of PE to impair CEC of HDL. Interestingly, a more pronounced impairment in the CEC of PE-containing HDL was observed in the presence of ARA-PE, suggesting additional deleterious effects mediated by ARA. Although the mechanism underlying such a role for ARA was not identified in our study, it is noteworthy that the enrichment of HDL with another omega-6 fatty acid, i.e. linoleic acid, led to a reduced CEC of HDL²⁹.

In addition to CEC, our work demonstrated that the addition of Soy-PE diminished the AOX of HDL. Seeking for mechanistic insights, we observed that PE-containing HDL exhibited a reduced capacity to acquire phospholipids from LDL compared to control HDL which could explain its diminished AOX. Indeed AOX requires the transfer of oxidized phospholipids, including lipid hydroperoxides, from LDL to HDL, which is proportional to HDL's surface fluidity, followed by their reduction via redox-active methionine (Met) residues of apoA-I^{43,50}. PC- and PE-containing HDL used in our *in vitro* experiments only differed in their head groups (choline vs ethanolamine). Ethanolamine head groups are considered to be more rigid than choline⁵¹, due to their capacity to form non-covalent bonds with neighboring lipids, restricting their movement^{51,52}. This rigidity could limit the incorporation

of exogenous phospholipids into PE HDL by increasing interactions with adjacent lipids on the HDL surface as observed in our experiments. Several reports indicated that PE has limited interactions with ApoA-I and can alter both the content and conformation of ApoA-I in rHDL^{20,49}. This could contribute to the diminished AOX of our PE rHDL by affecting the availability of apoA-I Met residues and HDL interactions with other lipoproteins, like LDL²⁰. In contrast, replacing Soy-PE with ARA-PE preserved the AOX of HDL, suggesting that ARA may restore the impaired AOX of PE-HDL. In this regard, we observed an enhanced transfer of phospholipids from LDL to ARA-PE HDL, likely reflecting its increased surface fluidity which could potentially account for its conserved AOX.

Finally, our data on the influence of PE and ARA-PE on the AIA of HDL in pro-inflammatory human macrophages treated with LPS demonstrated that the presence of PE abolished the capacity of HDL to decrease the expression and secretion of IL-1 β . Previous studies have established a strong link between CEC and AIA of HDL^{2,45}, suggesting that the impaired AIA of PE-containing HDL could result from its impaired CEC. In this regard, Sun et al. linked cholesterol accumulation in plasma membrane of macrophages with enhanced TLR-4 signaling and p38 MAPK activation⁵³. Although cell surface expression of TLR4 in human macrophages was not altered in the present study by the presence of PE in HDL, the phosphorylation of p38 MAPK and activation of other inflammatory signaling pathways (ERK, JNK) known to be modulated by HDL⁴⁵ was no longer attenuated by PE-containing HDL, providing mechanistic clues on the negative impact of PE on the AIA of HDL.

In human macrophages non-treated with LPS, the presence of PE converted HDL into pro-inflammatory particles, which are recognized as contributors of inflammation in atherosclerosis⁵⁴. Interestingly, although the enrichment of PE with ARA was without additional effect on the pro-inflammatory effects of PE-HDL in LPS-treated human macrophages, a potent increase in the expression and secretion of IL-1 β was detected in non-treated cells following the long-term incubation with ARA-PE HDL. We propose that the proinflammatory effect of ARA-PE HDL mainly resulted from an increased production of eicosanoids, including HETEs (5S, 11S, and 15S), PGE2, and TXB2, which

were found elevated in the present study when macrophages were incubated with ARA-PE HDL in comparison to others HDL particles. Indeed, ARA is the primary precursor for the pro-inflammatory eicosanoids which are potent lipid mediators implicated in the inflammatory process of ASCVD^{23,26}. Eicosanoid generation mainly involves ARA release from cell membrane phospholipids by phospholipase A2 followed by enzymatic metabolism of ARA by cyclooxygenases (COXs) generating PGs and TXs, and by lipoxygenases (LOXs) generating HETEs and leukotrienes²⁶. Our findings uncovered that HDL, primarily PE-containing HDL, can be a source of ARA in macrophages for inducing inflammation through eicosanoid production. Such a mechanism could play a major role in cellular IL-1 β secretion and the development of ASCVD.

Although omega-3 PUFAs, including the mixtures of EPA and DHA, were without effects on cardiovascular events⁵⁵⁻⁵⁷, the use of a highly purified EPA ethyl ester was reported to reduce the CV risk²⁵, suggesting a specific cardioprotective effect of EPA that would not be exerted by other omega-3 PUFAs. Our findings, thereby provide new mechanistic insights regarding the potential impact of EPA on ASCVD. Indeed, our data demonstrated that EPA was able to counteract the deleterious effects of PE, especially those of ARA-PE, on HDL functions. Thus, EPA-PE restored the altered AOX of PE-containing HDL to a higher degree than did ARA-PE. The positive influence of omega-3 PUFA, including EPA, on the AOX of both rHDL and plasma HDL has been well-documented^{28,29}. This effect can be attributed to the EPA structure composed of an additional double bond compared to ARA, rendering rHDL more fluid and improving their capacity to acquire lipids. Moreover, we observed that EPA-PE enhanced both surface and core fluidity of rHDL, this latter parameter being only increased by EPA-PE HDL in our experimental conditions.

Our study also demonstrated that EPA-PE restored the anti-inflammatory activity of rHDL in LPS-treated macrophages which was abolished in PE-containing HDL. Strikingly, such an effect of EPA-PE HDL on the inhibition of IL-1 β secretion was also observed in the absence of LPS when control HDL was without effect on inflammatory processes in macrophages. The well-established anti-

inflammatory effects of EPA^{23,47,58}, especially its capacity to attenuate LPS-induced IL-1 β secretion by macrophages⁵⁹, could contribute to the strong AIA of EPA-PE HDL. Tian et al. reported that dietary EPA-PC and EPA-PE attenuated the inflammatory phenotype of macrophages by reducing IL-1 β expression and stimulating M2 anti-inflammatory macrophage polarization⁶⁰ through a role of EPA in eicosanoid production⁶¹. In agreement with this study, we found that EPA-PE rHDL not only abolished the increased production of eicosanoids observed with ARA-PE rHDL, but also markedly reduced 5S-HETE levels in human macrophages compared to both PC- and PE-containing rHDL, further supporting the importance of PUFA composition in PE HDL for the production of eicosanoids in macrophages.

Finally, the presence of EPA-PE improved the CEC of HDL from macrophages, a metric of HDL function inversely associated with ASCVD⁴⁻⁶. A similar observation was reported by Tanaka et al. with EPA-PC enhancing the CEC of HDL compared to PC HDL²⁷, suggesting that the effect of EPA on the CEC of HDL can be independent of the nature of PL classes. In this context, the elevation of both the surface and core fluidity of HDL by EPA-PE compared to PC-containing HDL could potentially account for the enhanced CEC by facilitating cholesterol insertion at the particle surface. This hypothesis is consistent with the established role of HDL surface fluidity as a key determinant of this process^{10,62}. Moreover, the increased core fluidity of EPA-PE HDL could further facilitate cholesterol incorporation into HDL. Such profound fluidizing effects of EPA-PE are likely due to the high unsaturation level of EPA, conferring a better structural flexibility for adopting highly kinked conformations in the phospholipid monolayer of HDL⁶³.

Conclusion.

In conclusion, our study provides evidence that PE species, particularly ARA-PE, participate in the formation of dysfunctional HDL, consistent with the deleterious role of PE (36:5) in ASCVD reported by epidemiological studies. Circulating levels of PE (36:5) species, i.e. ARA-PE and EPA-PE, might therefore serve as novel biomarkers of HDL functionality and provide new approaches to the formulation of reconstituted HDL for therapeutic use in ASCVD³. Importantly, our data provide

mechanistic insights on the cardioprotective role of EPA in ASCVD by counteracting the PE-mediated dysfunctionality of HDL. Such a role might contribute to the reduction of cardiovascular events observed in patients consuming highly purified EPA ethyl ester in the REDUCE IT clinical trial²⁵, supporting the use of EPA-rich diets as a therapeutic strategy to enhance HDL-mediated cardiovascular protection.

Conflict of interest.

The authors declare no competing financial interests.

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Contributors.

MT, LMH, VDD, ML, HW, CR, CM, JPPDB, SG, EF, HD, PEK, EFV, PL, IG and WLG performed the research. MT, ML, MP, JPPDB, MG, AK, IG and WLG analyzed the data. TH generated and provided mouse models. MT, MG, AK, IG and WLG designed the study. AG, PG and EB recruited the patients. MT, IG and WLG wrote the manuscript. IG and WLG supervised the study. WLG funded the research. All authors read and approved the final version of the manuscript.

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Legends to Figures.

Figure 1. Total PE and PE (36:5) contents of HDL2 were associated with atherosclerosis in women with Metabolic Syndrome. Human HDL2 particles were isolated from plasma of 86 women with Metabolic Syndrome by sequential ultracentrifugation for 48 hours. Total PE and PE (36:5) contents in HDL2 were quantified by LC-ESI/MS/MS. Carotid Intima media thickness (cIMT) was evaluated by ultrasonography and correlated with total PE and PE (36:5) contents in HDL2. **A.** Unadjusted and adjusted linear regression analysis for the mean cIMT (in millimeter, mm) according to total PE content of HDL2 **B.** Unadjusted and adjusted linear regression analysis for the mean cIMT (mm) according to PE (36:5) content of HDL2. The analysis was adjusted for traditional risk factors, including age, diabetes mellitus, hypertension, smoking, plasma total cholesterol and triglycerides levels. HDL: high density lipoprotein; PE: Phosphatidylethanolamine.

Figure 2. Injection of ARA-PE rHDL did not reduce the size of aortic atherosclerotic plaque as compared to Soy-PC rHDL in CETP transgenic-LDLr KO female mice. **A.** CETP transgenic/ LDL receptor KO female mice were fed high cholesterol diet for 8 weeks before injection with 15mg/kg of Soy-PC rHDL (n=5) or ARA-PE rHDL (n=6), retro-orbitally, every second day. The mice received 10 injections of either treatment, and were maintained on a chow diet during rHDL treatment. The hearts were carefully dissected at the level of the aortic root, and embedded in OCT, then cut every 10µm from the aortic root at the level of the three valves. **B.** Sections were labeled with Oil red-O to quantify the atherosclerotic plaque size. The extent of the atherosclerotic plaque was quantified using ImageJ software.*p<0.001 vs. Soy-PC rHDL. CETP: cholesteryl ester transfer protein; HC: high cholesterol; rHDL: reconstituted high density lipoprotein; PC: phosphatidylcholine; ARE-PE: arachidonic acid-phosphatidylethanolamine.

Figure 3. Soy-PE reduced the CEC of Soy-PC rHDL, and ARA-PE exacerbated this effect while EPA-PE reversed it and even improved the CEC. THP-1 macrophages loaded with radiolabeled [3H] cholesterol were incubated with 5, 10, 20, or 50 µg ApoA-1/ml of Soy-PC, Soy-PE, ARA-PE, and EPA-PE rHDLs for 4 hours. At the end of incubation, cellular lipids were extracted, the radioactivity content in both cells

and media was measured, and the percentage of free [^3H] cholesterol efflux to different rHDLs was calculated. **A.** Phospholipidome composition of the rHDLs. **B.** The CEC of rHDLs. For cholesterol efflux data, statistical analysis done using multiple t-test, and significance determined by Holm-Sidak method, without assuming consistent SD. #vs. Soy-PC rHDL, ϕ vs. Soy-PE rHDL, δ vs. ARA-PE rHDL, with p values less than 0.05 (exact p values are indicated in the text). Values are shown from at least 3 independent experiments that were performed in triplicate for each condition.

Figure 4. Soy-PE impaired the AOX of rHDL compared to Soy-PC rHDL, while ARA-PE and EPA-PE reversed this effect. LDL was isolated from healthy individuals, and DCFH fluorescent probe was used to assess the oxidation of LDL induced by copper sulfate (CuSO_4). DCFH solution (0.2mg/ml) was dried and mixed with LDL (0.2 mg/ml of total cholesterol). Soy-PC, Soy-PE, ARA-PE, or EPA-PE rHDLs were added to the mixture (50 μg ApoA-I/ml), then CuSO_4 solution was added (0.05 μM), and the mixture volume was completed to 100 μl with PBS. Different mixtures were transferred to 96 well black microplates (100 μl /well), and the oxidation was assessed for 24 hours at 37 $^\circ\text{C}$ using Spectromax Gemini thermometer. **A.** Oxidation kinetics for different rHDLs. **B.** Amount of oxidized DCFH formed after 24 hours. **C.** Typical oxidation rate during the propagation phase. **D.** Lag phase duration, **E.** Oxidability of rHDL expressed as relative fluorescence units (RFU) for different rHDLs (50 μg ApoA-I/ml), treated with CuSO_4 , and DCFH solution (0.2mg/ml) in the absence of LDL. *vs. LDL+ Cu^{+2} , with p values <0.05 (exact p values being indicated in the text). Values are shown as mean \pm SEM from at least 3 independent experiments that were performed in triplicate for each condition.

Figure 5. Soy-PE diminished the capacity of rHDL to accept phospholipids from LDL, while ARA-PE and EPA-PE reversed this effect, potentially by enhancing the fluidity of HDL. To evaluate the capacity of rHDLs to acquire phospholipids (PL), LDL was labeled with Dil fluorescent probe (5 μM). Then, Dil-labelled LDL (7.5 mg phospholipid/dL) was mixed with rHDL (4 mg ApoA-I/dL), or reference ApoB deficient plasma (1:30 v/v), and incubated for 1 hour to achieve PL transfer. After that, mixtures were incubated with ApoB-depleting precipitant for 10 minutes, followed by centrifugation and aspiration

of the rHDL- containing supernatant, and the fluorescence intensity of Dil-rHDL was measured. For the fluidity assay, rHDL particles (3.3 mg ApoA-I/dL) and DPH (0.80 μ M) or TMA-DPH (0.32 μ M) were incubated at 37C° for 1 hour to achieve probe incorporation into the lipoproteins, and the anisotropy of the probe fluorescence was measured. **A.** The percentage of Dil-labelled PL transferred from LDL to rHDLs. **B.** Fluorescence anisotropy of TMA-DPH probe, which is inversely related to surface fluidity of rHDL. **C.** Fluorescence anisotropy of DPH probe, which is inversely related to core fluidity of rHDL. **D.** Scheme illustrates the influence of Soy-PE, ARA-PE and EPA-PE on the arrangement of the phospholipid monolayer of rHDLs, according to their head group and the unsaturation level in their fatty acid moieties. These data indicate that Soy-PE reduced the capacity of rHDL to accept phospholipids from LDL, while the fatty acid moieties of ARA-PE and EPA-PE enhanced it, which could result from their fluidizing effects. In addition, these data point out that EPA-PE rHDL represented the most fluid particles of all the rHDLs studied. *vs. ADP, #vs. Soy-PC rHDL, ϕ vs. Soy-PE rHDL, δ vs. ARA-PE rHDL, with p values <0.05 (exact p values being indicated in the text). Values are shown as mean \pm SEM from at least 3 independent experiments that were performed in triplicate for each condition. TMA-DPH: trimethylamine-diphenylhexatriene; DPH: diphenylhexatriene.

Figure 6. Soy-PE reduced the AIA of rHDL, and ARA-PE exacerbated this effect while EPA-PE reversed it and even improved the AIA of rHDL. AIA of rHDLs was evaluated in LPS-stimulated THP-1 macrophages. **A.** Relative mRNA and protein levels of IL-1 β after short term (4 hours), and **B.** long term (16 hours) treatments of THP-1 cells with 20 μ g ApoA-I/mL of different rHDL followed by 4 hour stimulation with 100 ng/mL of LPS. **C-E.** Western blot analysis for the phosphorylated and non-phosphorylated ERK, p38, and JNK MAPKs in THP-1 cells treated with 20 μ g ApoA-I/mL of rHDLs for 4 hours, followed by 0.5 hour stimulation with 100 ng/mL of LPS.*vs. LPS alone, #vs. Soy-PC rHDL, ϕ vs. Soy-PE rHDL, δ vs. ARA-PE rHDL, with p values less than 0.05 (exact p values being indicated in the text). Values shown are from at least 3 independent experiments that were performed in triplicate for each condition. IL-1 β : Interleukin-1 beta; LPS: lipopolysaccharide; JNK: Jun kinase; ERK: Extracellular signal-regulated kinase; MAPK: mitogen-activated protein kinase.

