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Functional activities of HDL particles in acute myocardial infarction: dissection of the roles of proteome vs. lipidome components

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Lists

AAPH	2,2′-azo-bis-(2-amidinopropane) hydrochloride
ANOVA	Analysis of Variance
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
apo	apolipoprotein
BMI	body-mass index
CETP	cholesteryl ester transfer protein
CRP	C-reactive protein
eNOS	endothelial nitric oxide synthase
HDL	high-density lipoprotein
HDL-C	HDL-cholesterol
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
HOMA	homeostatic model assessment
HPLC	high-performance liquid chromatography
hsCRP	high-sensitivity CRP
ICAM-1	intercellular adhesion molecule-1
IDL	intermediate-density lipoproteinIL, interleukin
LBP	lipopolysaccharide-binding protein
LCAT	lecithin:cholesterol acyltransferase
LC/MS	liquid chromatography/mass spectrometry
LDL	low-density lipoprotein
LDL-C	LDL-cholesterol

LOOH	lipid hydroperoxide
LpA-I	HDL particles containing only apoA-I
LpA-I:A-II	HDL particles containing both apoA-I and apoA-II
$LpPLA_2$	lipoprotein-associated phospholipase A_2
LPS	lipopolysaccharide
LXR	liver X receptor
MCP-1	monocyte chemotactic protein-1
NFkB	nuclear factor kappa B
NMR	nuclear magnetic resonance
NO	nitric oxide
PAF	platelet-activating factor
PAF-AH	platelet-activating factor-acetyl hydrolase
PAI-1	plasminogen activator inhibitor type 1
PLOOH	phospholipid hydroperoxide
PLPC	1-palmitoyl-2-linoleoyl phosphatidylcholine
PLTP	phospholipid transfer protein
POPC	1-palmitoyl-2-oleoyl phosphatidylcholine
PON	paraoxonase
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acid
RCT	reverse cholesterol transport
rHDL	reconstituted HDL

ROS	reactive oxygen species
RXR	retinoid X receptor
S1P	sphingosine-1-phosphate
SAA	serum amyloid A
SNP	single-nucleotide polymorphism
sPLA$_2$	secretory phospholipase A_2
SR-BI	scavenger receptor class B type I
TF	tissue factor
TFPI	tissue factor pathway inhibitor
tPA	tissue plasminogen activator
TNF-alpha	tumour necrosis factor-alpha
VCAM-1	vascular cell adhesion molecule-1
VLDL	very-low density lipoprotein

To my parents and sisters, and to Bertrand.

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Resumo

Rached FH. Atividades funcionais das partículas de HDL no Infarto Agudo do Miocárdio: Dissecção dos papéis de componentes proteoma vs lipidome. Bolsa de Doutorado em Ciência e Cardiologia pela Universidade de São Paulo e Université Pierre & Marie Currie - Universidade de Paris VI. 2013.

Baixos níveis plasmáticos de colesterol de lipoproteína de alta densidade (HDL-C) são achados frequentes no quadro de infarto agudo do miocárdio (IAM) e são bons marcadores de recorrência de eventos cardiovasculares. A HDL apresenta múltiplas atividades ateroprotetoras e é altamente heterogênea em sua estrutura, composição e função. A funcionalidade da HDL não é refletida através de medições clínicas de rotina de HDL-C e pode ser um biomarcador mais informativo do risco cardiovascular quando comparados ao HDL-C. No entanto, as possíveis relações entre as modificações na composição lipídica molecular e a funcionalidade das subpopulações das partículas de HDL em indivíduos normolipidémicos e em pacientes com infarto agudo do miocárdio não estão completamente compreendidas.

Plasmas e soros foram obtidos de sujeitos saudáveis e normolipidêmicos (n=14) e de pacientes com IAM com elevação do segmento ST (IAMCSST) nas primeiras 24 horas após o diagnóstico (n=16). Foram fracionados por ultracentrifugação com gradiente de densidade para isolar cinco subpopulações principais de HDL (as de maior tamanho e menor densidade: HDL2b, HDL2a e as de menor tamanho e maior densidade: HDL3a, HDL3b e HDL3c). O efluxo de colesterol celular foi avaliado em células THP-1 e a atividade antioxidante foi avaliada in vitro contra a peroxidação lipídica da LDL (oxidação da LDL de referência isolada ou em presença das HDL3b, HDL3c e HDLtotal); o lipidoma da HDL foi analisado por LC/MS/MS.

Em indivíduos normolipidémicos, o conteúdo do lisofosfatidilcolina e dos fosfolipides com carga negativa (fosfatidilserina e ácido fosfatídico) aumentou progressivamentente conforme o aumento da densidade das partículas de HDL, enquanto que o conteúdo de ceramida e esfingomielina reduziu. As atividades biológicas das subpopulações de HDL, como a capacidade de efluxo de colesterol em células THP-1 e a atividade antioxidante para a oxidação da LDL, foram predominantemente associadas às pequenas, densas e ricas em proteínas HDL3. A heterogeneidade no lipidoma HDL foi correlacionada à funcionalidade de HDL.

Os pacientes com IAM apresentaram baixos níveis de HDL-C (-31%, $p<0.001$) e alto grau de inflamação, (determinado pelos acentuados níveis plasmáticos de proteína C-reativa,

amilóide A sérica e interleucina-6) em comparação com controles pareados para idade e sexo. Nos pacientes, HDL total e suas subpopulações exibiram menor capacidade de efluxo de colesterol em células THP-1 (até -33%, p<0.001, com base em unidade de massa de fosfolipídios) vs controles. HDL total e as subpopulações pequenas e densas HDL3b e HDL3c dos pacientes com IAM exibiram redução da atividade antioxidante (até -68%, p<0.05, com base em unidades de massa de HDL) vs controles. As subpopulações de HDL no IAM apresentaram redução em apo A-I (até -23%, p<0,01) e enriquecimento em SAA (até 11,8 vezes, p<0.05). No, IAM, HDL total e os seus subpopulações estavam enriquecidas em dois lípidos bioactivos, pró-inflamatórios (lisofosfatidilcolina até 3,0 vezes, p<0.01) e ácido fosfatídico (até 8,4 vezes, p<0.01), tais alterações foram mais pronunciadas na pequena densa, subfração HDL3b. Curiosamente, tais importantes alterações no proteoma e no lipidome estavam intimamente correlacionadas à funcionalidade da HDL.

A análise da estrutura-função nestes estudos, portanto, revela que (i) o lipidoma da HDL pode impactar fortemente a funcionalidade ateroprotetora em indivíduos normolipidemicos, (ii) a carga do lipidome da HDL sofre profundas alterações no meio inflamatório do IAM, e (iii) tais modificações, juntamente com alterações concomitantes no proteoma da HDL, podem ter implicação direta na funcionalidade da HDL, diminuída na fase inicial do IAM.

Palavras-chave: HDL-colesterol; Lipoproteínas HDL; Metabolismo dos lipídeos/fisiologia; Fosfolipídeos/metabolismo; Esfingofosfolipídeos; Colesterol; Transportadores de cassetes de ligação de ATP/metabolismo; Antioxidantes/análise; Antioxidantes/química; Infarto do miocárdio; Inflamação; Marcadores biológicos/sangue.

Résumé

Rached FH. Les activités fonctionnelles des particules de HDL chez les pacients avec infarctus du myocarde: la dissection du rôle des composantes du protéome vs lipidome [thèse]. São Paulo: Faculté de Médecine, Université de São Paulo et Université Pierre et Marie Currie, Paris VI. 2013.

Des niveaux bas de cholestérol associé aux lipoprotéines de haute densité (HDL-C) sont caractéristiques d'infarctus du myocarde (MI) et prédisent des accidents cardiovasculaires récurrents. HDL possède de multiples activités athéroprotectives et sont hautement hétérogènes en structure, composition et fonctionnalité. Cette dernière n'est pas mise en évidence par les analyses cliniques classiques et serait pourtant plus informative que les taux circulants d'HDL-C. Cependant, les liens éventuels entre les modifications de composition moléculaire en lipides et la fonctionnalité des sous-populations d'HDL restent assez peu compris chez les sujets normolipidémiques et ayant subi un MI aigu.