Figure 7. Soy-PE converted rHDL into pro-inflammatory particles and ARA-PE exacerbated this effect while EPA-PE reversed it and even improved the basal AIA of rHDL. A. Pro-inflammatory eicosanoid production after 16 hours treatment of THP-1 cells with 20 μ g/mL of rHDLs. The pro-inflammatory effects of rHDLs were evaluated in THP-1 macrophages in the absence of LPS stimulation. **B.** Relative mRNA and protein levels of IL-1 β after short term (4 hours) and **C.** long term (16 hours) treatment of THP-1 cells with 20 μ g ApoA-I/mL of rHDL. *vs. None, #vs. Soy-PC rHDL, ϕ vs. Soy-PE rHDL, δ vs. ARA-PE rHDL, with p values less than 0.05 (exact p values being indicated in the text). Values are from at least 3 independent experiments that were performed in triplicate for each condition. HETE: hydroxyeicosatetraenoic acids; PGE2: prostaglandin E2, TXB2: thromboxane B2, LOX: lipoxygenase, and COX: cyclooxygenase enzymes.

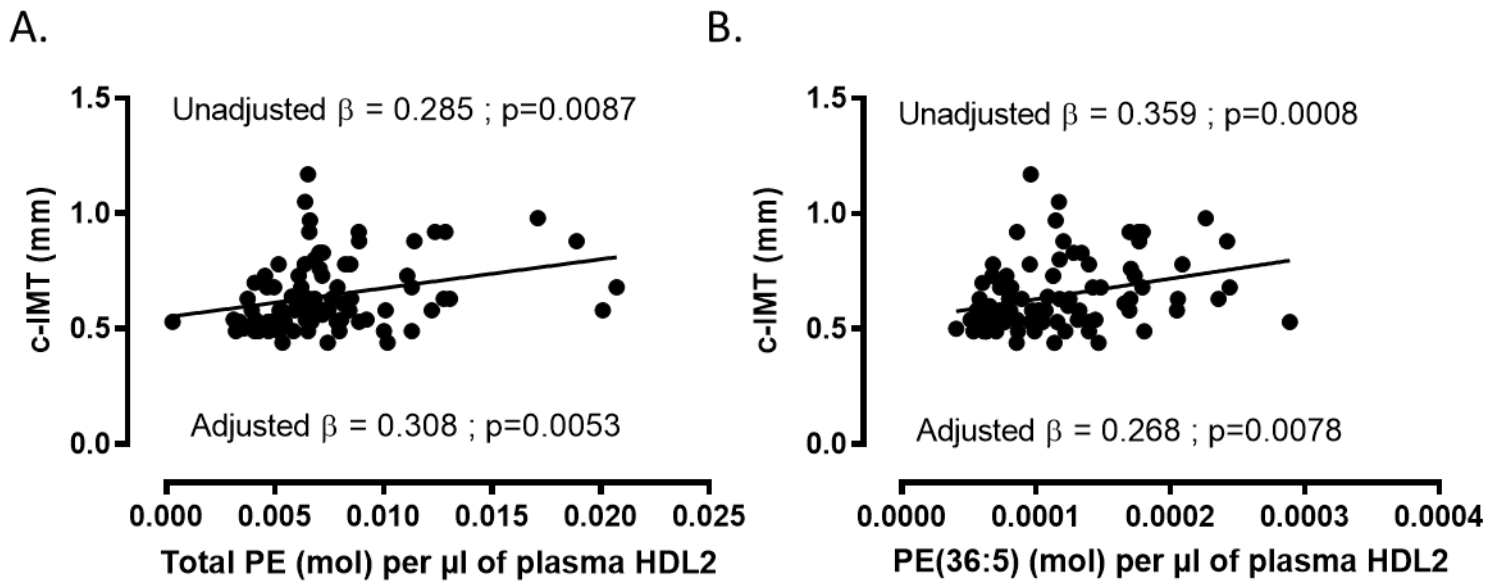
Figure 1 (figure 3.1 in the index of figures)

Figure 3.1. Total PE and PE (36:5) contents of HDL2 were associated with atherosclerosis in women with Metabolic Syndrome.

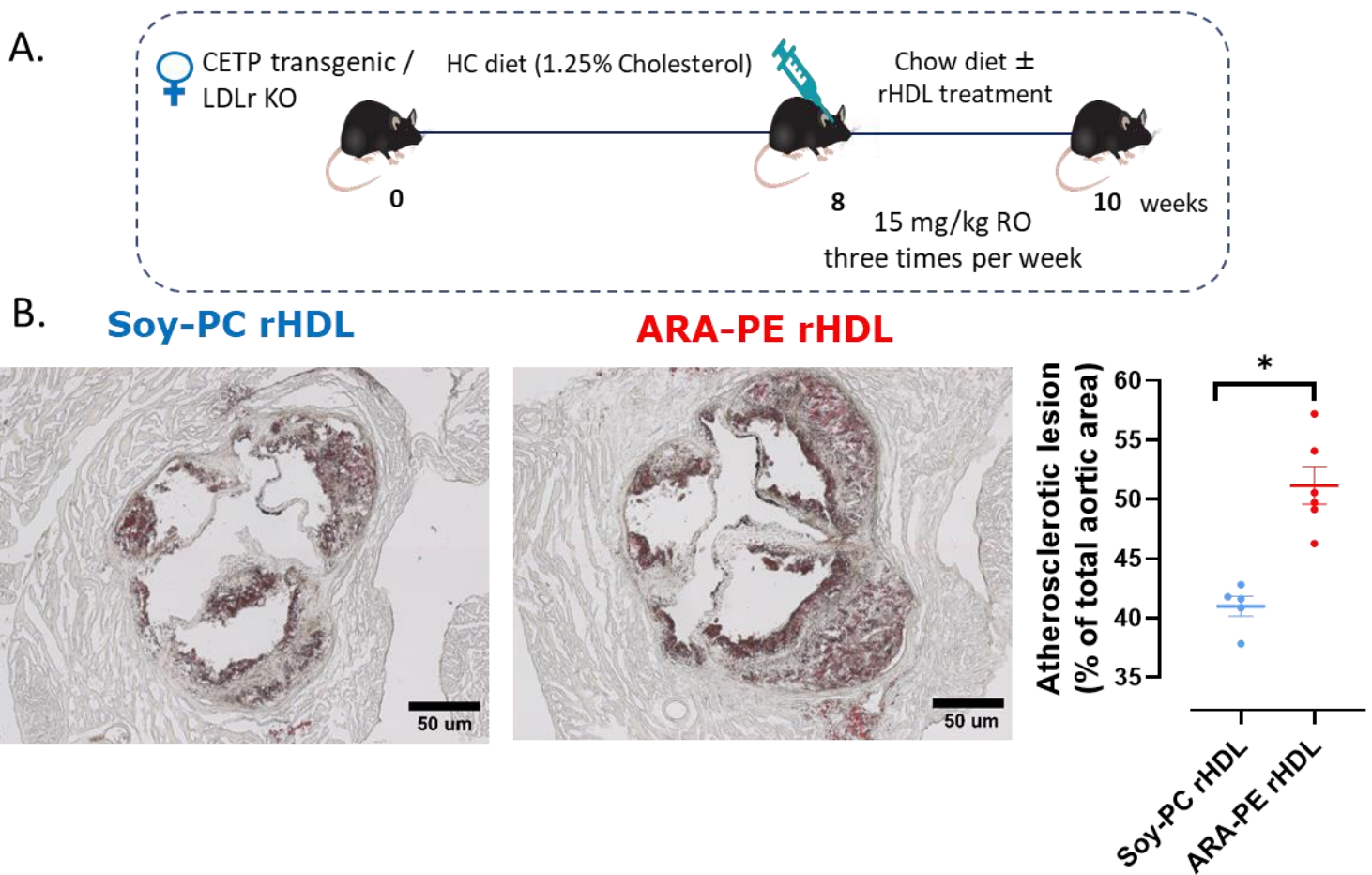
Figure 2 (figure 3.2 in the index of figures)

Figure 3.2. Injection of ARA-PE rHDL did not reduce the size of aortic atherosclerotic plaque as compared to Soy-PC rHDL in CETP transgenic-LDLr KO female mice.

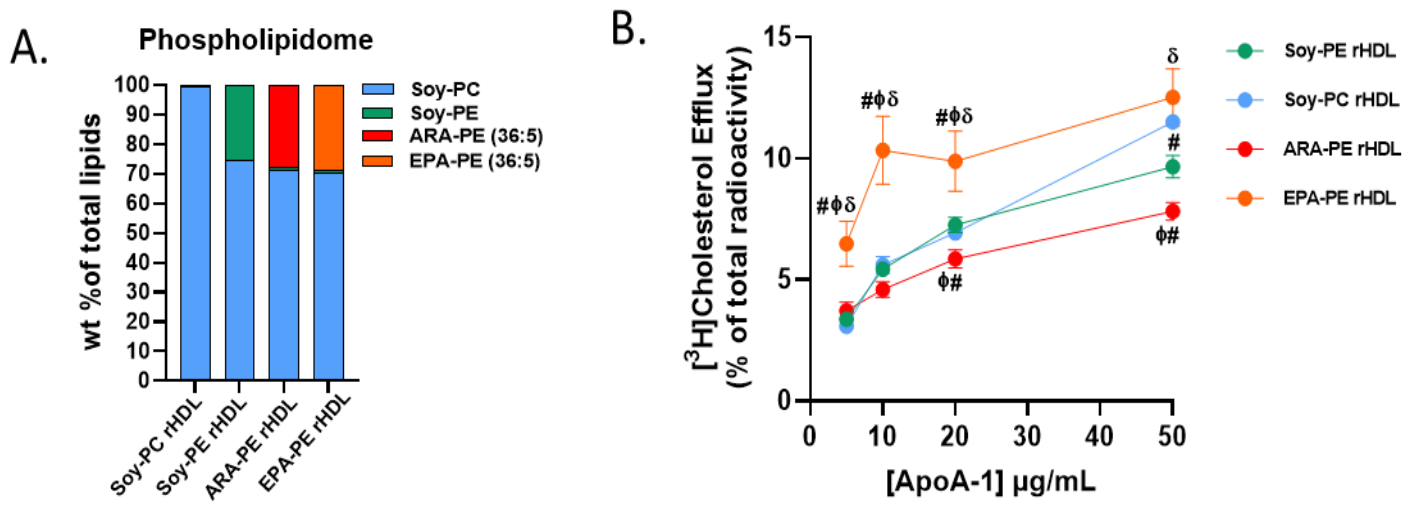
Figure 3 (figure 3.3 in the index of figures)

Figure 3.3. Soy-PE reduced the CEC of Soy-PC rHDL, and ARA-PE exacerbated this effect while EPA-PE reversed it and even improved the CEC.

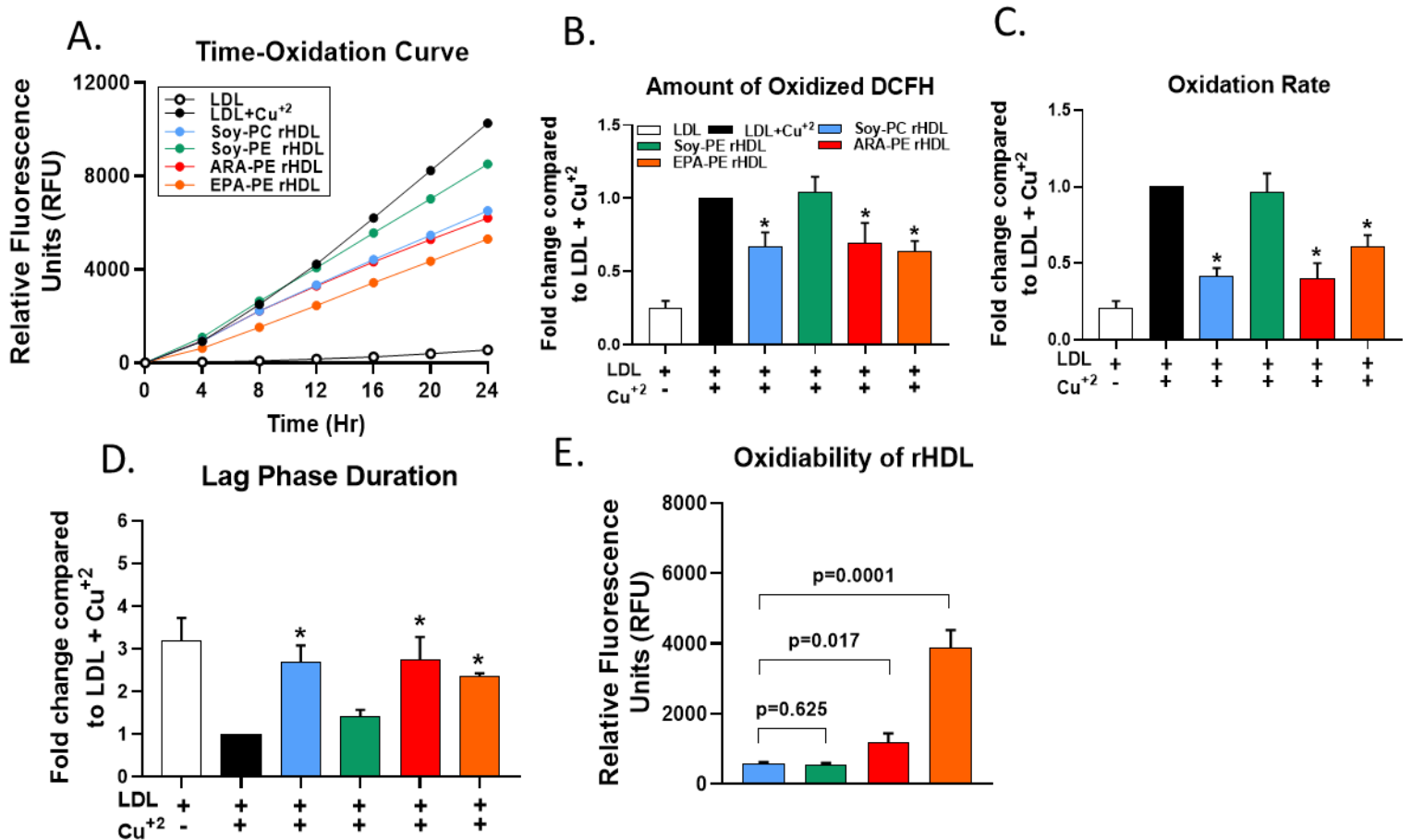
Figure 4 (figure 3.4 in the index of figures)

Figure 3.4. Soy-PE impaired the AOX of rHDL compared to Soy-PC rHDL, while ARA-PE and EPA-PE reversed this effect.

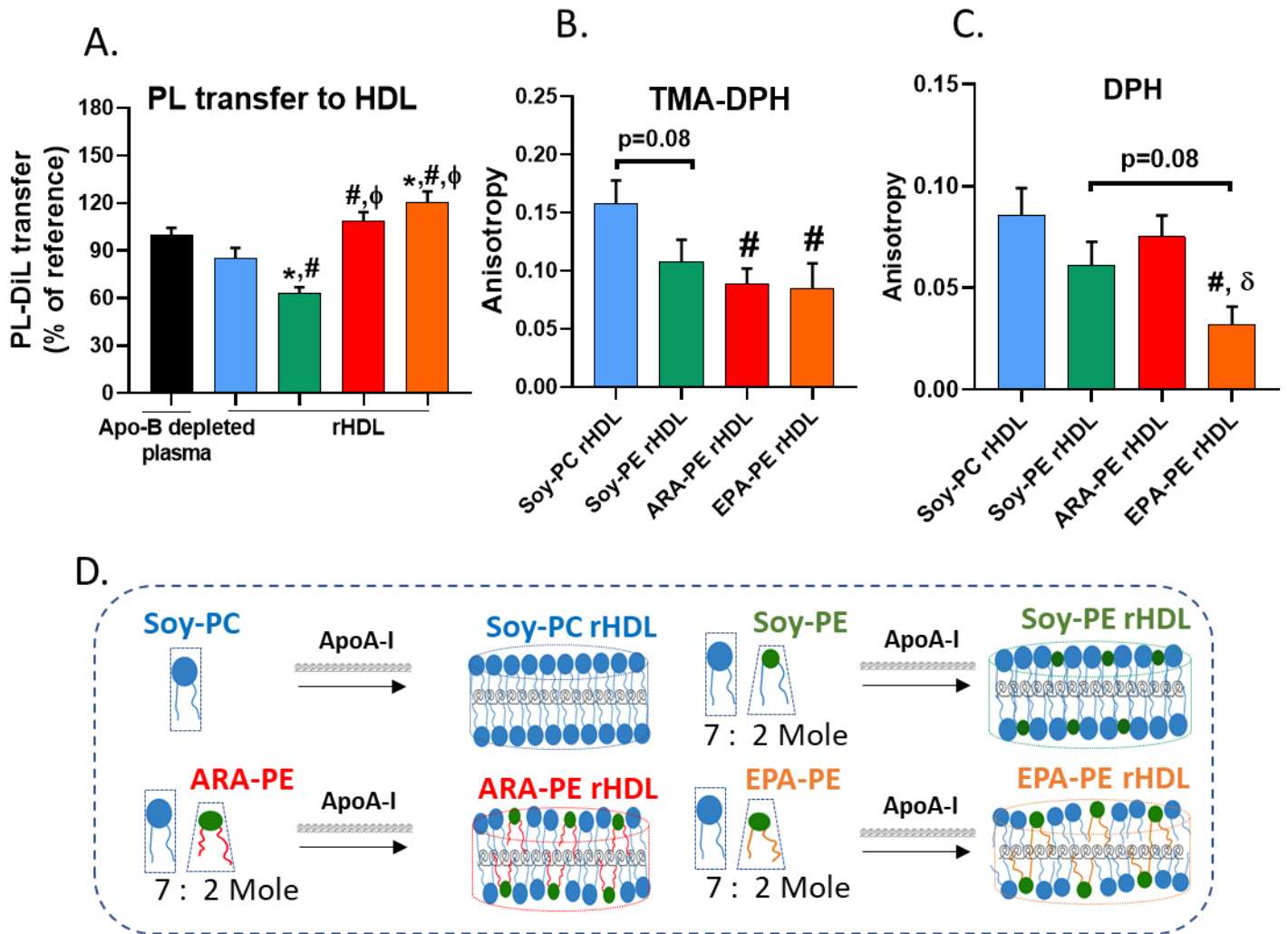
Figure 5 (figure 3.5 in the index of figures)

Figure 3.5. Soy-PE diminished the capacity of rHDL to accept phospholipids from LDL, while ARA-PE and EPA-PE reversed this effect, potentially by enhancing the fluidity of HDL.