Les plasmas de sujets contrôles, normolipidémiques, (n=14) et de patients ayant subi un infarctus du myocarde avec élévation de segment (STEMI) (n=16) obtenus dans les 24h suivant le diagnostic ayant des âges et sexes homogènes dans les deux groupes furent fractionnés par gradient de densité. Cinq sous-populations de HDL furent ainsi isolées : les grandes et légères HDL2b et HDL2a et les petites et denses HDL3a, 3b et 3c. La capacité de ces HDL à réaliser l'efflux de cholestérol fut mesuré sur des cellules THP-1 et leur activité antioxydante évaluée in vitro contre la peroxydation lipidique des LDL (LDLde référence seul ou en présence de HDL3b, HDL3c ou HDLtotal); le lipidome des HDL fût analysé par LC/MS/MS.

Dans les HDL de sujets normolipidémiques, la concentration en lysophosphatidylcholine et en phospholipides négativement chargés (phosphatidylsérine et acide phosphatidique) augmente avec la densité des sous-populations de HDL, alors que les contenus en sphingomyéline et céramide diminuent.

Les activités biologiques clés des sous-populations de HDL, et plus particulièrement la capacité à réaliser l'efflux de cholestérol et à protéger les LDL contre l'oxydation, furent associées majoritairement avec les petites, denses et riches en protéines HDL3c. L'hétérogénéité du lipidome de HDL fût corrélée à leurs fonctionnalités.

Chez les patients STEMI ayant des taux de HDL-C bas (-31%, $p<0.001$) et une inflammation aigue (déterminée par une élévation marquée de la C-reactive protein, serum amyloid A (SAA) et interleukine-6), comparé aux sujets normolipidémiques de même âge et sexe, leurs HDL plasmatiques et sous-populations montrent une capacité d'efflux de cholesterol des

cellules THP-1 atténuée (jusqu'à -33%, $p<0.001$, pour une même masse de phospholipides) vs contrôles.

Les HDL et les sous-populations petites et denses, HDL3b et 3c plasmatiques montrèrent une activité antioxydante réduite (jusqu'à -68%, $p<0.05$, pour une même masse totale de HDL). Les sous-populations de HDL chez les patients STEMI furent appauvries en apoA-I (jusqu'à -23%, $p<0.01$) et enrichies en SAA (jusqu'à +11.8 fois, $p<0.05$). Les HDL de STEMI et leurs sous-populations furent enrichies en deux lipides pro-inflammatoires: lysophosphatidylcholine (jusqu'à 3 fois, $p<0.01$) et en acide phosphatidique (jusqu'à 8.4 fois, $p<0.01$). Ces changements furent plus prononcés dans les petites et denses HDL3b. De manière intéressante, de telles altérations du protéome et lipidome furent intimement liées aux fonctionnalités des HDL.

L'analyse structure-fonction dans cette étude révèle que (i) le lipidome de HDL peut agir de manière significative sur leurs fonctions athéroprotectives chez les sujets normolipidémiques, (ii) le lipidome de HDL subit d'importantes modifications dans un environnement inflammatoire aigu tel que celui du STEMI, and (iii) de telles altérations associées à des changements du protéome de HDL peuvent être directement impliquées dans la détériorationdiminution de la fonctionnalité des HDL lors des premières phases de STEMI.

Mots clés: HDL-cholestérol; Lipoproteínes HDL; Métabolisme des lipides ; Phospholipides; Sphingolipides; Cholestérol; Transporteurs à ATP Binding Cassette; Antioxydants; Infarctus aigu du myocarde; Inflammation; Marqueurs biologiques.

Abstract

Rached FH. Functional activities of HDL particles in Acute Myocardial Infarction: Dissection of the roles of proteome vs. lipidome components. Doctoral Fellowship in Science and Cardiology from São Paulo University and Pierre & Marie Currie University - Paris VI University. 2013.

Low plasma levels of high-density lipoprotein-cholesterol (HDL-C) are typical of acute myocardial infarction (MI) and predict risk of recurrent cardiovascular events. HDL displays multiple atheroprotective activities and is highly heterogeneous in structure, composition and function. The functionality of HDL particles is not reflected by routine clinical measurements of HDL-C and could be more informative biomarker of CV risk as compared to circulating HDL-C levels. However, the potential relationships between modifications in the molecular lipid composition and the functionality of HDL particle subpopulations in normolipidemic subjects and in patients with acute MI are incompletely understood.

Plasmas of healthy normolipidemic controls (n=14) and of age- and sex-matched ST segment elevation MI (STEMI) patients obtained within 24h after diagnosis (n=16)- were fractionated by density gradient ultracentrifugation to isolate five major HDL subpopulations (large, light HDL2b and HDL2a and small, dense HDL3a, HDL3b, and HDL3c). Cellular cholesterol efflux was assessed in THP-1 cells from THP-1 cells and antioxidative activity was evaluated in vitro towards reference low-density lipoprotein (LDL) oxidation; the HDL lipidome was analysed by LC/MS/MS.

In normolipidemic subjects, the contents of lysophosphatidylcholine and of negatively-charged phosphatidylserine and phosphatidic acid increased progressively with increase in hydrated density of HDL, whereas the proportions of sphingomyelin and ceramide decreased. Key biological activities of HDL subpopulations, notably cholesterol efflux capacity from THP-1 cells and antioxidative activity towards LDL oxidation, were predominantly associated with small, dense, protein-rich HDL3. The heterogeneity in the HDL lipidome was correlated to HDL functionality.

In STEMI patients, who featured low HDL-C (-31%, $p<0.001$) and acute-phase inflammation (determined as marked elevations in C-reactive protein, serum amyloid A (SAA) and interleukin-6), as compared to age- and sex-matched controls, plasma HDL and its subpopulations displayed attenuated cholesterol efflux capacity from THP-1 cells (up to -33%, $p<0.001$, on a unit phospholipid mass basis) vs. controls. Plasma

HDL and small, dense HDL3b and 3c subpopulations from STEMI patients exhibited reduced anti-oxidative activity (up to -68%, $p<0.05$, on a unit HDL mass basis). HDL subpopulations in STEMI were depleted in apoA-I (up to -23%, $p<0.01$) and enriched in SAA (up to +11.8-fold, $p<0.05$). STEMI plasma HDL and its subpopulations were enriched in two proinflammatory bioactive lipids, lysophosphatidylcholine (up to 3.0-fold, $p<0.01$) and phosphatidic acid (up to 8.4-fold, $p<0.01$); such changes were most pronounced in the small, dense, HDL3b subfraction. Interestingly, the alterations in the proteome and lipidome were intimately related to HDL functionality.

The structure-function analysis performed in these studies thereby reveals that (i) the HDL lipidome may strongly impact atheroprotective functionality in normolipidemic subjects; (ii) the HDL lipidome undergoes profound alterations in its cargo in the inflammatory milieu of STEMI and (iii) such modifications, together with concomitant alterations in HDL proteome, can be directly implicated in impaired HDL functionality in the early phase of STEMI.

Keywords: HDL-cholesterol; HDL lipoproteins; Lipid metabolism; Phospholipids; Sphingolipids, Cholesterol; Acute myocardial infarction; Inflammation; ATP-Binding Cassette Transporters; Antioxidants; Biological markers.

Preface

It has long been known that there is an inverse relationship between plasma levels of high density lipoprotein cholesterol (HDL-C) and the risk of cardiovascular disease.(1) Specifically, prospective data from the Framingham Heart(2) and ARIC(3) studies revealed that subjects with HDL-C levels in the lowest quintiles exhibited the highest frequency of cardiovascular events. The prevalence of low HDL-C levels can vary from 20% in a general population to up to 60% in patients with established coronary heart disease.(4) Low HDL-C levels not only are associated with an increased incidence of coronary heart disease but also with a more aggressive progression of angiographically-defined coronary artery disease and with a greater risk for carotid atherosclerosis and ischemic stroke mortality.(5, 6)

The relative increase in atherogenic lipoproteins in the bloodstream (very low density lipoprotein, VLDL, low-density lipoprotein, LDL) compared to anti-atherogenic high-density lipoprotein (HDL) is associated with endothelial dysfunction, which is intimately linked to the local chronic inflammation and oxidative stress.(7) The endothelial dysfunction results from excessive penetration and retention of LDL in the subendothelial space and the recruitment of monocytes and their differentiation into macrophages. This leads to local inflammatory state with increased generation of reactive oxygen, nitrogen and chlorine species.(8) In this context, LDL is the major substrate of oxidative attack in the arterial wall; indeed, analysis of chemical composition of atherosclerotic plaques shows the presence of lipid and protein oxidation products, attesting firstly the existence of oxidative stress(9) and secondly the presence of oxidized LDL (oxLDL). LDL becomes oxidized following penetration into the arterial intima, being capable of inducing activation of several cell types in the arterial wall. Thus, oxLDL induces: (i) activation of endothelial cells, resulting in the expression of adhesion molecules on their surface, (ii) recruitment and adhesion of monocytes and their differentiation into macrophages in the arterial intima, (iii) formation of foam cells, and (iv) endothelial cell apoptosis.(7) All these effects of oxLDLs make these particles the main actors involved in atherogenesis, in concert with macrophages and foam cells.

Among factors that critically influence the process of atherogenesis, the presence of low levels of HDL is widely recognized. The main mechanisms potentially underlying the cardioprotective effects of HDL are: (i) reverse transport of cellular cholesterol, (ii) protection of LDL against oxidative stress, (iii) anti-inflammatory, (iv) anti-thrombotic,(v) cytoprotective, (vi) vasodilatory, and (vii) anti-infectious activities. Such a variety of biological activities can be explained by the heterogeneity of the HDL fraction as a consequence of structural changes and compositional transformations of HDL particles during their intravascular metabolism. Indeed, HDL can be separated by different methods into several subpopulations. One example involves separation by density gradient ultracentrifugation, which isolates five HDL subfractions, large, light, lipid- HDL2b and 2a and small, dense, protein-rich HDL3a, 3b and 3c.

The multiple biological functions of HDL particles are an integral reflection of their bioactive protein and lipid components.(10) Among HDL particle subpopulations, small, dense, protein-rich HDLs are distinguished by a specific proteome(11) and lipidome,(12) unique particle structure(13) and potent anti-atherogenic properties.(10) Such activities can be compromised under conditions of atherogenic dyslipidemia and acute or chronic inflammatory state. Indeed, cholesterol efflux capacity(14) and anti-inflammatory(15) and anti-apoptotic activities(16, 17) of HDL are attenuated in patients with cardiovascular and metabolic diseases.

Acute myocardial infarction (MI) features myocardial ischemia, which involves oxidative stress and acute-phase inflammation. Low plasma levels of HDL-C are typical of acute MI, and predict risk of recurrent cardiovascular (CV) events.(18) This relationship may reflect alterations in both the circulating levels and functionality of HDL, which either precede the coronary event, or which occur during the acute phase, or both. Indeed, defects in the antiatherogenic activities of HDL particles may in principle include cellular cholesterol efflux together with antioxidative, anti-apoptotic, vasodilatory and antithrombotic actions.(19) All

such activities are potentially relevant to the pathophysiology of acute MI. Low plasma HDL-C concentrations in acute MI reflect profound alterations in lipoprotein metabolism in an inflammatory milieu, which may in turn lead to modification in both the lipid and protein components of HDL particles(20) and alter their antiatherogenic activities. Indeed, HDL from acute MI patients is defective in its capacity to stimulate endothelial NO production,(21) to reduce prothrombotic activation in endothelial cells(22) and to protect endothelial cells from apoptosis.(17) Potential structural and functional alterations in HDL particle subpopulations in acute MI and their interrelationships are therefore of special interest, as they may impact both the extent of ischemia and the degree of damage to cardiac tissue, thereby indirectly modulating the intensity of the acute-phase response.

Comprehensive structural and compositional analyses of HDL may provide key information to identify subpopulations displaying specific biological functions and acquiring deficient functionality in specific clinical situations, with the potential to reveal novel biomarkers of cardiovascular risk and new pharmacological targets. As a first step in the evaluation of the compositional origin of defective HDL function in acute MI, key features of the HDL lipidome in normolipidemic subjects were quantitated and their potential relationship to HDL functionality was assessed. This aspect is the first part of these studies. Based on the data obtained, in the second part of this work, integrated analyses of the lipidome, proteome and functionality of total HDL and the major plasma HDL particle subpopulations were undertaken in ST elevation myocardial infarction (STEMI) patients displaying low HDL-C levels in the acute phase (<24h after diagnosis).This thesis is organised into three sections, Introduction, Experimental and Discussion. The Introduction section consists of two chapters. The first chapter introduces normal functional HDL, highlighting its physicochemical properties, intravascular metabolism, biological activity and heterogeneity. The second chapter focuses on functionally defective HDL. At the end of this section, key questions and objectives of this study are presented.

Three chapters constitute the Experimental section. The first chapter lists materials and methods used in this work. The second is devoted to the data obtained in normolipidemic subjects. The third part, the most extensive and important one, describes data obtained in patients with acute myocardial infarction.

Finally, the Discussion, Conclusions and Perspectives section is dedicated to the discussion of the results obtained and to the conclusions and perspectives raised in this work.

Section I:

1. Introduction

1.1 Chapter I) Normal functional HDL

1.1.1 Lipoprotein definition, composition and metabolism

Cholesterol is essential for all animal life by being required among others in synthesis of cell membranes, bile acids and steroid hormones. Since cholesterol is insoluble in water, it is transported in the circulatory system within lipoproteins.

Lipoproteins are plurimolecular, quasi-spherical, pseudomicellar complexes composed of lipids solubilised by proteins having distint structure and function, the apolipoproteins (apo). They consist of a surface monolayer of phospholipids (PL), free cholesterol (FC) and proteins primarily apolipoproteins and enzymes, and a core comprising hydrophobic apolar lipids (triglycerides (TG), and cholesterol esters, (CE)). The percentage of lipid and protein vary depending on the class of lipoproteins. Major lipoproteins in human plasma are, in the order of increasing density and decreasing size, chylomicrons, VLDL, IDL, LDL and HDL. These lipoproteins differ in molecular mass and apolipoprotein composition as shown in Table 1. ApoB is the predominant protein component of pro-atherogenic, cholesterol-rich LDL, VLDL, VLDL remnants and intermediate-density lipoproteins (IDL), whereas apoA-I is the major protein component of anti-atherogenic HDL.

During the transport of cholesterol in the circulatory system, lipoproteins undergo complex transformations and lipid and protein exchange as well as enzymatic changes occur that affect their composition, size, structure and function. At the cellular level, lipoproteins are picked up by specific or non-specific receptors and some of them (LDL) are internalized and degraded in order to provide the cells with cholesterol. Intracellular cholesterol is then used by the cell for synthesis of various endogenous substances such as steroid hormones

and bile acids, or stored as cholesterol esters. Lipoprotein metabolism (Figure 1) is the result of a perpetual dynamic equilibrium reflecting their high level of heterogeneity.

Chylomicrons, the least dense type of cholesterol transport particles, are aggregated with apoB-48 as nascent chylomicrons. They are the transporters that carry dietary lipids (composed of triglycerides 80-95% total weight, phospholipids 3-6% total weight, free cholesterol 2-7% total weight) from the intestine to muscle and adipose tissues for energy and storage. VLDLs particles which contain mainly endogeneous triglycerides (55% of total weight) together with cholesterol (cholesteryl esters; 12% of total weight, and free cholesterol; 7% of total weight) and phospholipids (18% of total phospholipids) are secreted by the liver with apoB-100. Both chylomicrons and VLDL particles follow similar metabolism. In the bloodstream, HDL particles donate apoC-II and apoE to these nascent chylomicrons or to VLDL. ApoC-II activates LPL, causing hydrolysis of the chylomicrons and VLDLs particle and the release of glycerol and fatty acids which can be absorbed by adipose tissue and muscle. The hydrolyzed chylomicrons, called chylomicron remnants, are subsequently taken up by the liver via an apoE receptor(23). Under the action of LPL, VLDLs are converted to IDLs. The predominant remaining proteins are apoB-100 and apoE. IDLs particles can be, then, absorbed by the liver via an apoE receptor or they can be further hydrolyzed by hepatic lipase (HL) releasing glycerol and fatty acids and converting IDLs to LDLs.