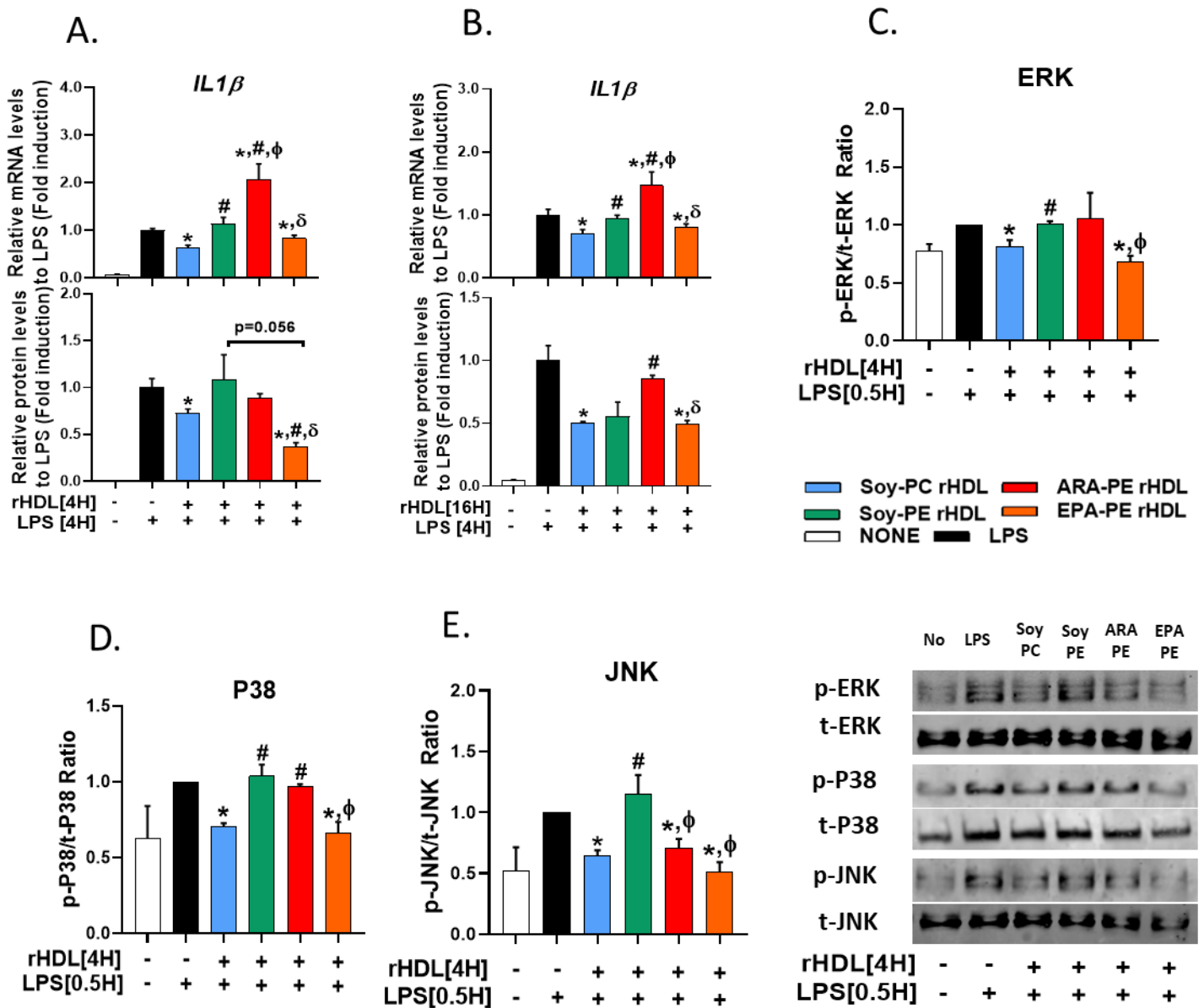
Figure 6 (figure 3.6 in the index of figures)

Figure 3.6. Soy-PE reduced the AIA of rHDL, and ARA-PE exacerbated this effect while EPA-PE reversed it and even improves the AIA of rHDL.

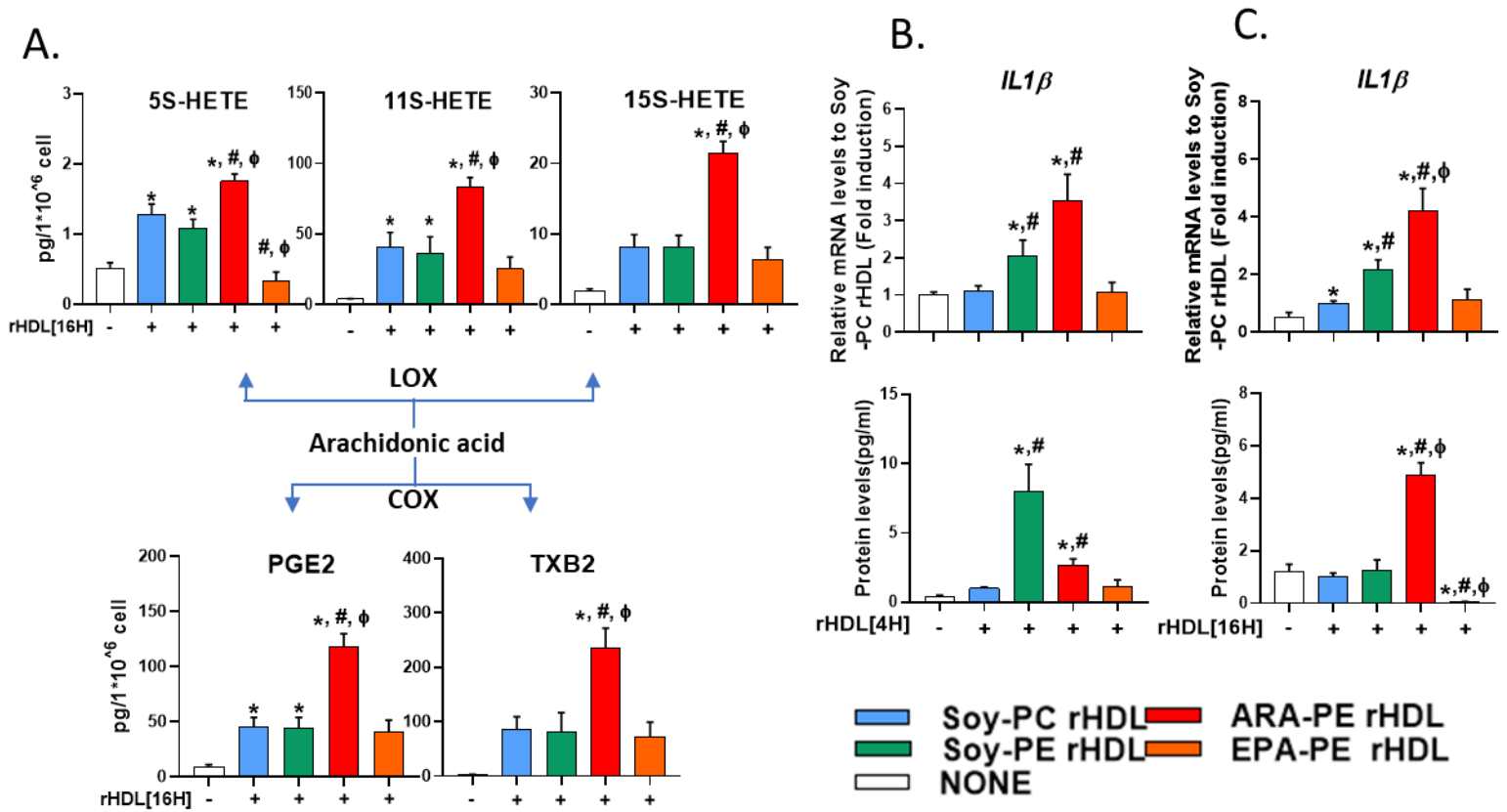
Figure 7 (figure 3.7 in the index of figures)

Figure 3.7. Soy-PE converted rHDL into pro-inflammatory particles and ARA-PE exacerbated this effect while EPA-PE reversed it and even improved the basal AIA of rHDL.

Supplemental data.

Table and Figure Legends.

Supplemental Table 1. Clinical data of female patients with metabolic syndrome.

Supplemental Table 2. Phospholipid: protein ratio in rHDL and their double bond content.

The rHDL particles were prepared by mixing human ApoA-I and SOY-PC without or with either SOY-PE, ARA-PE (16:1/20:4) or EPA-PE (16:0/20:5). **A.** Theoretical ApoA-I and phospholipid amounts used to prepare rHDL particles. **B.** Actual ApoA-I and phospholipid composition of the rHDL particles. ApoA-I: apolipoprotein A-I; DB: double bond; EPA: eicosapentaenoic acid; ARA: arachidonic acid; rHDL: reconstituted HDL; PC: phosphatidylcholine; PE: phosphatidylethanolamine.

Supplemental Table 3. List of primers used in this study. The mRNA levels of all the genes tested were normalized to the mean expression of three housekeeping genes, including NONO, TUBA and HSP90AB1.

Supplemental Table 4. Subspecies of PE (36:5) in human plasma. A. Blood concentrations of the six putative molecular subspecies of PE (36:5) predicted from the quantified individual fatty acid chains that construct these phospholipids in normolipidemic adults (both male + females). **B.** Chemical structure of PE (36:5) subspecies and the fatty acid moieties that constitute the six PE (36:5) subspecies. PE: phosphatidylethanolamine. *gamma-Linolenic (omega-6) vs alpha-Linolenic (omega-3) acid moiety.

Supplemental Figure 1. Quality control of ApoA-I and rHDL particles. A. Denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis to control the purity of ApoA-I containing fractions performed using 20% denaturing polyacrylamide gel revealed

with Coomassie blue. **B.** Nondenaturing PAGE analysis to control the size of rHDLs performed using 4–20% gradient polyacrylamide gel revealed with Coomassie blue. Standard proteins of known diameters were used for the calibration. ApoA-I: apolipoprotein A-I; ApoA-II: apolipoprotein A-II; EPA: eicosapentaenoic acid; ARA: arachidonic acid; rHDL: reconstituted HDL; PC: phosphatidylcholine; PE: phosphatidylethanolamine; Std: standard.

Supplemental Figure 2. Injection of Soy-PC rHDL reduced the size of aortic atherosclerotic plaque in CETP transgenic-LDLr^{-/-} female mice. **A.** Plasma levels of human ApoA-I at various time points after one injection of Soy-PC rHDL (n=3) or PBS (n=3) to validate the effectiveness of RO injection **B.** Plasma cholesterol levels after 8 weeks of HCD diet to validate the effectiveness of HCD. **C.** CETP transgenic/ LDL receptor KO female mice were fed high cholesterol diet (HCD) for 8 weeks before injection with 15 mg/kg of Soy-PC rHDL (n=2) or PBS (n=2), retro- orbitally (RO), every second day. The mice received 12 injections of either treatment, and were maintained on chow diet during rHDL treatment. The hearts were carefully dissected at the level of the aortic root, and embedded in tissue-tek optimum cutting temperature (OCT) medium, then cut every 10 µm from the aortic root at the level of 3 valves. **D.** Sections were labeled with Oil red-O to quantify the atherosclerotic plaque size. The extent of the atherosclerotic plaque was quantified using ImageJ software. *p<0.05 vs Soy-PC rHDL. CETP: cholesteryl ester transfer protein; HC: high cholesterol; rHDL: reconstituted high density lipoprotein; PC: phosphatidylcholine; PBS: Phosphate buffered saline.

Supplemental Figure 3. Compositional analysis of rHDL formulations used in this study. **A.** Fatty acid composition of Soy-PC, and Soy-PE (Avanti Polar Lipids) that were used to produce rHDLs. Two forms of PE (36:5) including ARA-PE (PE (16:1/20:4)), and EPA-PE (PE (16:0/20:5)), produced by UMR5246 Institute, were also used to produce rHDLs. **B.** LC/MS/MS Lipidomic analysis of the rHDL formulations including Soy-PC rHDL, Soy-PE rHDL, ARA-PE rHDL, and EPA-

PE rHDL. Data are shown for a representative preparation of rHDL. ARA: arachidonic acid; EPA: eicosapentaenoic; FA: fatty acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine.

Supplemental Figure 4. The impact of rHDLs on the gene expression of the major lipid transporters involved in cholesterol efflux pathways as well as on the ABCA1 and TLR-4 surface expression.

A-C. To evaluate the impact of rHDLs on the expression of the major genes involved in cholesterol efflux pathways, THP-1 cells were treated with 20 µg ApoA-1/ml of different rHDLs for 4 hours and the mRNA levels of ABCA1, ABCG1, and SRB1 were evaluated.

D. To evaluate the impact of rHDLs on the surface expression of ABCA1 in THP-1 macrophages, cells treated with 20 µg ApoA-1/ml of rHDL for 4 hours were collected and incubated with 100 µl of the ABCA1 rabbit primary antibody diluted in FcR blocking reagent containing PBS-FBS 5% (1:50 v/v) for 30 minutes. Then cells were stained with 100 µl of a secondary fluorescent antibody (Alexa-fluor 594 goat anti-rabbit IgG; 1:250 v/v) for additional 30 minutes. At the end of the incubation, cells were fixed using FOXP3 fixation diluent before they were subjected to flow cytometry analysis. **E.** TLR-4 cell surface expression. *vs. None (no rHDL was added), [#] vs. Soy-PC rHDL, ^φ vs. Soy-PE rHDL, ^δ vs. ARA-PE rHDL, with p values less than 0.05 (exact p values being indicated in the text). Values are shown from at least 3 independent experiments that were performed in triplicate for each condition.

Supplemental Figure 5. Oxidation parameters calculated from biphasic oxidation kinetics to evaluate the extent of copper-induced LDL oxidation.

The extent of LDL oxidation induced by copper ions was evaluated from the kinetic curve of the fluorescence of oxidized dichlorofluorescein diacetate (DCFH) registered during 24 hours, and expressed as relative fluorescence units (RFU). The kinetics displayed two characteristic phases, known as the lag and propagation phases. **A.** The lag phase represents a period of slow oxidation; its duration determined by extrapolating two lines: one originating from the curve's beginning (green line),

and the other aligning the rapid increase in the oxidation (orange line). **B.** The propagation phase signifies a period of rapid oxidation that starts after the lag phase and continues either the end of the experiment. The slope observed during this phase reflects the oxidation rate. **C.** The final measurement of RFU indicates the accumulated amount of oxidized DCFH during the entire duration of the experiment. Shorter lag phase, higher oxidation rate in propagation phase and higher amount of oxidized DCFH at the end of the experiment all indicate a higher level of LDL oxidation.

Supplemental table 1. (Table 3.1 in the index of tables)

Women with MetS (n=86)

	Median [Q1-Q3]
Age (year)	61.0 [53.0-66.0]
Weight (Kg)	60.65 [54.80-72.73]
BMI (Kg/m ²)	24.2 [21.2-28.65]
Waist (cm)	87.75 [87.00-97.00]
HbA1c (%)	5.80 [5.60-6.20]
TC (mg/dL)	230.5 [199.5-264.5]
TG (mg/dL)	95.0 [69.0-144.3]
Fasting glucose (mmol/L)	4.90 [4.47-5.40]
LDL-C (mg/dL)	147.0 [116.5-174.8]
HDL-C (mg/dL)	63.5 [53.0-74.0]
ApoA-I (g/L)	1.64 [1.49-1.87]
ApoB (g/L)	1.03 [0.93-1.21]
Lp(a) (mg/dL)	22.0 [9.0-59.7]
c-IMT	0.62 [0.54-0.75]
CAC score	22.7 [0.0-125.8]
Lipid-lowering therapy (%)	57

Table 3.1. Clinical data of female patients with metabolic syndrome.