LDLs contain relatively high cholesterol content (42% of cholesteryl esters and 8% of free cholesterol). Phospholipids and trglycerides are also lipid components of LDL particles (22% and 6% of the total weight, respectively). The exclusive apolipoprotein of LDLs is apoB-100. The uptake of LDLs occurs predominantly in liver (75%), adrenals and adipose tissue via LDL receptor-mediated endocytosis. LDL can be modified or oxidized and taken up by macrophages by scavenger receptors SRAI and CD36, which become engorged and form foam cells. These cells often become trapped in the walls of blood vessels and contribute to artherosclerotic plaque formation.(24)

Table 1 - Physical and chemical properties of human plasma lipoproteins (25, 26)

Lipoproteins	Density (g/ml)	Diameter (nm)	Molecular mass (Da)	Major apolipoproteins
Chylomicrons	0.93	75-1200	50-1000 x 10^6	B48, E, C, A-I
VLDL	0.93-1.006	30-800	10-80 x 10^6	B100, E, C
IDL	1.006-1.019	27-35	5-10 x 10^6	B100, E
LDL	1.019-1.063	18-27	2.3 x 10^6	B100
HDL2	1.063-1.125	9-12	360 x 10^3	A-I, A-II, C
HDL3	1.125-1.210	7-9	175 x 10^3	A-I, A-II, C
Pre-β-HDL	1.210-1.240	<7 (discoidal)	25-70 x 10^3	A-I

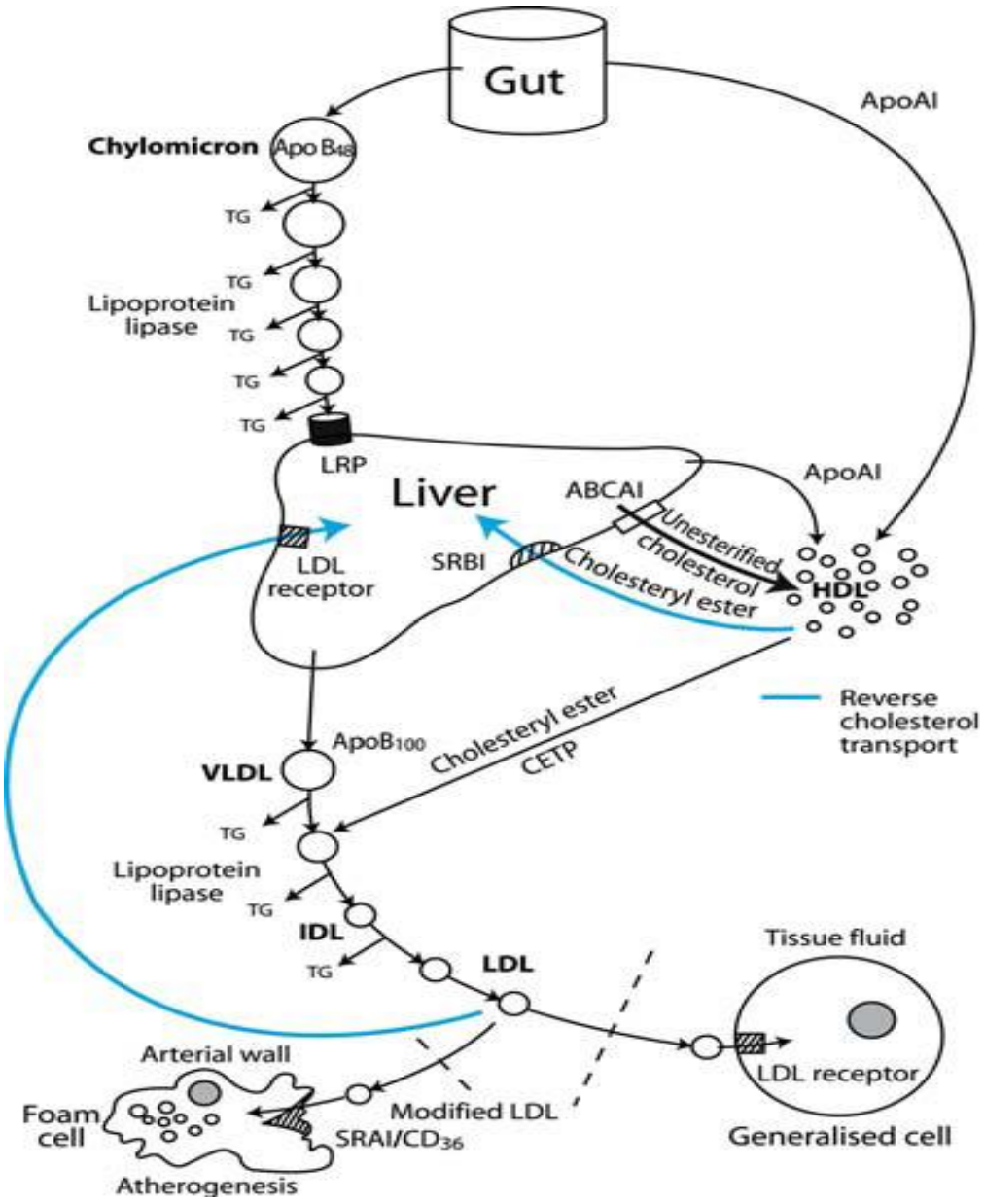

Figure 1- Metabolism of human plasma lipoproteins.(27)

1.1.2 HDL Composition, Structure and Heterogeneity

1.1.2.1 HDL Composition

Plasma HDL (hydrated density, 1.063–1.21 g/ml) is a heterogeneous group of small discoid and spherical particles (7–12 nm diameter), representing the smallest, densest and protein-richest lipoproteins due to elevated protein content (>30% by weight) compared with other lipoproteins(28). HDL proteins have traditionally been divided into four groups: apolipoproteins, enzymes, lipid transfer proteins and minor proteins (<5% of total HDL protein). Whereas apolipoproteins and enzymes have now been widely recognized for their key role in HDL metabolism and functionality, the importance of the minor proteins, as the acute phase response proteins and those involved in complement regulation, has received increasing attention over the last years.

ApoA-I is the major structural HDL apolipoprotein, a 28 kDa molecular mass protein that contains 8 amphipathic alpha-helicoidal domains of 22 amino acids each and accounts for about 70% of total HDL protein. ApoA-I plays a key role in the biogenesis and function of HDL. The second major HDL apolipoprotein, apoA-II, represents about 20% of total HDL protein. Minor HDL apolipoprotein components include apoE, apoA-IV, apoA-V, apoJ, apoM, apoC-I, apoC-II, and apoC-III. Plasma HDL particles also carry enzymes involved in lipid metabolism, including lecithin/cholesterol acyltransferase (LCAT), enzymes with plausible antioxidative activities, such as platelet-activating factor-acetyl hydrolase (PAF-AH, also called lipoprotein-associated phospholipase A_2), paraoxonase 1 (PON1) and glutathione selenoperoxidase (GSPx), and other proteins and peptides, such as serum amyloid A (SAA), a major positive acute-phase reactant, amyloid-β, the principal constituent of senile plaques

in Alzheimer's disease, or α-1-antitrypsin, a potent inhibitor of serine proteinases. Recent proteomic studies have identified up to 50 distinct proteins associated with HDL isolated by ultracentrifugation.(11, 29)

Beyond carrying a large number of proteins, HDL particles have roughly half of their total mass accounted for by lipid components. Phospholipids predominate in the HDL lipidome, accounting for 40 to 60% of total lipid, with lesser proportions of cholesteryl esters (30 to 40%), triglycerides (5 to 12%) and free cholesterol (5 to 10%). Recent lipidomic analyses allowed the identification of more than 200 individual molecular species of lipids in the HDL lipidome.(30)

1.1.2.2 HDL Structure and Heterogeneity

Plasma HDL particles are highly heterogeneous in their physicochemical properties, metabolism, and biological activity (Figure 2). The heterogeneity in HDL size and structure is intimately related to the amphiphathic, highly dynamic, helical structure of apoA-I that allows apoA-I to switch between conformations as a function of the quantity of attached lipids (31).

Discoid HDL, called pre-β HDL, are small (≤ 8 nm diameter) and lipid-poor (lipid content ≤ 30%) particles made up of apolipoproteins, primarily apoA-I, embedded with small amounts of lipid constituted of PL and free cholesterol (Figure 2A). Spherical HDL particles are larger (> 8 nm diameter) and additionally contain a hydrophobic core of cholesteryl esters and triglyerides. Two spherical HDL subpopulations can be distinguished: HDL2 (density 1.063 – 1.125 g/ml), light, large, lipid-rich HDL subfraction (triglycerides, cholesteryl esters, free cholesterol and phospholipids represent 9%, 18%, 6% and 25% of total weight, respectively) and HDL3 (density 1.125-1.21 g/ml), dense, small, lipid-poor HDL subfraction

(triglycerides, cholesteryl esters, free cholesterol and phospholipids represent 7%, 14%, 3% and 23% of total weight, respectively). (10)

Methods to fractionate HDL into subfractions were developed on the premise that assays involving HDL subfractions might be superior to HDL-C levels in assessing CVD risk. HDL can be separated by different techniques, developed over years, into distinct subclasses according to physicochemical properties and chemical composition.

Density and size

HDL particles can be separated using ultracentrifugation on the basis of density into two main subfractions, large, light, lipid-rich HDL2 and small, dense, protein-rich HDL3.(32) HDL2 and HDL3 can be further subfractionated by isopycnic density gradient ultracentrifugation into five distinct subpopulations of decreasing size, HDL2b, HDL2a, HDL3a, HDL3b and HDL3c (Figure 2C).(32),(33) This last approach to assess HDL particle heterogeneity was developed in our laboratory(34) and employed in these studies.

In addition, a novel, gas-phase differential electrophoretic macromolecular mobility-based method termed “ion mobility” allows direct quantification of large, medium and small HDL.(32) A nuclear magnetic resonance (NMR) spectroscopy-based methodology is another way to quantify plasma levels of large, intermediate and small HDL particles.

Electrophoretic mobility

HDL2 and HDL3, separated on the basis of density, can be further subfractionated on non-denaturing polyacrylamide gradient gel into five subpopulations; HDL2b, HDL2a, HDL3a, HDL3b and HDL3c as shown above (Figure 2C).

Another electrophoretic approach to studying HDL heterogeneity involves two-dimensional electrophoresis, which allows separation by charge and size of more than 10 HDL subspecies (Figure 2D). These subspecies include the discoid precursor pre-β (very

small pre-β1 which contains apoA-I and phospholipids and large pre-β2 and pre-β3), α (very small discoid α4, which contains apoA-I, phospholipids and free cholesterol; small spherical α3, which contains apoA-I, apoA-II, phospholipid, free cholesterol, cholesteryl ester, and triglyceride; medium-sized spherical α2, which contains the same constituents as α3 HDL; and large spherical α1, which contains the same constituents as α3 and α2 HDL, except the near absence of apoA-II) and pre-α (pre-α1, pre-α2 and pre-α3) particles that are of similar size as α particles, but are present in lower amounts and do not contain apoA-II (Figure 2D).(32)

Composition

HDL can be immunoseparated on the basis of apolipoprotein composition into particles containing only apoA-I (LpA-I), both apoA-I and apoA-II (LpA-I:A-II) (Figure 2B), or apoE.(32) HDL subpopulations may also markedly differ in the content of minor proteins which can be semi-quantitatively evaluated using proteomic approaches.(35)

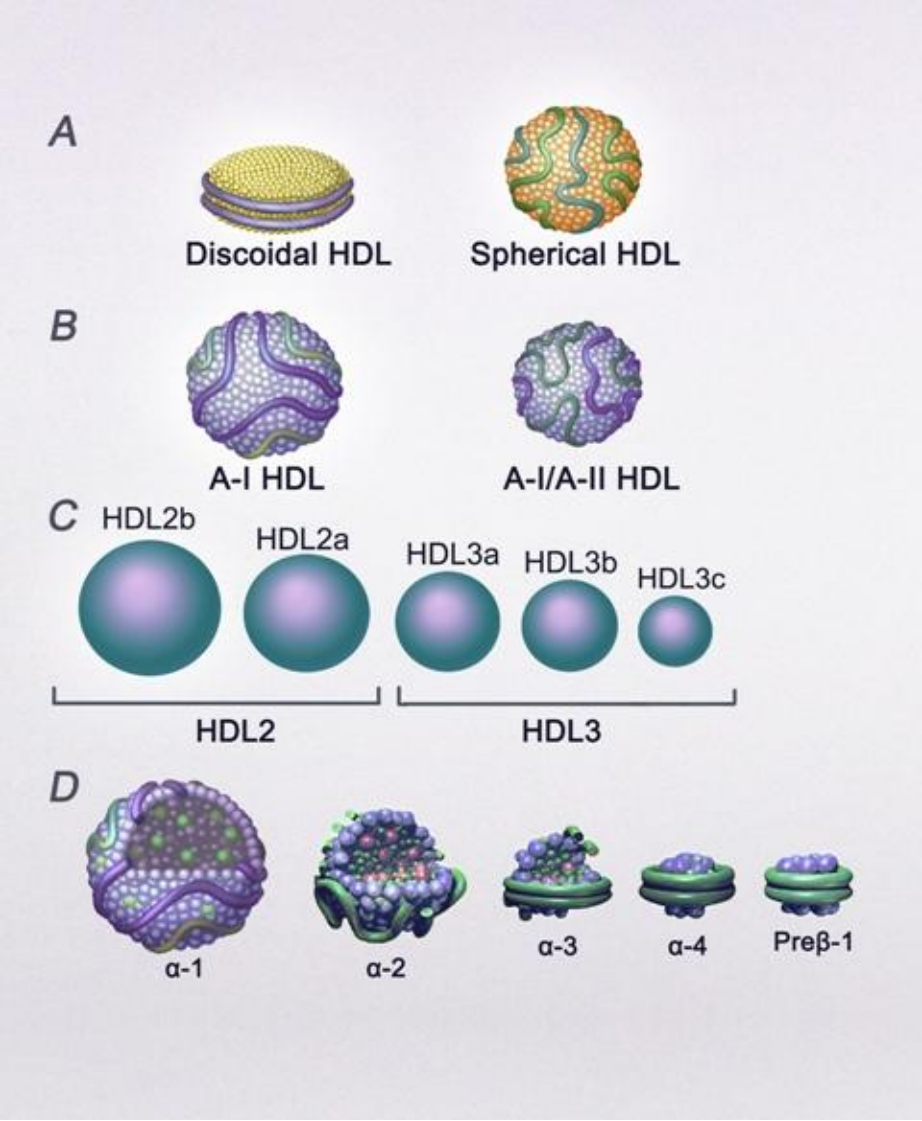

Figure 2- Heterogeneity of HDL particles. Major subpopulations of HDL particles differing in shape (A), apolipoprotein composition (B), density and size (C), and electrophoretic mobility (C,D) are shown as revealed by gel electrophoresis (A), immunoaffinity chromatography (B) and ultracentrifugation (C,D). Using 2D-gel electrophoresis, particles are separated by size in the vertical dimension and by charge in the horizontal dimension into particles of pre-β, α, and pre-α mobility. (Adapted from Rye et al (36) and Schaefer et al (37))

1.1.3 HDL Metabolism

HDL particles are being constantly remodelled as they transport cholesterol and other lipids between cells and other lipoproteins (Figure 3). The majority of plasma HDL is in the form of spherical particles, which are produced by intravascular processes from lipid-free apoA-I or lipid-poor pre-β HDL.(38) Such small HDL precursors are primarily generated as nascent HDL by the liver or intestine; they are unstable and readily acquire lipids via the ATP binding cassette transporter (ABC) A1-mediated efflux of cholesterol and phospholipids from cells.(39) Discoid HDLs are also generated from redundant surface components of triglyceride-rich lipoproteins following triglyceride hydrolysis by lipoprotein lipase (LPL). Finally, they can be generated during the interconversion of HDL3 and HDL2 mediated by PLTP. (39) These particles represent an excellent substrate for LCAT. This enzyme transfers a fatty acid residue from the position sn-2 of phosphatidylcholine (lecithin) to the hydroxyl group of cholesterol, resulting in the formation of cholesteryl esters and lysophosphatidylcholine. LCAT generates most of the cholesteryl esters present in plasma.

Cholesteryl esters are extremely hydrophobic and sequester into the core of the particles as they are formed; this effect converts discoid HDL into the large spherical HDL particles. Such HDLs can undergo further remodelling via particle fusion and surface remnant transfer mediated by PLTP.(39) Large HDL2 can, in turn, be converted into small HDL3 upon CETP-mediated transfer of cholesteryl ester from HDL to apoB-containing lipoproteins, upon SR-BI-mediated selective uptake of cholesteryl ester by the liver and steroidogenic organs, and upon hepatic lipase-mediated and endothelial lipase-mediated hydrolysis of core triglyceride.(40) When CETP-mediated transfer of cholesteryl ester occurs between HDL and triglyceride-rich lipoproteins, triglyceride-rich HDLs are generated which can be further hydrolysed by hepatic lipase to small, triglyceride-rich particles.(41) The concerted action of CETP and hepatic lipase promotes 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.(42) HDL lipids are catabolised primarily in the liver, either separately from HDL proteins by selective uptake, via CETP-mediated transfer to VLDL and LDL, or as holoparticles via uptake through LDL receptors for apoE-containing HDL and through receptors for HDL holoparticles.