Supplemental table 2. (Table 3.2 in the index of tables)

A.

rHDL	Composition/(Mole)					DB in 1 Mole PC	DB in 1 Mole PE	DB in total PL/1 Mole ApoA-I	% DB relative to Soy-PC rHDL
	ApoA-I	Soy-PC	Soy-PE	ARA-PE	EPA-PE				
Soy-PC	1	90	-	-	-	1.54	-	139	-
Soy-PE	1	70	20	-	-	1.54	1.58	140	+ 0.72%
ARA-PE	1	70	-	20	-	1.54	2.50	158	+ 13.7%
EPA-PE	1	70	-	-	20	1.54	2.50	158	+ 13.7%

B.

rHDL	ApoA-I (mg/ml)	Phospholipid (mg/ml)	Phospholipid/apoA-I weight ratio
Soy-PC	5.86	10.6	1.82
Soy-PE	6.15	8.63	1.40
ARA-PE	5.58	8.06	1.44
EPA-PE	5.22	8.10	1.55

Table 3.2. Phospholipid: protein ratio in rHDL and their double bond content.

Supplemental table 3. (Table 3.3 in the index of tables)

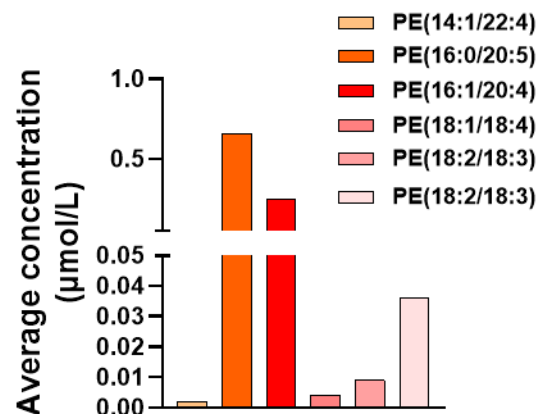
Gene	Full Name	Forward (5'-3')	Reverse (3'-5')
HSP90AB1	heat shock protein 90kDa alpha (cytosolic), class B member 1	CTC TGT CAG AGT ATG TTT CTC GC	GTT TCC GCA CTC GCT CCA CAA A
NONO	non-POU domain-containing octamer-binding housekeeping gene	CAT CAA GGA GGC TCG TGA GAA G	TGG TTG TGC AGC TCT TCC ATC C
TUBA	α -tubulin	GAT GCT GCC AAT AAC TAT GCC CGA G	GAA AAC CAA GAA GCC CTG AAG ACG G
ABCA1	ATP-binding cassette A1	GCC AAG GAC CAA AGT GAT G	CAC CCC GTA TGA ACA GGA TT
ABCG1	ATP-binding cassette G1	CCG ACC GAC GAC ACA GAG A	CTG AGC ACG AGA CAC CCA CCA CA
SRB1	Scavenger receptor class B type 1	CAT CTA CCC ACC CAA CGA AG	TGA GGA AGT GAG GAT GGG AG
IL1B	Interleukin 1 beta	TCC AGG GAC AGG ATA TGG AG	TCA TCT TTC AAC ACG CAG GA

Table 3.3. List of primers used in this study.

Supplemental table 4. (Table 3.4 in the index of tables)

A.

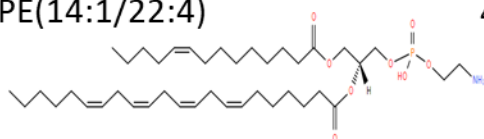
PE (36:5) subspecies	Most probable $\mu\text{mol/l}$	Upper-limit $\mu\text{mol/l}$
1. PE(14:1/22:4)	0.000 – 0.004 μM	0.278 – 0.856 μM
2. PE(16:0/20:5)	0.453 – 0.865 μM	6.093 – 10.107 μM
3. PE(16:1/20:4)	0.111 – 0.385 μM	0.883 – 3.383 μM
4. PE(18:1/18:4)	0.000 – 0.008 μM	0.009 – 0.125 μM
5. PE(18:2/18:3)*	0.007 – 0.011 μM	0.109 – 0.225 μM
6. PE(18:2/18:3)*	0.032 – 0.040 μM	0.58 – 0.696 μM



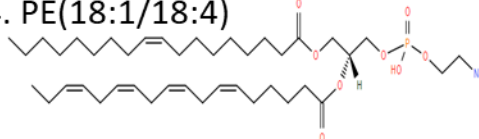
B.

Chemical structure of PE (36:5) subspecies

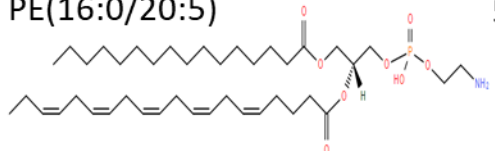
1. PE(14:1/22:4)



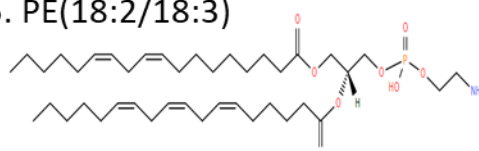
4. PE(18:1/18:4)



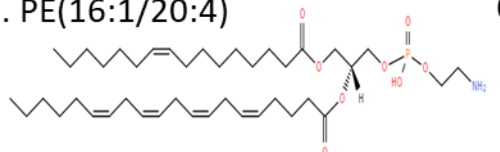
2. PE(16:0/20:5)



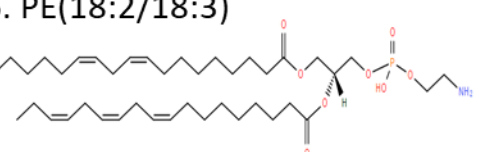
5. PE(18:2/18:3)



3. PE(16:1/20:4)



6. PE(18:2/18:3)



C14:1 = Myristoleic acid

C16:0 = Palmitic acid

C16:1 = Palmitoleic acid

C18:1 = Oleic acid

C18:2 = Linoleic acid

C18:3 = alpha-Linolenic acid

C18:3 = gamma-Linolenic acid

C18:4 = Stearidonic acid

C20:4 = Arachidonic acid

C20:5 = Eicosapentaenoic acid

C22:4 = Adrenic acid

Table 3.4. Subspecies of PE (36:5) in human plasma.

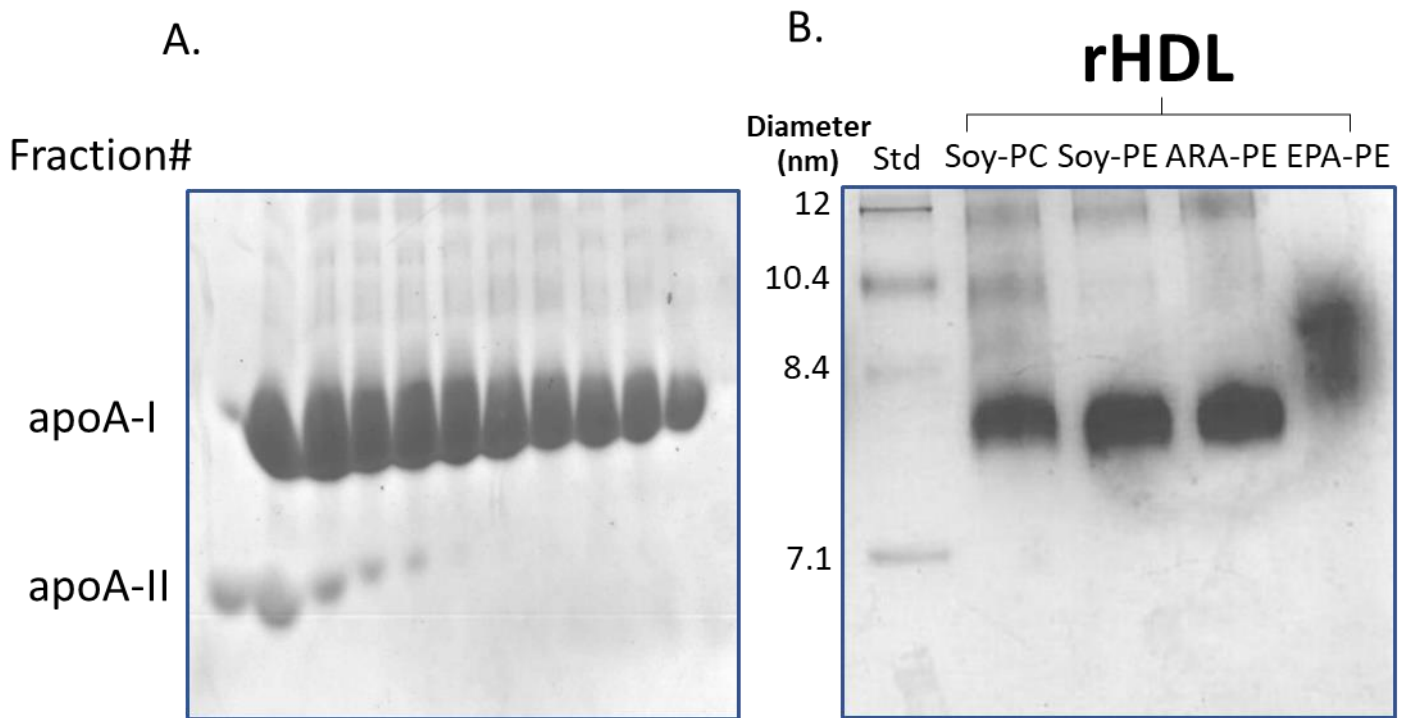
Supplemental Figure 1. (Figure 3.8 in the index of figures)

Figure 3.8. Quality control of ApoA-I and rHDL particles.

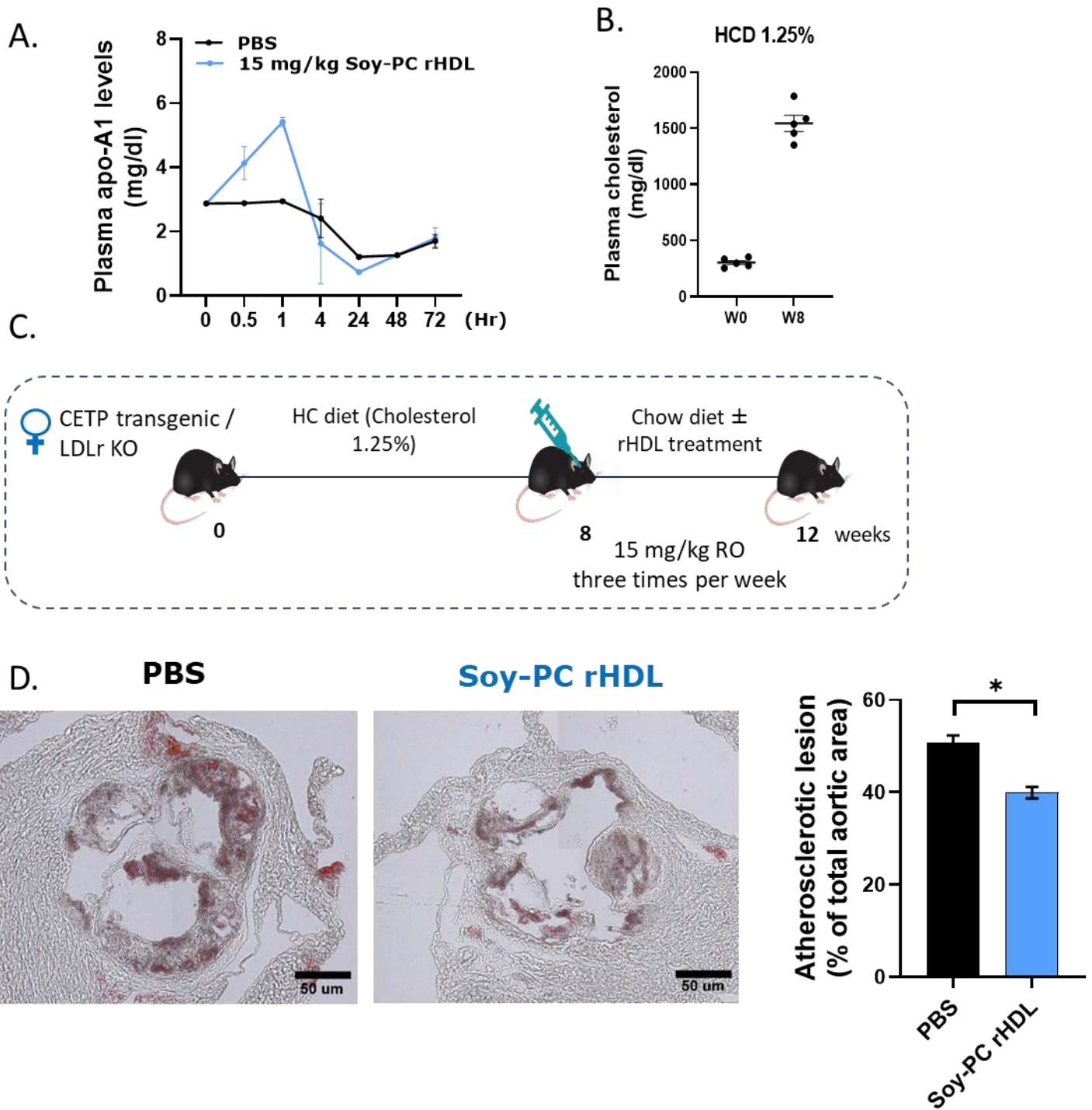
Supplemental Figure 2. (Figure 3.9 in the index of figures)

Figure 3.9. Injection of Soy-PC rHDL reduced the size of aortic atherosclerotic plaque in CETP transgenic-LDLr $-/-$ female mice.

Supplemental Figure 3. (Figure 3.10 in the index of figures)

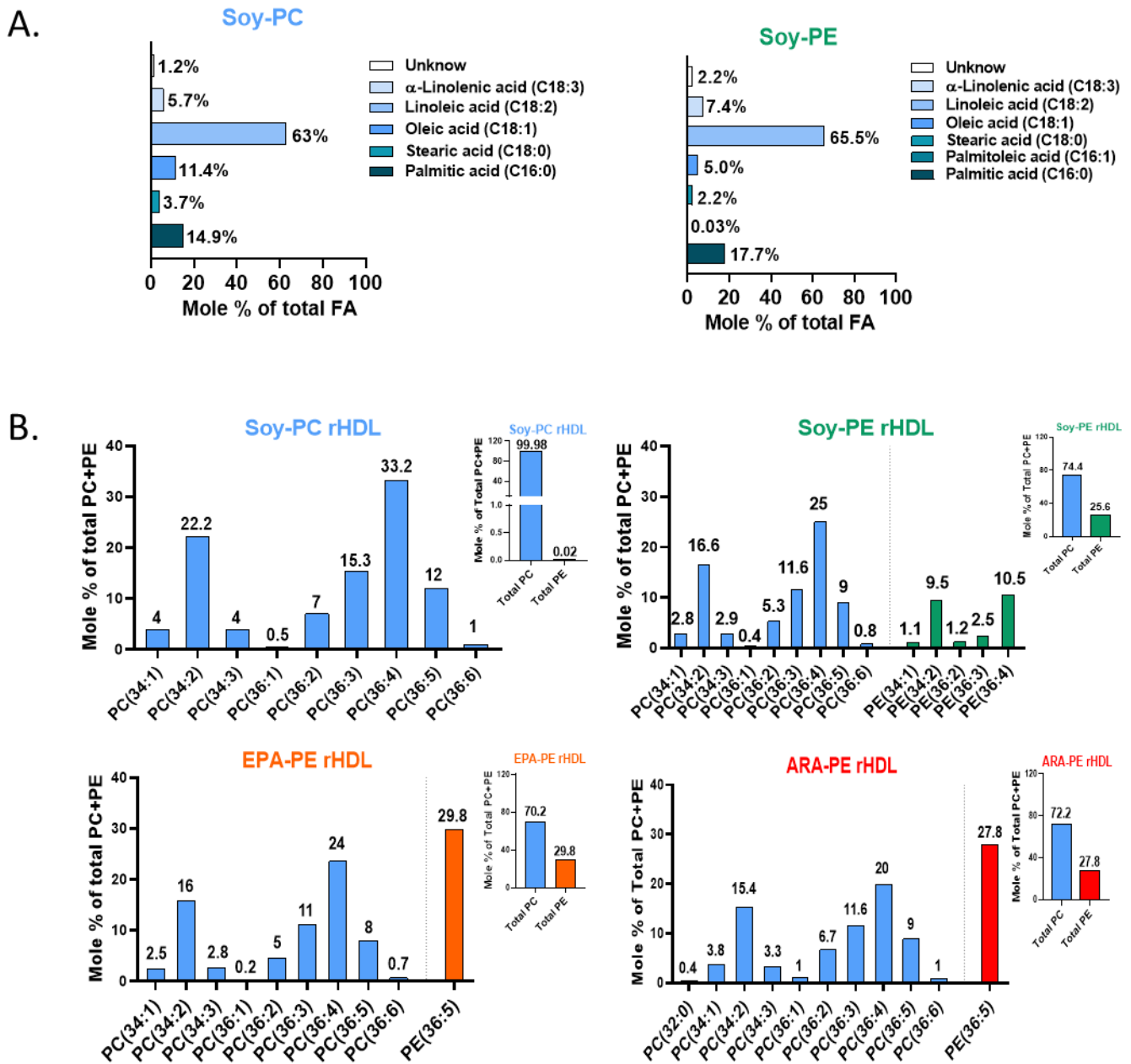


Figure 3.10. Compositional analysis of rHDL formulations used in this study.