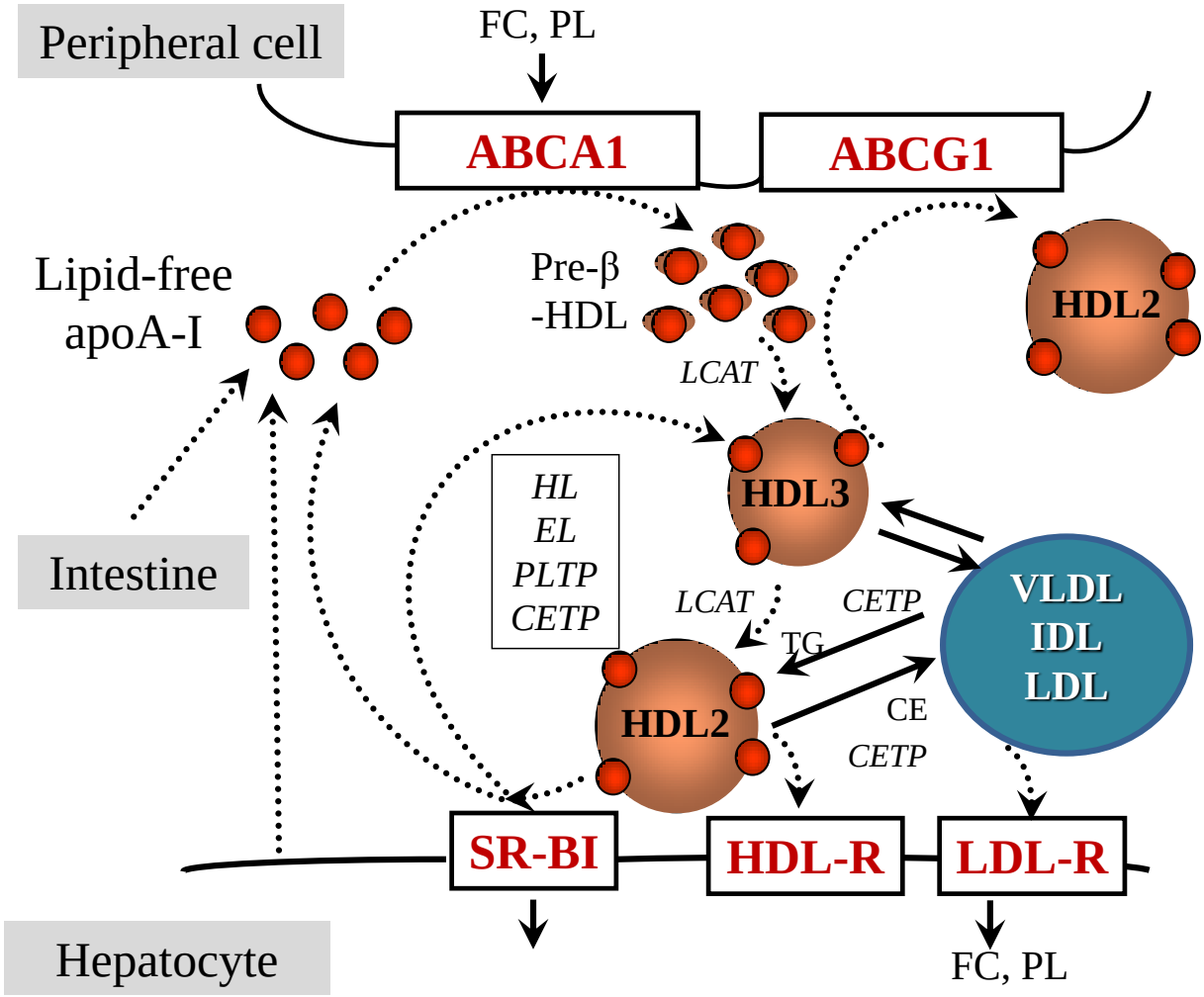

Figure 3 – Intravascular HDL particle remodeling and metabolism in normolipidemia.(43)

1.1.4 Biological Activities

HDL possesses a number of anti-atherogenic activities which involve cholesterol efflux from cells as well as antioxidative, anti-inflammatory, cytoprotective, vasodilatory, anti-thrombotic and anti-infectious activities.(43, 44) In this part, cholesterol efflux capacity and antioxidative activity, which have been in a focus of this thesis, will be discussed.

1.1.4.1 Cholesterol efflux capacity

It has been known for many years that HDL can beneficially impact atherosclerotic plaques. The key anti-atherosclerotic pathway mediated by HDL is the reverse cholesterol transport (RCT), which represents transfer of excess cholesterol from peripheral cells, including arterial wall macrophages, to the liver for excretion into the bile. This pathway is considered as the classical protective function of HDL toward atherosclerosis. The macrophages which capture cellular debris, as well as modified or aggregated lipoproteins, contain excess of cholesterol which leads to increased cytotoxicity. For this reason, cholesterol efflux from macrophages represents an important cytoprotective pathway which can occur by several mechanisms, including the following: (i) unidirectional ATP-dependent pathway mediated by the ABCA1 transporter; (ii) unidirectional ATP-dependent pathway mediated by the ATP binding cassette G1 transporter (ABCG1); (iii) ATP-independent, bidirectional pathway involving SR-BI; and (iv) receptor-independent passive diffusion according to cholesterol concentration gradient (Figure 4).(45, 46)

The pathway most studied is ABCA1-mediated cholesterol efflux to lipid-poor apolipoproteins. Indeed, abnormalities of ABCA1 gene are the cause of severe deficits in HDL, as in patients suffering from Tangier disease or other forms of family hypoalphalipoproteinemia.(47) In addition, the ability of apoAI to promote cholesterol efflux is reduced by inhibition of ABCA1 transporter and increased by its overexpression.(48) The main regulators of the expression of ABCA1 gene are nuclear receptors LXR, "Liver X receptors" type α and β, which act as a heterodimer with their partner, RXR "Retinoid X Receptor".(49) The LXRs are activated by endogenous oxysterols, which are generated enzymatically from cholesterol. Oxysterols and LXR agonists regulate transcription of

ABCA1 in macrophages and cause an increase in cholesterol efflux to lipid-poor apo AI (Figure 4).

However, *in vivo*, most of the apo AI are not lipid-poor but exists as mature HDL particles, HDL2 and HDL3. Mature HDLs are also able to promote cholesterol efflux from macrophages. It has recently been established that another member of the family of ABC transporters, ABCG1 is able to promote cholesterol efflux from macrophages to mature HDL(50, 51) by a mechanism that remains poorly characterised up to now. In macrophages, the transcription of ABCG1 as well as its translocation to the plasma membrane is regulated by cholesterol overload and/or by the action of LXR agonists.(52)

It was also reported that there may be other pathways for cholesterol efflux, for example, SR-BI is expressed in macrophages and can promote cholesterol efflux to mature HDL.(53) However, it is not certain that in macrophages, SR-BI participates quantitatively in cholesterol efflux. However, bone marrow cells overexpressing SR-BI in LDL-receptor or apoE-deficient mice,(54),(55) reduce atherosclerosis, consistent with an atheroprotective role of SR-BI in macrophages.

Role of HDL Components

Proteins appear to provide a major contribution to the cholesterol efflux capacity. Lipid-free apoA-I, apoA-II, apoE, and other HDL apolipoproteins induce fast, saturable, unidirectional and LCAT-independent efflux of cellular cholesterol and PL;(40) as a consequence, HDL particles efficiently acquire cholesterol in the extravascular compartment.