Supplemental Figure 4. (Figure 3.11 in the index of figures)

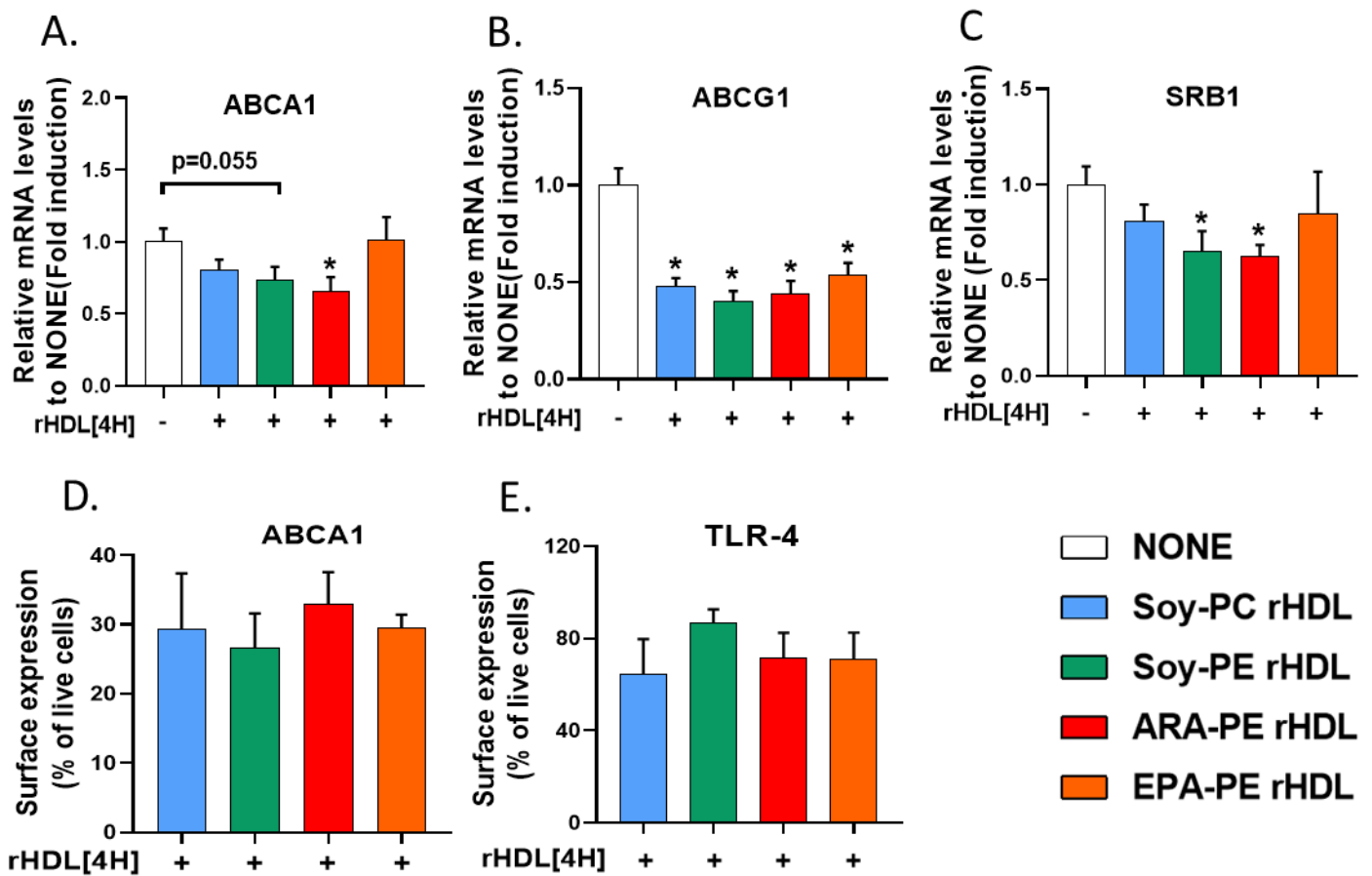


Figure 3.11. The impact of rHDLs on the expression of the major genes involved in cholesterol efflux pathways as well as on the ABCA1 and TLR-4 surface expression.

Supplemental Figure 5. (Figure 3.12 in the index of figures)

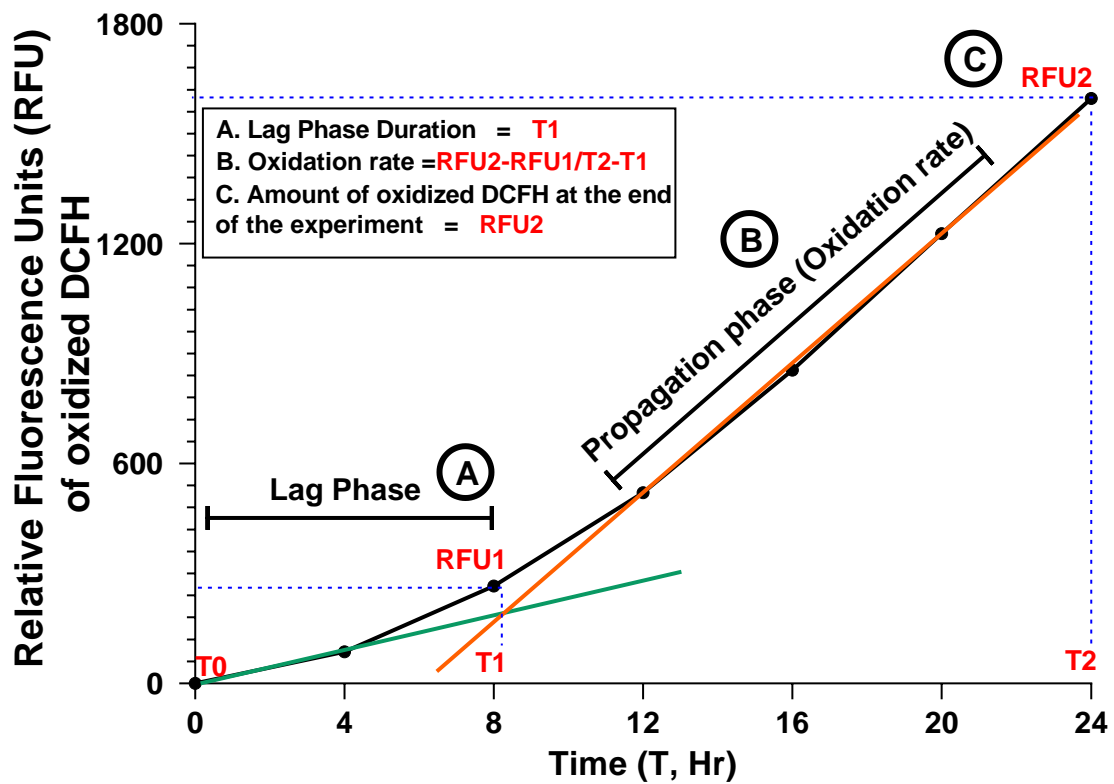


Figure 3.12. Oxidation parameters calculated from biphasic oxidation kinetics to evaluate the extent of copper-induced LDL oxidation.

DISCUSSION AND PERSPECTIVES

4

"Discovery consists of seeing what everybody has seen and thinking what nobody has thought." - Albert Szent-Györgyi

Chapter 4. General discussion and Perspectives

In general, my research work provided valuable insights into the relationship between various PE species and HDL functionality, shedding light on their implications in ASCVD.

PE in HDL: Current Insights, Associations with ASCVD, and potential Implications for HDL Functionality

Atherosclerosis, a chronic inflammatory disease of arteries, is the primary driver of ASCVD. Among the key features of atherosclerotic plaque are LDL retention and oxidation, intravascular inflammation, and the formation of foam macrophages within the arterial wall (32). Among plasma lipoproteins, HDL is the only one that counteracts atherosclerosis via its capacity to facilitate cholesterol efflux from arterial foam macrophages (i.e. RCT pathway) or to prevent its influx into arterial macrophages (i.e. RRT pathway). In addition, HDL is well-known for its protective anti-inflammatory, and anti-oxidative effects (49,173). A growing body of evidence emphasizes the pivotal role of HDL's composition (mainly proteins and lipids) in determining its functions. Notably, phospholipids (\pm 35-50% of HDL lipidome), such as PC, PE, PS, and PI, largely influence HDL functionality based on their physicochemical properties and quantity. Under pathological conditions, both composition and functions of HDL particles are susceptible to alterations, rendering HDL with attenuated functions or even becoming proatherogenic (251). In this regard, studies highlighted that altered abundance of specific phospholipid species could contribute to HDL's dysfunctionality. While several lipidomic studies demonstrated a positive association between plasma PE, notably PE (36:5), and ASCVD, the underlying mechanisms remain unknown (15,16). Plasma PE (36:5) exists as EPA-PE and ARA-PE, with the latter predominating in CMD and ASCVD.

During my research work, I addressed the hypothesis that PE species, especially PE (36:5), within HDL particles might attenuate their anti-atherogenic functions. The objectives of my research were threefold; first, to establish potential associations between PE species content in HDL and atherosclerosis in patients with MetS; second, to assess the influence of ARA-PE (36:5) on the capacity of HDL to reduce atherosclerosis in mice model; third, to investigate *in vitro* how PE species, including ARA-PE and EPA-PE might alter the structural and functional properties of HDL. My research work revealed positive links between total PE and PE (36:5) contents in HDL with atherosclerosis, and identified PE species, notably PE (36:5), as novel

biomarkers of HDL functionality. In addition, I shed light on potential novel pathways by which PUFA associated to PE (i.e. ARA-PE and EPA-PE) could impact ASCVD, enhancing our comprehension of their potential roles in disease development and treatment (refer to **Figure 2.1**).

Total PE and PE (36:5) and atherosclerosis: Bridging the knowledge gap

In our clinical cohort, we established for the first time a strong link between the content of PE species in HDL and their implication in ASCVD. Earlier lipidomic studies have identified a positive association between plasma levels of PE species and ASCVD with PE (36:5) species emerged as a lipid signature to predict ASCVD events, and mortality (refer to **Section 1.7**) (15, 16). In this study, we revealed a strong positive correlation between total PE and PE (36:5) contents in HDL from women with MetS and cIMT, a surrogate marker for atherosclerosis (307), reinforcing the notion of the potential proatherogenic roles of PE species in ASCVD. Interestingly, our study found that the positive association between total PE and PE (36:5) contents in HDL and atherosclerosis remained significant after adjusting for various risk factors including age, T2D, hypertension, smoking, and plasma levels of total cholesterol and triglycerides. This imply that total PE and PE (36:5) contents in HDL could serve as independent markers for assessing ASCVD risk. Compared to early studies, our findings provide novel insights via linking the association of plasma PE and PE (36:5) with ASCVD to their contents in HDL, hinting a mechanistic link for such association.

The work of Khere et al., demonstrated a strong inverse correlation between the CEC of HDL from macrophages and cIMT, independently of the HDL-C levels, signifying that higher CEC of HDL aligns with lower cIMT measurements (6). Our findings propose a novel perspective: the content of PE, notably PE (36:5), within HDL might exert negative influence on the CEC of HDL, which could potentially contribute to atherosclerosis development, as evidenced by the positively correlated cIMT measurements. Thus, we propose that measuring HDL-PE together with HDL-C and their ratio (i.e. HDL-PE to HDL-C ratio) rather than only HDL-C levels could help to assess the quality of HDL and could serve as novel marker to detect HDL dysfunctionality. These insights suggest that modulation of HDL functionality by PE species could participate to their positive association with ASCVD.

Moreover, our study revealed a positive correlation between total PE and PE (36:5) contents in HDL from women with MetS and both the CAC score >100 and the presence of

atherosclerotic plaque in carotid arteries as illustrated in **Annex 1**. However, after adjusting for age, the correlation was no longer significant, likely due to the strong impact of age on atherosclerosis development and the limited size of our cohort, indicating the importance of validating our findings in larger cohorts.

These observations suggest that total PE and PE (36:5) contents in HDL may contribute to atherosclerosis development, hinting the potential clinical relevance of PE composition of HDL in ASCVD risk assessment.

PE (36:5) in HDL and atherosclerosis: *In vivo* mechanistic insights

As we established the positive association of PE (36:5) with atherosclerosis in human, we further validated this association in mice model. While prior research hinted at the significance of PE (36:5)'s fatty acid composition in driving its association with ASCVD, the precise molecular subspecies remained undetermined (537). In this context, two subspecies of PE (36:5) including EPA-PE and ARA-PE, exist in human plasma. Collectively, the deleterious role of ARA in ASCVD, the higher prevalence of ARA-associated phospholipids in obese women with metabolic dysfunction and in patients with ASCVD (538,539), and the increased levels of ARA-containing PE species in HDL from rheumatoid arthritis patients that were linked to inflammation and increased CVD risk (540), suggest a likely prevalence of ARA-PE subspecies in HDL from women with MetS.

Thus, we investigated the impact of ARA-PE presence in HDL on atherosclerosis development. Using rHDL approach in a pertinent mouse model of atherosclerosis, we observed a diminished capacity of ARA-PE rHDL to reduce atherosclerosis when compared to the control Soy-PC rHDL. Previous studies employing rHDLs, such as CSL-111 and CSL-112, have demonstrated their capacity to induce rapid regression of atherosclerotic plaque via enhancing cholesterol removal from arterial wall macrophages (451). Additionally, these rHDLs have demonstrated potent anti-inflammatory and antioxidant properties in both animal models and human subjects (458–462). Given the similar composition of our control Soy-PC rHDL to CSL-111, and CSL-112 rHDLs, the effectiveness of our control in reducing atherosclerosis reflects its efficient anti-atherogenic functions. In contrast, the diminished capacity of ARA-PE rHDL to reduce atherosclerosis suggests a potential impairment in its functions.

The association between the total PE content in HDL and atherosclerosis suggests deleterious effects from the ethanolamine (Etn) head group, while the association between PE (36:5) in HDL and atherosclerosis suggest additional deleterious effects from its lipid part (i.e. ARA). Thus, we explored independently the influence of Etn head group and of the lipid part of PE on the functions of rHDL. To study the impact of Etn head group, we used Soy-PC and Soy-PE. These molecules differ solely in their head groups (Choline versus Etn). On the other hand, to focus on the lipid part, we employed ARA-PE and EPA-PE. These molecules are distinct from Soy-PE only in their fatty acid composition, namely ARA, and EPA, respectively.

PE and ARA-PE (36:5) in HDL: Attenuating HDL-Functions

Our *in vitro* investigations identified PE in general (i.e. Soy-PE), and ARA-PE in specific, as novel biomarkers for main dysfunctions of HDL particles including their cholesterol efflux capacity (CEC), antioxidant activity (AOX), and anti-inflammatory activity (AIA), further emphasizing the critical role of HDL phospholipidome in determining its functions (10).

Beginning with the impact of PE's head group, our study revealed that the presence of PE in rHDL particles reduced their CEC from human macrophages compared to PC rHDL, without affecting the expression of the lipid transporters involved in this process. Thus, we proposed that the negative influence of PE on the CEC of rHDL may be attributed to the lower affinity of its Etn head group toward cholesterol compared to the Choline head group of PC (541), implying reduced cholesterol insertion into PE rHDL. Moreover, PE was shown to alter the dynamic structure and interactions of rHDL particles with cell membrane receptors like ABCA1(18,445). In line with our findings, Fournier et al., reported an inverse correlation between total PE content of HDL and cellular cholesterol efflux to human serum (19). In addition, PE also impaired the AOX of rHDL, likely by diminishing its capacity to acquire phospholipids from LDL (343,542). This effect may be due to the rigidity of the Etn head group (543,544), which could limit the incorporation of phospholipids into PE rHDL by increasing interactions with adjacent lipids on rHDL surface. Furthermore, PE also attenuated the AIA of rHDL by impairing its capacity to inhibit both the LPS-induced IL-1 β expression and secretion and the LPS-induced ERK, JNK, and p38 MAPK activation in macrophages. Even more, we also noticed short term pro-inflammatory effects of PE rHDL in non-activated human macrophages with LPS, manifesting as increased IL-1 β expression and secretion. Our data also revealed similar pro-inflammatory effects with PE rHDL at the protein levels of MCP-1, IL-6, IL-8, and IL-12 cytokines in activated and resting macrophages as illustrated in **Annexes 3 and**

5. In addition, we observed an impaired capacity of PE rHDL to reduce the mRNA levels of VCAM-1, ICAM-1, MCP-1, and IL-6 in TNF α -activated HUVECs compared to PC rHDL as illustrated in **Annex 2**. Together, our findings indicate that the presence of PE, independently of its lipid part, deleteriously impacts the CEC, AOX, and AIA of HDL particles, and render them pro-inflammatory.

Moving to the impact of PE's lipid part, our study revealed that the replacement of Soy-PE with ARA-PE further impaired the CEC of rHDL. Up to date, only one study reported a reduced CEC of rHDL due to free linoleic acid (an omega-6 fatty acids) (545), with no data available on the impact of ARA. Unlike Soy-PE, our study highlighted the capacity of ARA-PE to effectively preserve the AOX of PE-containing rHDL, potentially by enhancing its capacity to acquire phospholipids from LDL, likely via increasing the surface fluidity of rHDL. Moreover, our findings revealed higher susceptibility of ARA-PE rHDL to oxidation as illustrated in **Annex 7**, which potentially enhance its oxidants-scavenging capacity and overall antioxidant potential. While ARA-PE impaired the AIA of rHDL similarly as Soy-PE in LPS-activated macrophages (except for the inhibition of JNK activation), we noticed that ARA-PE rHDL increased the production of pro-inflammatory eicosanoids, and induced long term IL-1 β expression and secretion in non-activated macrophages. This long term pro-inflammatory effect was also confirmed at the protein levels of TNF α , MCP-1, IL-6, and IL-12 as illustrated in **Annexes 6**. Given that ARA is the primary precursor for pro-inflammatory lipid mediators (538,546), the increased production of eicosanoids suggest that macrophages used ARA-PE derived from rHDL as a source of ARA. Finally, we observed that ARA-PE rHDL failed to reduce the mRNA levels of VCAM-1, ICAM-1, MCP-1, and IL-6 in TNF α -activated HUVECs, while increased the mRNA levels of VCAM-1 in non-activated HUVECs compared to Soy-PC HDL as illustrated in **Annex 2**. Our findings indicate that the presence of ARA in the lipid part of PE, exacerbates its deleterious effects on the CEC and AIA of HDL particles, and render them more pro-inflammatory, whilst preserve their AOX.

In conclusion, our findings highlighted the capacity of PE to impair the anti-atherogenic functions of HDL, and the fact that ARA-PE (36:5) exacerbates this deleterious effect, and convert rHDL into pro-atherogenic particles, providing mechanistic insights into how the contents of total PE and PE (36:5) in HDL are linked to ASCVD.

EPA-PE (36:5) in HDL: Novel mechanisms for EPA-mediated atheroprotection.

Remarkably, our *in vitro* investigations allowed also to identify EPA-PE, the other major subspecies of PE (36:5), as an enhancer of HDL's anti-atherogenic functions, potentially counteracting the negative effects of PE and ARA-PE and providing valuable mechanistic insights into the EPA-mediated atheroprotection.

Our study revealed that the replacement of Soy-PE with EPA-PE completely abolished the deleterious effects of PE on all HDL functions, while concomitantly enhanced the functions of HDL compared to Soy-PC rHDL. First, we observed that EPA-PE robustly enhanced the CEC of rHDL compared to Soy-PC rHDL. Such effect of EPA has been reported with EPA-PC compared to PC rHDL (547). We showed also the capacity of EPA-PE to improve both the surface and core fluidity of rHDL. Such profound fluidizing effects of EPA-PE are likely due to EPA's high unsaturation level, conferring higher structural flexibility for adopting highly kinked conformations in the phospholipid monolayer of rHDL (548). This could facilitate cholesterol insertion and provides more space for cholesterol incorporation into rHDL, confirming the established role of HDL's surface fluidity as a key determinant of this process (212,213). We revealed also the capacity of EPA-PE to preserve the AOX of PE-containing rHDL, likely via enhancing both the capacity of rHDL to acquire phospholipids from LDL, and its susceptibility to oxidation as illustrated in **Annex 7**, thereby enhancing its oxidant-scavenging capacity. In line with our findings, the positive influence of free omega-3 PUFA (i.e. EPA, docosahexaenoic acid (DHA), and α -linolenic acid) on the AOX of both plasma HDL and rHDL and has been well-documented (545,549). The presence of EPA-PE enhanced also the capacity of rHDL to inhibit LPS-induced IL-1 β expression and secretion, while exhibited similar inhibitory effects on LPS-induced ERK, JNK, and p38 MAPK activation compared to Soy-PC rHDL in human macrophages. In parallel, EPA-PE rHDL reduced both the production of pro-inflammatory eicosanoid (i.e. 5S-HETE) and the secretion of IL-1 β in resting human macrophages. To strengthen these AIA properties, EPA-PE rHDL reduced the mRNA levels of VCAM-1, ICAM-1, and IL-6 in resting HUVECs compared to Soy-PC rHDL as illustrated in **Annex 2**. Such effect was reported with EPA-PC rHDL in activated endothelial cells compared to PC rHDL (547). Worth mentioning is that earlier studies have demonstrated similar effectiveness of EPA-PC and EPA-PE in promoting anti-inflammatory effects in macrophages (550), suggesting that the beneficial effects of EPA are independent of the associated

phospholipid. Our data indicate that the presence of EPA in PE abolished its deleterious effects on HDL functions, and potentially improved them.

In conclusion, our finding suggested the cardioprotective potential of EPA-PE (36:5) via reversing the deleterious effects of PE on HDL functions and potentially improving them. Such findings reinforce the therapeutic significance of EPA in managing ASCVD, potentially via enhancing HDL-mediated atheroprotection.

Study limitations

As with any research, our study raises new questions warranting further exploration. First, the relatively small sample size in our cohort may affect the data robustness and their generalizability, warranting validation in larger, diverse cohorts including both genders. Second, understanding the precise mechanisms underlying the impact of PE, ARA-PE, and EPA-PE on HDL functions requires more investigations. Third, differences between the *in vitro*, *in vivo* models and human biology necessitate clinical validation. Finally, the structural complexity of plasma HDL may influence its functionality, emphasizing the need to explore how PE, ARA-PE, and EPA-PE affect the structural and functional properties of plasma HDL isolated from individuals with CMD.

Future perspectives

In our study, we uncovered a new mechanism linking plasma PE, especially PE (36:5), to atherosclerosis. We found that PE has deleterious effects on the anti-atherogenic functions of HDL, and these effects are worsened in the presence of ARA in PE. In contrast, we identified the presence of EPA in PE as a rescue for HDL functions, reversing the PE's negative effects and potentially enhancing them. Our research highlights the significance of PE, and notably PE (36:5), in influencing HDL functions, providing novel insights for ASCVD management. Our findings also pave the way for future research to deepen our understanding of how PE, ARA-PE, and EPA-PE affect HDL functions in ASCVD.

Our *in vitro* data emphasize the need to validate the effects of PE-containing rHDLs on HUVECs, particularly regarding VCAM-1 and ICAM-1 protein levels and cell surface expression using flow cytometry analysis. We are also interested in assessing how these rHDLs impact HUVECs' ability to bind THP-1 monocytes through cell adhesion assays. Additionally, it is interesting to investigate the influence of PE-containing rHDLs on the ER stress response

via IRE1a /ASK1/p38 MAPK signaling pathway, known to mediate pro-inflammatory effects by rHDL (368). Furthermore, it is essential to determine whether rHDLs serve as a source of EPA and ARA for cells by assessing their incorporation into the cell membrane of macrophages. Beyond our *in vitro* focus on HDL functions such as CEC, AOX, and AIA, HDL particles exhibit several other beneficial effects, including anti-apoptotic, anti-thrombotic, and vasodilatory properties, all contributing to cardiovascular protection. Therefore, it is valuable to explore how PE species influence these functions using endothelial cell lines and platelets, the main cellular targets of HDL to exert these effects. Given the crucial role of HDL in accepting cholesterol from TGRL during their lipolysis (i.e. RRT) for atheroprotection, it's vital to assess how PE, ARA-PE, and EPA-PE influence this function. This could offer valuable insights if the presence of PE in HDL contributes to the observed U-shaped relationship between HDL-C levels and CVD risk, which our lab previously linked to this function.

To deepen our understanding of how ARA-PE rHDL impacts the atherosclerosis *in vivo*, we plan to investigate its effect on cholesterol removal from peripheral cells by assessing the CEC of mice plasma from cholesterol-loaded macrophages. Additionally, we will evaluate its impact on atherosclerotic plaque's inflammation by examining macrophage infiltration and endothelial VCAM-1 expression in aortic sections using immunostaining. We will also measure systemic inflammatory markers like plasma IL-6 and IL-1 β levels. Furthermore, it is important to explore the effects of PE and EPA-PE on the ability of rHDL to reduce atherosclerosis and inflammation in mice model, to validate their *in vitro* effects.

Furthermore, to validate the positive associations between total PE and PE (36:5) contents in HDL and atherosclerosis, we have access to a cohort of patients with familial hypercholesterolemia (n=300) with controlled LDL-C levels by statin treatment. In this cohort, we have complete cardiac phenotyping, including cIMT, CAC score and also a CT scan of the coronary arteries (coroscanner). The latter provides information on the volume of the plaque, the degree of cellular infiltration, and the level of calcification of the arteries. Additionally, it is equally important to analyze the correlations between total PE and PE (36:5) contents and the functions of isolated HDL particles from those patients. This could potentially enhance our understanding of the impaired HDL functionality observed in familial hypercholesterolemia patients in earlier studies (551,552).

The most significant aspect of our research lies in its clinical translation. The contrasting effects of ARA-PE and EPA-PE on HDL functions, propose valuable insights into the clinical

relevance of plasma EPA/ARA ratio in ASCVD. Although a lower EPA/ARA ratio is a marker of inflammation and increased ASCVD risk (538), no available data regarding this ratio in the context of plasma lipoproteins, and the phospholipids to which EPA and ARA are associated. Our data propose a link between EPA/ARA ratio, plasma PE, and HDL functions, suggesting a potential mechanism by which this ratio may influence ASCVD. Building upon this, future clinical studies are essential to investigate the impact of increasing plasma EPA/ARA ratio on HDL functions. In addition, investigating the impact of different EPA-PE/ARA-PE ratios on rHDL functions is equally important.

Given the significance of plasma EPA/ARA ratio in ASCVD (538), several therapeutic interventions have been made to raise this ratio aiming to reduce CVD risk and CAD events. Among these, the REDUCE IT and EVAPORATE trials have demonstrated high effectiveness of Icosapent Ethyl (a highly purified EPA ethyl ester) in reducing major ASCVD events, and atherosclerotic plaque volume (refer to **Section 1.1.5.2**) (139,523). The high effectiveness of Icosapent Ethyl in REDUCE IT trial, led some to suggest that a portion of the observed effects might be due to the theoretical deleterious effects of the mineral oil placebo rather than the beneficial effects of EPA (544,545). However, recent review demonstrated that the mineral oil is biologically inert at the quantities used in REDUCE-IT trial (553). Furthermore, the JELIS trial further supported the effectiveness of highly purified EPA in reducing CVD events without using mineral oil as placebo (554). Thus, omega-3 fatty acids (i.e. EPA) emerged as another option for reducing the burden of ASCVD. However, combining EPA with DHA, an another omega-3 fatty acid, in clinical trials such as STRENGTH trial failed to show any reduction in major CVD events in statin-treated patients received a 1g/day of a mixed carboxylic acid formulation of EPA plus DHA, compared with corn oil placebo (555). In another study, low-dose EPA-DHA (400 mg/day) failed also to reduce the major CVD events in patients with a history of MI (556). These conflicting results raised questions about the effectiveness of EPA alone but not with DHA in reducing CVD events. Indeed, several studies reported distinct effects of EPA and DHA on lipoproteins profile, inflammation, lipid oxidation, and cholesterol metabolism, supporting the notion that EPA has unique benefits in CVD, based on its unique physicochemical properties (557–559).

Strikingly, our findings led us to propose novel perspectives into the ongoing debate regarding the effectiveness of omega-3 PUFA (i.e. EPA & DHA), in reducing CVD. The beneficial effects of EPA-PE on HDL functions pave the way to a research area of significant

importance. Despite the importance of HDL in cardiovascular health, no prior research has delved deeply into the potential distinct effects of EPA and DHA on HDL functions, leaving a significant gap of knowledge. Thus, we plan to expand our future research to address this gap comprehensively. Our approach will involve producing various types of rHDL, including EPA-PE rHDL, DHA-PE rHDL, and EPA-PE/DHA-PE rHDL, and investigating their anti-atherogenic functions. In addition, we will conduct dose-dependent functional assays for these particles to elucidate the impact of low versus high doses of omega-3 fatty acids on HDL functions. Furthermore, we plan to assess the ability of these particles to reduce atherosclerosis in animal model, thereby solidifying their potential clinical relevance.

CONCLUSION | 5

"The journey of a thousand miles begins with a single step." - Lao Tzu

Chapter 5. Conclusion

In summary, our study establishes a strong link between HDL's PE content, particularly PE (36:5), and atherosclerosis, independent of traditional ASCVD risk factors. This highlights the potential use of the content of PE species, particularly PE (36:5), in HDL as novel markers for ASCVD risk assessment, with implications for improved risk prediction and prevention. Furthermore, our study added novel information to this association. Using rHDL containing ARA-PE, the predominant form of PE (36:5) in CMD and ASCVD, we showed that ARA-PE diminished the ability of rHDL to reduce atherosclerosis, shedding light on how ARA-PE (36:5) may contribute to atherosclerosis development, via impairing HDL-mediated atheroprotection. Our *in vitro* experiments reveal that the presence of PE, attenuate the functions of HDL, and the presence of ARA in PE worsened these effects and converted HDL into pro-inflammatory particles. These findings provide mechanistic insights into the positive associations between total PE and PE (36:5) contents in HDL and atherosclerosis, and potentially explain the strong link between PE (36:5) and ASCVD. Conversely, our *in vitro* findings highlight the significant beneficial impact of EPA-PE (36:5) on rHDL functions. EPA-PE not only enhances the CEC of rHDL, but also preserves/improves its AOX and AIA, respectively. Furthermore, EPA-PE (36:5) rHDL attenuate inflammation by reducing pro-inflammatory eicosanoids production, suggesting a novel mechanism for the cardioprotective effects of EPA. The contrasting effects of EPA-PE and ARA-PE on HDL functions propose that increasing plasma EPA/ARA ratio could enhance HDL functions, and protect against atherosclerosis. Our findings also have clinical implications, as they align with the remarkable effectiveness observed with Icosapent Ethyl in reducing cardiovascular events, as evidenced in the REDUCE IT clinical trial. In conclusion, our study advances our understanding of the role of PE species in atherosclerosis pathogenesis and ASCVD, by identifying them as novel biomarkers for HDL dysfunctionality. These findings may have significant implications for ASCVD risk assessment, and therapeutic management. Further research is needed to validate our findings and to explore their clinical application in practical healthcare settings.

ANNEXES

Annexes

Legends

Annex 1. Total PE and PE (36:5) contents of HDL2 are positively correlated with atherosclerosis in women with metabolic syndrome in unadjusted analysis. Human HDL2 particles were isolated from plasma of 86 women with metabolic syndrome by sequential ultracentrifugation. Total PE and PE (36:5) contents in HDL2 were quantified by LC-ESI/MS/MS and then correlated with coronary artery calcium (CAC) score, and the presence of atherosclerotic (AS) plaque in carotid arteries. **A.** Odds Ratios for CAC score > 100 according to total PE (left chart) and PE (36:5) (right chart) contents of HDL2. **B.** Odds Ratios for the presence of atherosclerotic (AS) plaques in carotid arteries according to total PE (left chart) and PE (36:5) (right chart) contents in HDL2. Statistical analysis was performed using R statistical software-version 3.3.2. HDL: high density lipoprotein; PE: Phosphatidylethanolamine.

Annex 2. The impact of PE-containing rHDLs on the gene expression of adhesion molecules and MCP-1 chemokine and IL-6 cytokine in both non-activated and activated HUVECs with TNF α . **A-D.** Relative mRNA levels of VCAM-1, ICAM-1, MCP-1, and IL-6, respectively, after 2 hours treatment of HUVECs with different rHDL followed by three hours of stimulation with 20 ng/mL of TNF α . **E-H.** Relative mRNA levels of VCAM-1, ICAM-1, MCP-1, and IL-6, respectively, after 2 hours treatment of HUVECs with different rHDL. Statistical analysis done using unpaired t-test with Welch's correction, without assuming consistent SD.*vs. TNF α only or none (no rHDL), #vs. Soy-PC rHDL, ^φvs. Soy-PE rHDL, ^δvs. ARA-PE rHDL, with p values<0.05. Values are shown as from at least 3 independent experiments that were performed in triplicate for each condition. TNF α : tumor necrosis factor alpha; VCAM-1: vascular cell adhesion molecule 1; ICAM-1: intercellular Adhesion Molecule 1; IL-6: interleukin 6; MCP-1: Monocyte Chemoattractant Protein-1.