ApoA-I plays a central role in the first step of cholesterol transport to the liver from macrophages. Indeed, mice overexpressing apoA-I display accelerated RCT.(56) ApoA-I solubilises and transports cholesterol and phospholipid and adopts a conformation with high affinity for membranes in small, cholesterol-poor HDL, thereby facilitating cholesterol

efflux.(57) Following cholesterol enrichment, a conformational change occurs in apoA-I which decreases its affinity for membranes, allowing lipoprotein particle release. ApoA-II is equally able to act as a primary acceptor and to efficiently remove cholesterol from macrophages in vivo.(58) Other apolipoproteins, including apoA-IV, apoC-I, apoE and apoM, are equally efficient acceptors of cellular cholesterol.(40, 59, 60)

Enzymes carried by HDL are also of major relevance for cholesterol efflux and RCT. LCAT is a key player in the net transfer of cholesterol from peripheral cells to the liver. PON1 is also implicated in cholesterol efflux. This enzyme stimulates HDL capacity to promote cholesterol efflux through enhanced HDL binding to macrophages,(61) an effect which is potentially related to cellular accumulation of lysophosphatidylcholine, which may stimulate cholesterol efflux via the ABCA1 pathway.(62)

The role of HDL lipids for HDL-mediated cholesterol efflux is also important. Despite their relatively minor contribution to the total surface area of spherical HDL, lipids provide an environment for the acquisition of exogeneousr lipid molecules, primarily of cholesterol, thereby assuring cellular lipid efflux. As a direct consequence, properties of HDL surface lipids may strongly impact efflux. First, the cholesterol efflux capacity of HDL via SR-BI dose-dependently increases in parallel with increasing HDL content of phospholipids, the major surface lipid component.(63) Qualitatively, the ability of HDL to accept cellular cholesterol depends on the physical state of surface phospholipids, with liquid-crystal unsaturated phospholipids forming the most efficient cholesterol acceptor particles.(64)

Heterogeneity of HDL particles in cholesterol efflux capacity

The efficacy of different HDL subpopulations in promoting cholesterol efflux via the receptor-mediated pathways depends on the receptors involved.

Lipid-free and/or lipid-poor HDL apolipoproteins, primarily apoA-I, potently and dose-dependently induce cholesterol efflux via interaction with ABCA1. ABCA1-mediated cellular cholesterol efflux can be efficiently driven not only by lipid-free/lipid-poor apoA-I but also by small discoid reconstituted HDL (rHDL) particles of 7.8 nm diameter resembling plasma pre-β1 HDL (65). Indeed, small HDL particles play a key role in cellular cholesterol efflux, consistent with the capacity of ABCA1 to account for the greater part of cholesterol efflux from macrophages as compared to other HDL receptors.(46, 66) The potent capacity of small HDL to cellular cholesterol efflux is driven by their low lipid content and high surface lipid fluidity which can induce conformational changes in apoA-I compared to large, light HDL, as explained above, resulting in enhanced exposure of the protein to the aqueous phase, increased capacity of HDL to acquire large amounts of lipids and enhancement of LCAT activity.(12, 67)

In spite of the key role of ABCA1 and small HDL in cholesterol efflux from lipid-loaded cells, pathways promoted by large HDL via SR-BI and ABCG1 may also contribute significantly to net cholesterol efflux. ABCG1 efficiently transports sterols, including cholesterol and 7-ketocholesterol, to mature α-HDL.(39) Similarly, large, lipid-rich HDL2 also potently promote cholesterol efflux via ABCG1.(65) Moreover, large, lipid-rich HDL particles represent a more efficient ligand for cellular uptake of cholesteryl ester mediated by SR-BI as compared to small, lipid-poor HDL.(39) The higher phospholipid content in large HDL, which results in greater lipid surface, is probably the reason why large HDL2 is more potent than small HDL3 in mediating cholesterol efflux via SR-BI.(39) By contrast, different HDL subclasses appear to represent similarly effective acceptors via the receptor-independent, passive diffusion pathway.(39)

When comparing cholesterol efflux properties of HDL subpopulations, it is essential to keep in mind the concentration basis employed for such comparison. Thus, on the basis of phospholipid content, small, dense HDLs more potently promote cholesterol efflux via SR-BI and ABCG-1, whereas on a particle number basis, large HDLs are more effective.(63, 68)

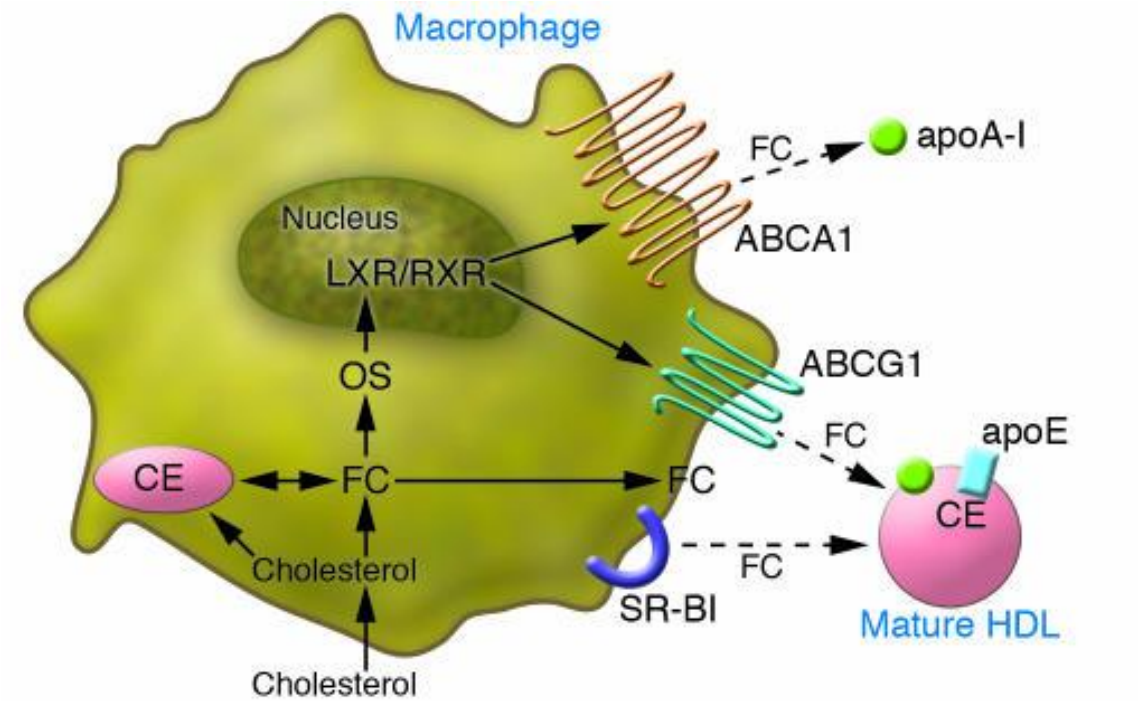

Figure 4 - The main pathways of cholesterol efflux from macrophages.(69)

1.1.4.2 Antioxidative activity

As described earlier, the oxidation of LDL appears to represent a major factor in the early stages of the development of atherosclerosis in humans. HDL antioxidative activity is typically observed as inhibition of LDL oxidation by HDL and LDL is thought to represent the major physiological target of HDL antioxidative action in vivo.(70) HDL antioxidative activity is therefore typically measured in vitro as inhibition of LDL oxidation by HD.(71, 72)

HDL can protect LDL from oxidative stress induced by various oxidants, which include one- and two-electron species. One-electron free-radical oxidantsoxidise both lipid and protein moieties of HDL which in turn propagate further oxidation of HDL components to stable termination products.(73) HDL particles potently protect LDL from oxidation by free radicals and inhibit accumulation of products of both lipid and protein oxidation, including oxidised phospholipids, short-chain aldehydes and oxidised amino acid residues.(71, 72) Such protective capacity is mediated by several HDL components and involves distinct molecular pathways.(74) One of them, evidenced in our lab, advocates that HDL protects LDL from oxidative damage by one-electron oxidants via a two-step mechanism which involves the initial transfer of phospholipid hydroperoxides (PLOOHs) from LDL to HDL, which is governed by the rigidity of the surface monolayer of HDL, and the subsequent reduction of PLOOH by redox-active methionine residues of apoA-I, with the formation of redox-inactive phospholipid hydroxides (fFgure 5).(74, 75)

By contrast, HDL-mediated protection of LDL from oxidation by two-electron oxidants, such as hypochlorite, which mainly modify the protein moiety of LDL, appears unspecific and probably reflects direct oxidant scavenging by HDL.(76)

Role of HDL Components

Both protein and lipid components of HDL particles determine their antioxidative activity.