Annex 3. The impact of PE-containing rHDLs on the gene expression and protein secretion of different pro-inflammatory cytokines and chemokines in LPS-activated macrophages after short term treatment. AIA of rHDLs was evaluated in LPS-stimulated THP-1 macrophages. **A-E.** Relative mRNA and protein levels of TNF α , MCP-1, IL-6, IL-8, and IL-12, after short term (4 hours) treatment of THP-1 cells with 20 μ g/mL of different rHDL

followed by 4 hour stimulation with 100 ng/mL of LPS. **F.** Summary for the effects of each rHDL treatment on the protein levels of different cytokines in LPS-stimulated THP-1 cells. Statistical analysis done using unpaired t-test with Welch's correction, without assuming consistent SD.*vs. LPS, #vs. Soy-PC rHDL, φ vs. Soy-PE rHDL, δ vs. ARA-PE rHDL, with p values <0.05. Values are shown as from at least 3 independent experiments that were performed in triplicate for each condition. LPS: lipopolysaccharide; TNF α : tumor necrosis factor alpha; MCP-1: Monocyte Chemoattractant Protein-1; IL: interleukin.

Annex 4. The impact of PE-containing rHDLs on the gene expression and protein secretion of different pro-inflammatory cytokines and chemokines in LPS-activated macrophages after long term treatment. AIA of rHDLs was evaluated in LPS-stimulated THP-1 macrophages. **A-E.** Relative mRNA and protein levels of TNF α , MCP-1, IL-6, IL-8, and IL-12, after long term (16 hours) treatment of THP-1 cells with 20 μ g/mL of different rHDL followed by 4 hour stimulation with 100 ng/mL of LPS. **F.** Summary for the effects of each rHDL treatment on the protein levels of different cytokines in LPS-activated THP-1 cells. Statistical analysis done using unpaired t-test with Welch's correction, without assuming consistent SD.*vs. LPS, #vs. Soy-PC rHDL, φ vs. Soy-PE rHDL, δ vs. ARA-PE rHDL, with p values <0.05. Values are shown as from at least 3 independent experiments that were performed in triplicate for each condition. LPS: lipopolysaccharide; TNF α : tumor necrosis factor alpha; MCP-1: Monocyte Chemoattractant Protein-1; IL: interleukin.

Annex 5. The impact of PE-containing rHDLs on the gene expression and protein secretion of different pro-inflammatory cytokines and chemokines in non-activated macrophages after short term treatment. **A-E.** Relative mRNA and protein levels of TNF α , MCP-1, IL-6, IL-8, and IL-12, after short term (4 hours) treatment of THP-1 cells with 20 μ g/mL of different rHDL. **F.** Summary for the effects of each rHDL treatment on the protein levels of different cytokines in non-activated THP-1 cells with LPS. Statistical analysis done using unpaired t-test with Welch's correction, without assuming consistent SD.*vs. none (no rHDL), #vs. Soy-PC rHDL, φ vs. Soy-PE rHDL, δ vs. ARA-PE rHDL, with p values <0.05. Values are shown as from at least 3 independent experiments that were performed in triplicate for each condition. LPS: lipopolysaccharide; TNF α : tumor necrosis factor alpha; MCP-1: Monocyte Chemoattractant Protein-1; IL: interleukin.

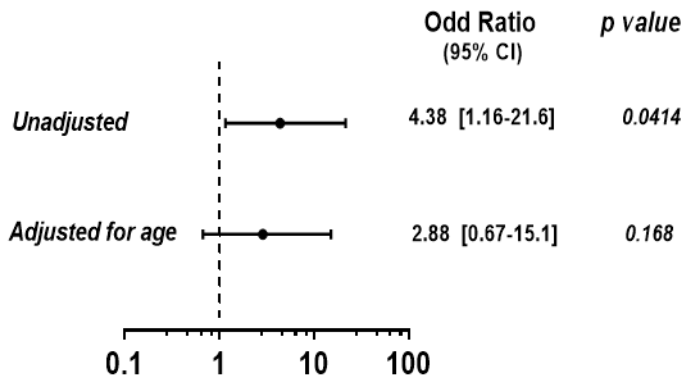
Annex 6. The impact of PE-containing rHDLs on the gene expression and protein secretion of different pro-inflammatory cytokines and chemokines in non-activated macrophages after long term treatment. AIA of rHDLs was evaluated in resting THP-1 macrophages. **A-E.** Relative mRNA and protein levels of TNF α , MCP-1, IL-6, IL-8, and IL-12, after short term (16 hours) treatment of THP-1 cells with 20 μ g/mL of different rHDL. **F.** Summary for the effects of each rHDL treatment on the protein levels of different cytokines in non-activated THP-1 cells with LPS. Statistical analysis done using unpaired t-test with Welch's correction, without assuming consistent SD. *vs. none (no rHDL), #vs. Soy-PC rHDL, °vs. Soy-PE rHDL, δ vs. ARA-PE rHDL, with p values <0.05. Values are shown as from at least 3 independent experiments that were performed in triplicate for each condition. LPS: lipopolysaccharide; TNF α : tumor necrosis factor alpha; MCP-1: Monocyte Chemoattractant Protein-1; IL-interleukin.

Annex 7. Oxidation parameters calculated from biphasic oxidation kinetics to evaluate oxidability of rHDLs. To evaluate the oxidability of rHDLs, different rHDLs (50 μ g ApoA-I/ml), treated with/without CuSO₄ (0.05 μ M), and DCFH solution (0.2mg/ml) in the absence of LDL. Different mixtures were transferred to 96 wells black plate (100 μ l/well), then the oxidation was assessed for 24 hours at 37C° using Spectromax Gemini device. **A.** Time-Oxidation curve for different conditions. **B.** Amount of oxidized DCFH formed after 24 hours. **C.** The oxidation rate during the propagation phase. **D.** Lag phase duration. Statistical analysis done using unpaired t-test with Welch's correction, without assuming consistent SD. Values are shown as mean \pm SEM from at least 3 independent experiments that were performed in triplicate for each condition.

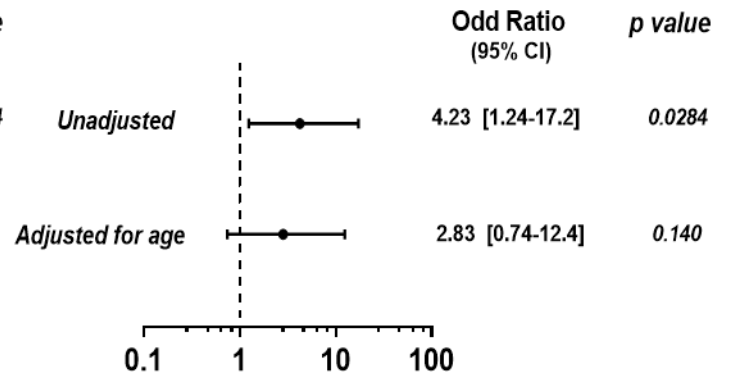
Annex 1.

A.

Odds Ratios for CAC score > 100 according to total PE content of HDL2

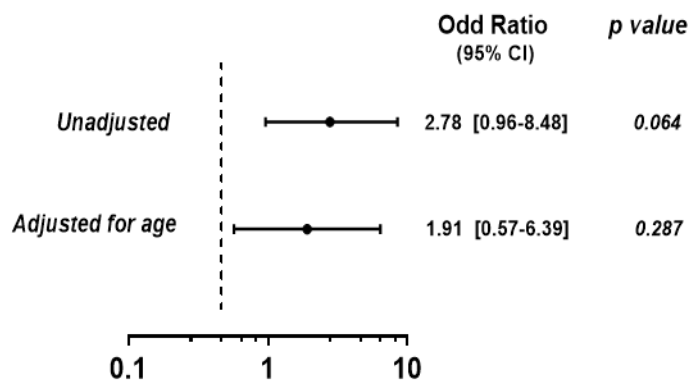


Odds Ratios for CAC Score >100 according to PE (36:5) content of HDL2

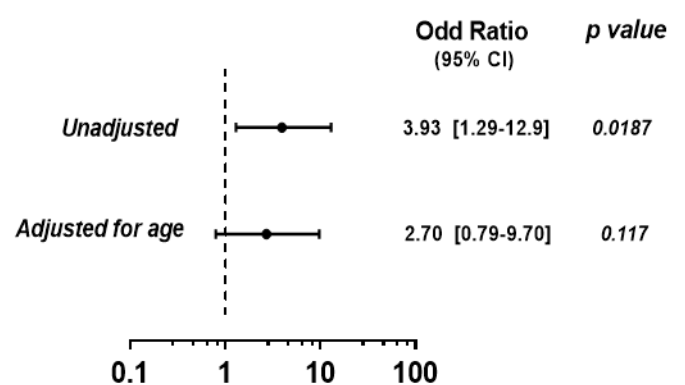


B.

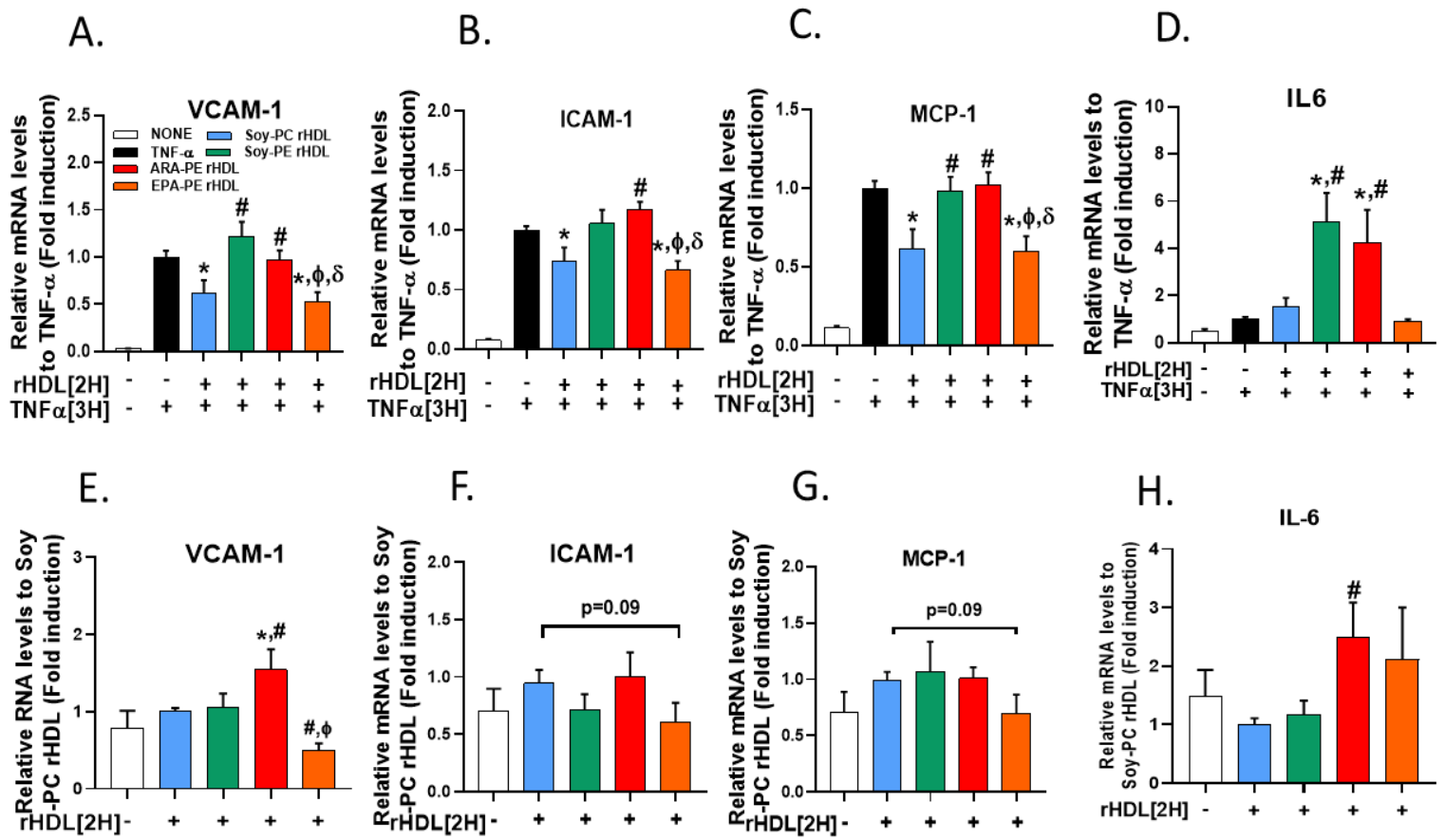
Odds Ratios for the presence of AS plaques in carotid arteries according to total PE content of HDL2



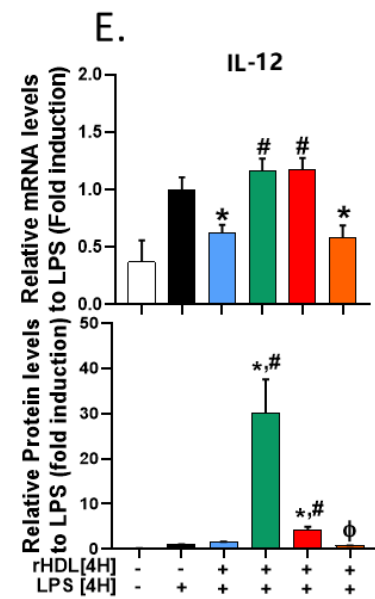
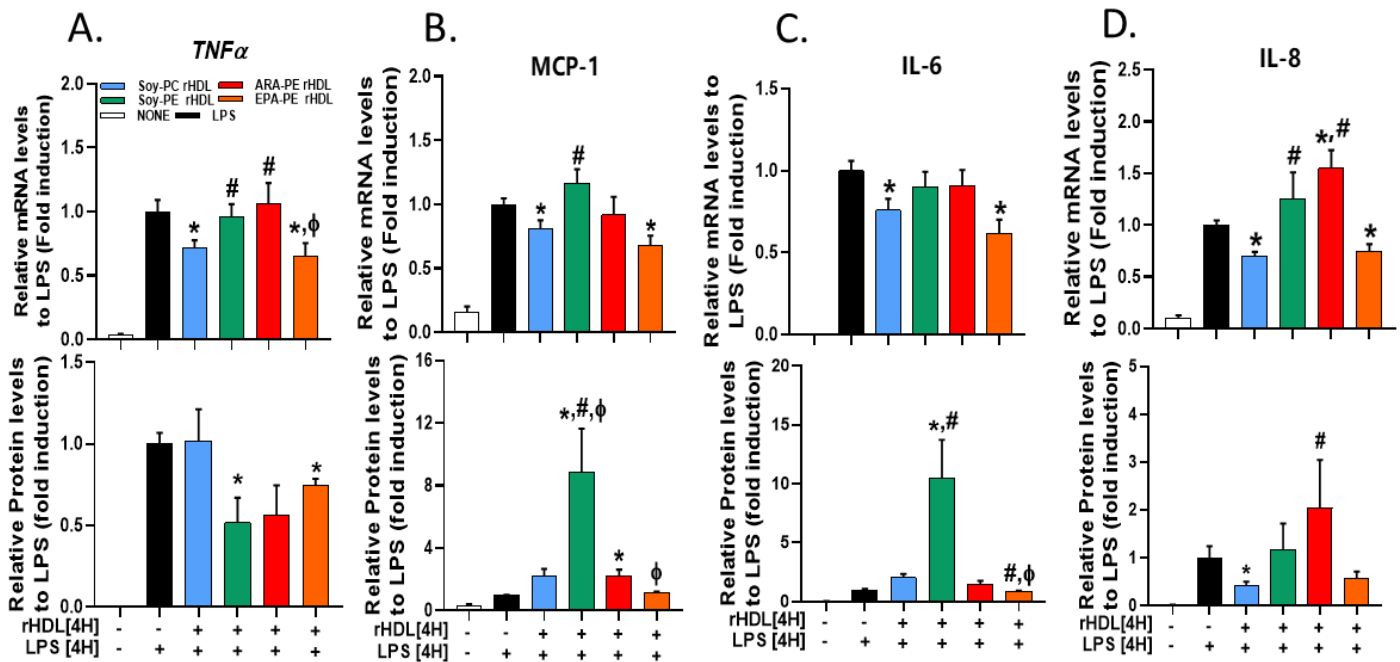
Odds Ratios for the presence of AS plaques in carotid arteries according to PE (36:5) content of HDL2



Annex 2.



Annex 3.

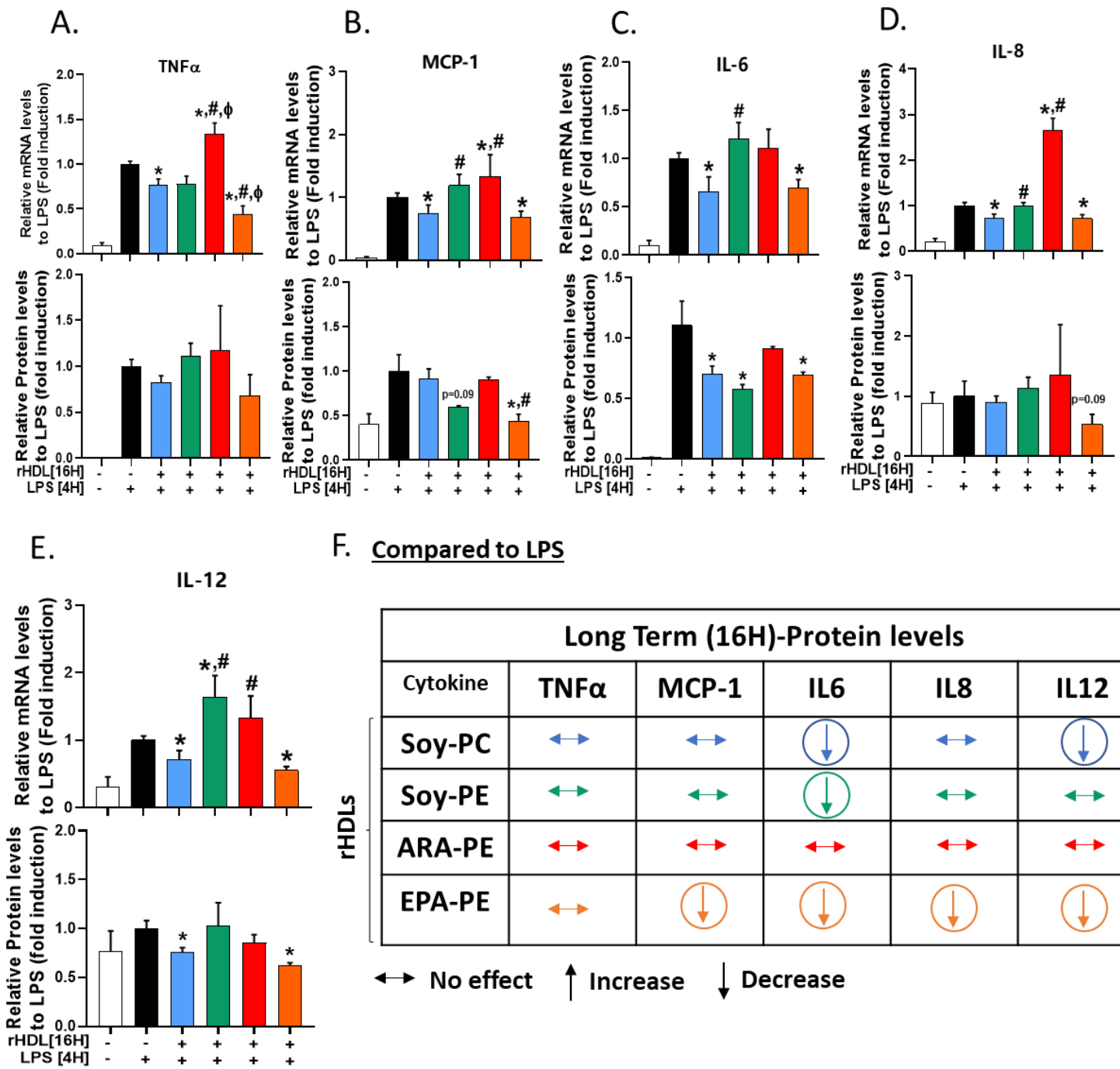


F. Compared to LPS

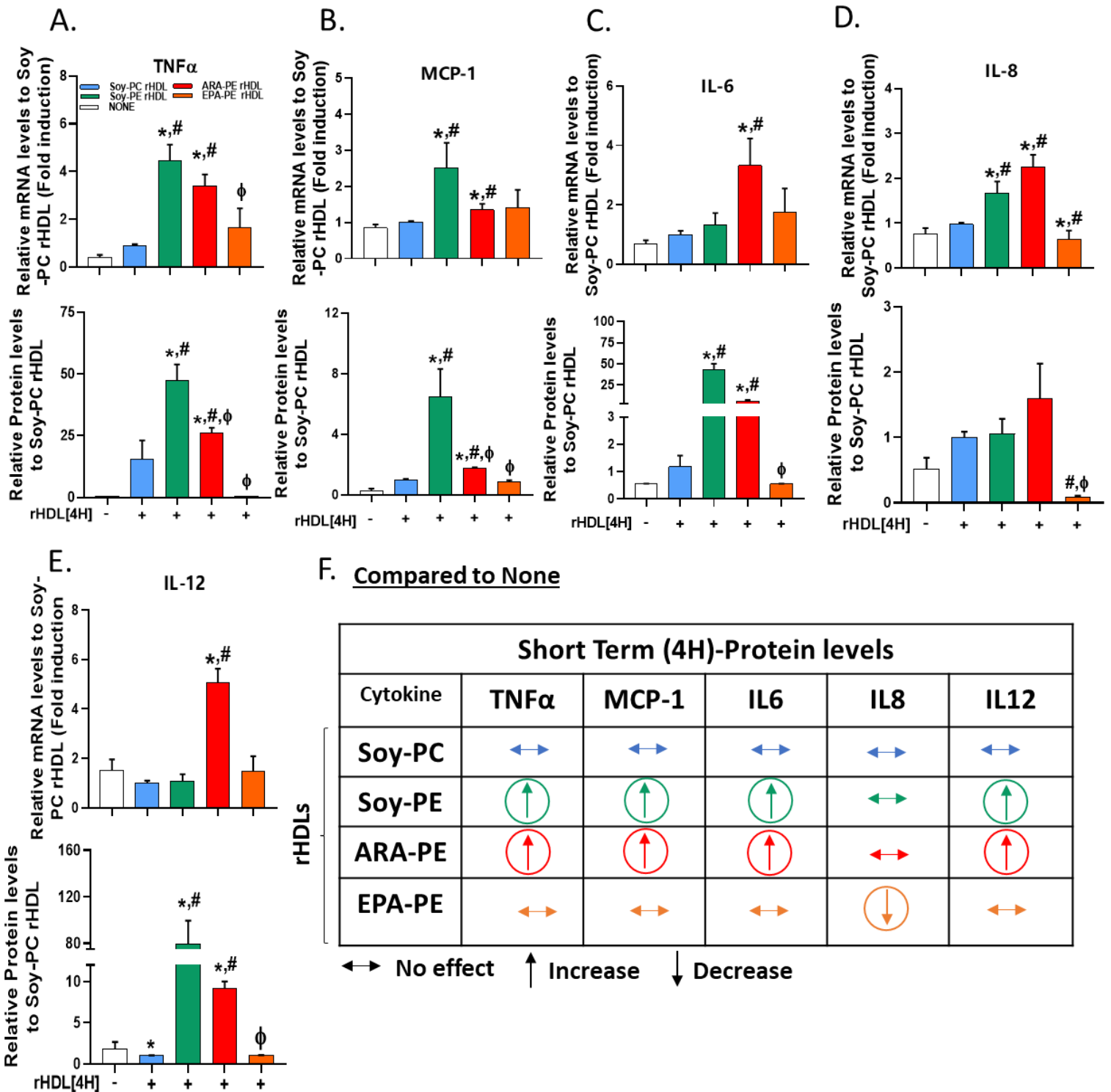
Short Term (4H)-Protein levels						
Cytokine	TNF α	MCP-1	IL6	IL8	IL12	
rHDLs	Soy-PC	↔	↔	↔	↓	↔
	Soy-PE	↓	↑	↑	↔	↑
	ARA-PE	↔	↑	↔	↔	↑
	EPA-PE	↓	↔	↔	↔	↔
	LPS	↑	↑	↑	↑	↑

↔ No effect ↑ Increase ↓ Decrease

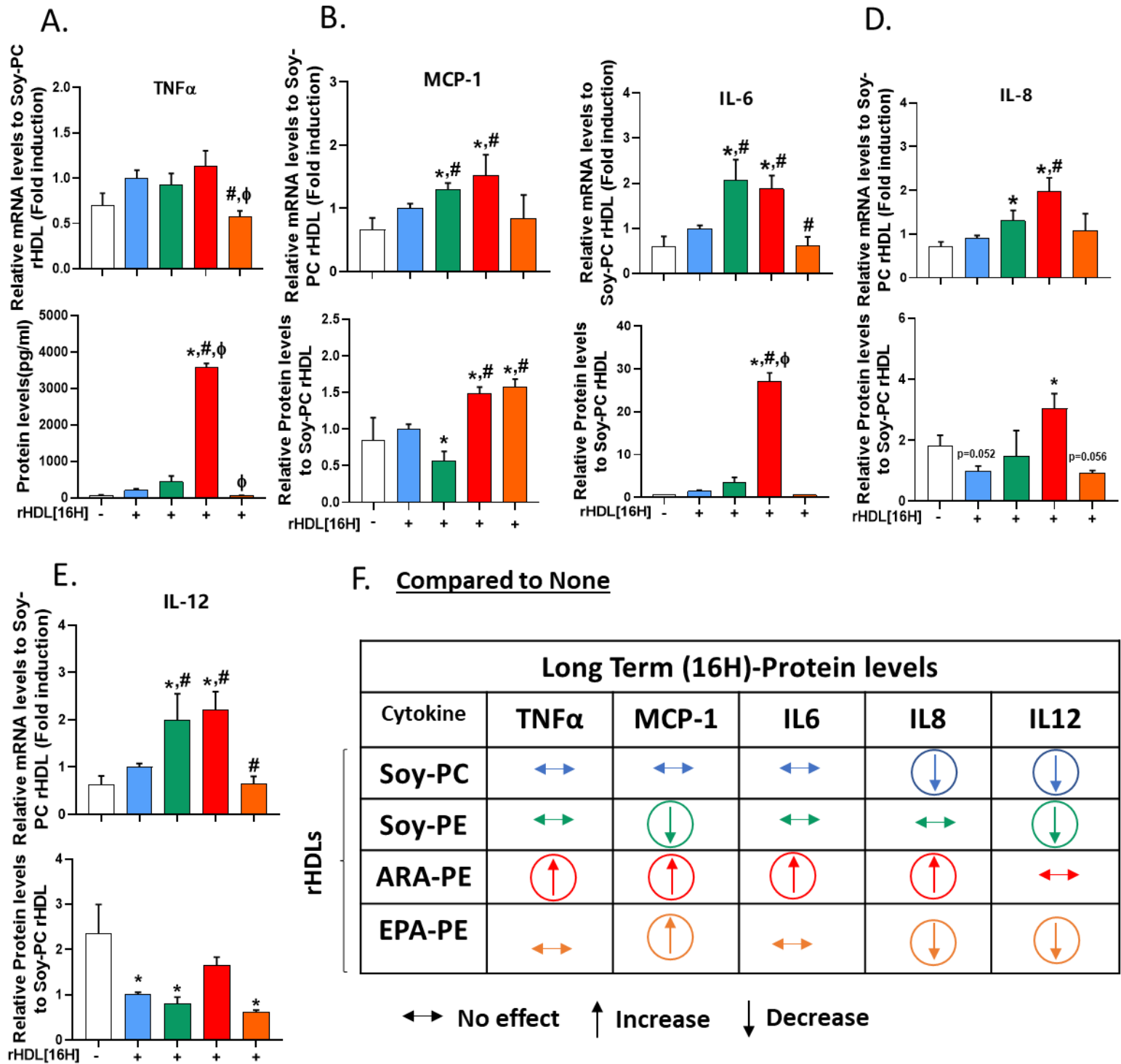
Annex 4.



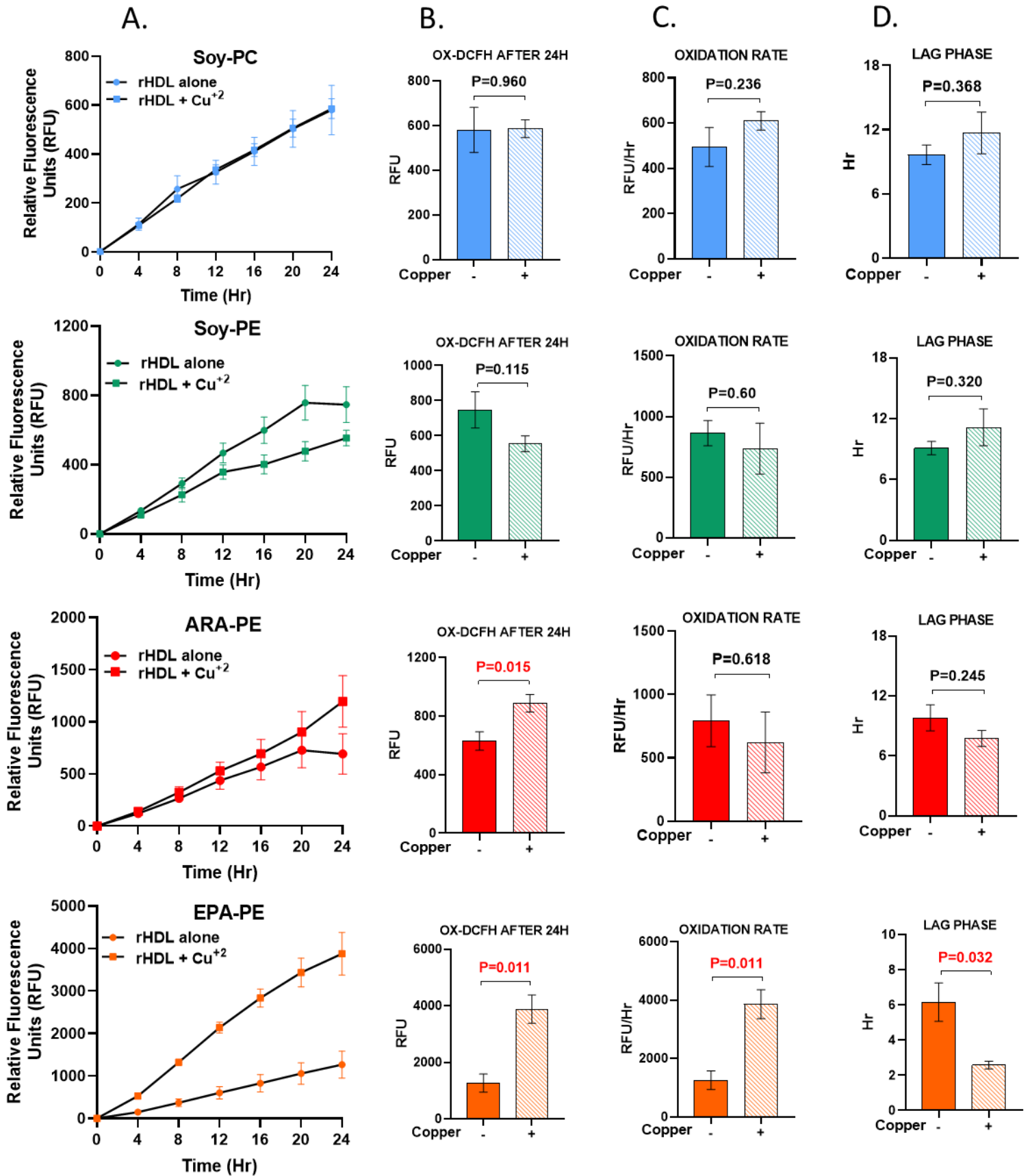
Annex 5.



Annex 6.



Annex 7.



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Abstract

HDL, or High-Density Lipoproteins, are often called "good cholesterol" by playing a vital role in reducing the risk of atherosclerotic cardiovascular diseases (ASCVD). HDL exert several atheroprotective effects, including their capacity to promote cholesterol efflux (CEC) from arterial macrophages, anti-inflammatory activities (AIA), and anti-oxidative activities (AOX). Among HDL components, phospholipids are considered the major determinants of HDL functions. Here, we aimed to identify new phospholipid species implicated in determining the atheroprotective functions of HDL. Higher plasma levels of phosphatidylethanolamine (PE) species are strongly linked to an increased risk of ASCVD, however, the underlying mechanisms remain unclear. Among PE species, PE (36:5) is recognized as a predictive lipid signature for ASCVD events. In human plasma, PE (36:5) exist as eicosapentaenoic (EPA, C20:5)-PE and arachidonic acid (ARA, C20:4)-PE. In this doctoral dissertation, we established a strong positive association between total PE and PE (36:5) contents in HDL and atherosclerosis in women with metabolic syndrome. Furthermore, our study added new information to this association in vivo, using ARA-PE, the predominant form of PE (36:5) in cardiometabolic diseases (CMD) and ASCVD. By infusing reconstituted HDL (rHDL) in an animal model with atherosclerosis, we revealed the inability of ARA-PE rHDL to reduce atherosclerosis compared to control rHDL, indicating an impaired rHDL-mediated atheroprotection. Our in vitro experiments revealed that PE, and more profoundly ARA-PE, attenuated the functions of rHDL and converted them into pro-inflammatory particles. Conversely, our in vitro findings highlighted the significant beneficial impact of EPA-PE on rHDL functions. EPA-PE not only enhanced the CEC of rHDL, but also preserved and improved its AOX and AIA, respectively. Furthermore, our findings revealed that EPA-PE rHDL attenuated inflammation by reducing pro-inflammatory eicosanoid production, suggesting a novel mechanism for the cardioprotective effects of EPA.

In conclusion, this research work advances our understanding regarding the role of PE species in atherosclerosis by identifying them as novel biomarkers for HDL functions. These findings could significantly impact risk assessment and therapeutic management in the context of ASCVD.