The antioxidative activity of HDL is related to the presence in HDL particles of several apolipoproteins and enzymes with antioxidative properties. Apolipoproteins that possess antioxidative activity include apoA-I, apoA-II, apoA-IV, apoE, apoJ, and apoM.

It appears that a major component of the antioxidative activity of HDL can be assigned to apoA-I which can prevent and/or delay free-radical-induced LDL oxidation by removing oxidized PL, such as 1-palmytoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine and 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine, from LDL and from arterial wall cells.(77)

The capacity of apoA-II to protect LDL from oxidation is however questionable, given the fact that over-expression of human apoA-II in dyslipidemic mice accelerates atherosclerosis in parallel with alterations in the HDL proteome that include displacement of antioxidative apoA-I and PON1 by apoA-II from HDL particles.(78, 79) Antioxidative properties have been equally reported for apoA-IV.(80) ApoE possesses established anti-atherosclerotic activity which is normally ascribed to its lipid-transport properties.(81) HDL-associated apoJ can attenuate oxidation of LDL by artery wall cells; (82) in addition, apoJ is cytoprotective at low physiological levels.(83) Moreover, apoM possesses antiatherogenic properties suggested by experiments in transgenic mice; and the possible mechanisms underlying this association may include antioxidative activity.(84)

Enzymatic components potentially contributing to antioxidative properties of HDL include PON1, PAF-AH and LCAT, all of which are proposed to hydrolyse pro-inflammatory short-chain oxidised phospholipids.(43, 44)

HDL lipids can significantly modulate antioxidative activities displayed by the protein components. Indeed, the rigidity of the phospholipid monolayer of HDL particles is a key modulator of the transfer efficiency of PLOOH from LDL to HDL.(75)

Heterogeneity of HDL particles in antioxidative activity

HDL particles are also heterogeneous in their capacity to protect LDL from oxidative damage induced by one-electron-free radicals oxidants. A non-uniform distribution of apolipoproteins, enzymes and lipids across the HDL particle spectrum may underlie this observation. Thus, small, dense, protein-rich HDL act as potent protectors of LDL from oxidation by inactivating lipid hydroperoxides, which are primary products of LDL lipid peroxidation.(85) As a consequence, accumulation of secondary lipid peroxidation products, such as aldehydes and short-chain oxidised phospholipids, is potently inhibited by small, dense HDL3.(85) Furthermore, as aldehydes interact with amino acid residues of apoB to form protein adducts, covalent oxidation of the LDL protein moiety is also significantly retarded by HDL3.(85) Consistent with these data, small, dense HDL3 is more resistant to oxidative modification as compared to large, light HDL2.(86)

Small, dense HDL3 particles may equally be superior to large, light HDL2 in terms of their capacity to remove oxidised lipids from other lipoproteins and cellular membranes. The diminished content of sphingomyelin and free cholesterol in small, dense HDL(12) may result in increased fluidity of the surface lipid monolayer, thereby facilitating incorporation of oxidised lipids of exogenous origin, such as those derived from oxidised LDL.(74) Second, hydrolysis of short-chain oxidised phospholipids by HDL-associated hydrolytic enzymes also appears to be enhanced in small, dense HDL3.(85) Enrichment of enzymatic activities of PON1, LCAT and PAF-AH in HDL3 may account for this effect. Furthermore, enzymatic activities might be beneficially influenced by the lipidome of small, dense HDL3 which is distinct in displaying a low sphingomyelin/phosphatidylcholine (SM/PC) ratio.(12) Indeed,

sphingomyelin belongs to structural lipids which exert a positive impact on surface rigidity and a negative impact on LCAT activity.(74) Finally, the unique proteome of HDL3c may have implications for its antioxidative activity, which is highly correlated with the enrichement in apoJ, apoM, SAA4, apoD, apoL-1, PON1/3 and PLTP.(11)

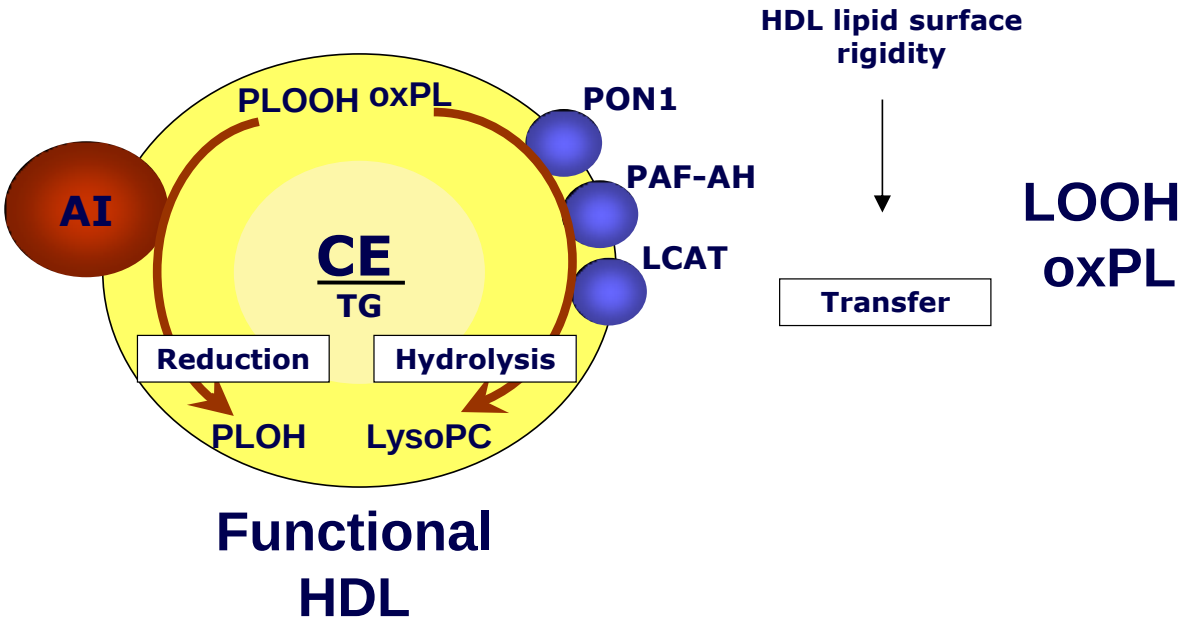

Figure 5 - Two-step mechanisms of inactivation of oxidised lipids by HDL.(75)

1.1.4.3 Other anti-atherogenic activities (Figure 6).

HDLs display multiple anti-inflammatory effects including:(87, 88) (i) inhibition of cytokine-induced adhesion molecule expression in endothelial cells; (ii) inhibition of monocyte adhesion to the endothelium; (iii) inhibition of monocyte activation, resulting in

reduced in pro-inflammatory cytokine and chemokine production; and (iv) reduction of neutrophil activation in the arterial wall. The anti-inflammatory activities of HDL appear to be primarily mediated by apoA-I, with a contribution of phospholipids, including S1P and sphingosylphosphorylcholine.(89)

HDLs equally display cytoprotective effects, such as: (i) promoting efflux of oxidized sterols via ABCG1; (ii) decreasing intracellular production of free radicals; (iii) reducing concentration of caspases (enzymes involved in apoptosis); and (iv) maintaining mitochondrial integrity. These effects are attributed to apoA-I, apoE and HDL-associated lipids, primarily S1P.(89) The functional heterogeneity of cytoprotective activities of HDL particles remains poorly characterised. Recent studies reveal that small, dense, protein-rich HDL3 potently protects human microvascular endothelial cells against primary apoptosis induced by oxidised LDL.(90, 91)

HDLs can contribute to the maintenance of vascular endothelium function by stimulating nitric oxide (NO) release and production of prostacyclin PGI_2 by endothelial cells.(92) Activation of NO production involves HDL binding to SR-BI, which activates the phosphatidyl-inositol 3-kinase (PI3K)/Akt signalling pathway and the phosphorylation of eNOS; this activation also depends on S1P receptors.(93, 94)

Anti-thrombotic activity of HDL is observed as inhibitory actions on platelet activation(95) as well as on factors that promote blood coagulation, including tissue factor, and factors X, Va, and VIIIa.(92, 96)

Finally, HDLs play a major role in the binding and clearance of circulating LPS to the bile and thereby inhibit endotoxin-induced cellular activation, resulting in potent anti-infectious activity. The inactivation of LPS by HDL is mediated by direct interaction with apoA-I and involves reduced CD14 expression on monocytes as a key step.(97)

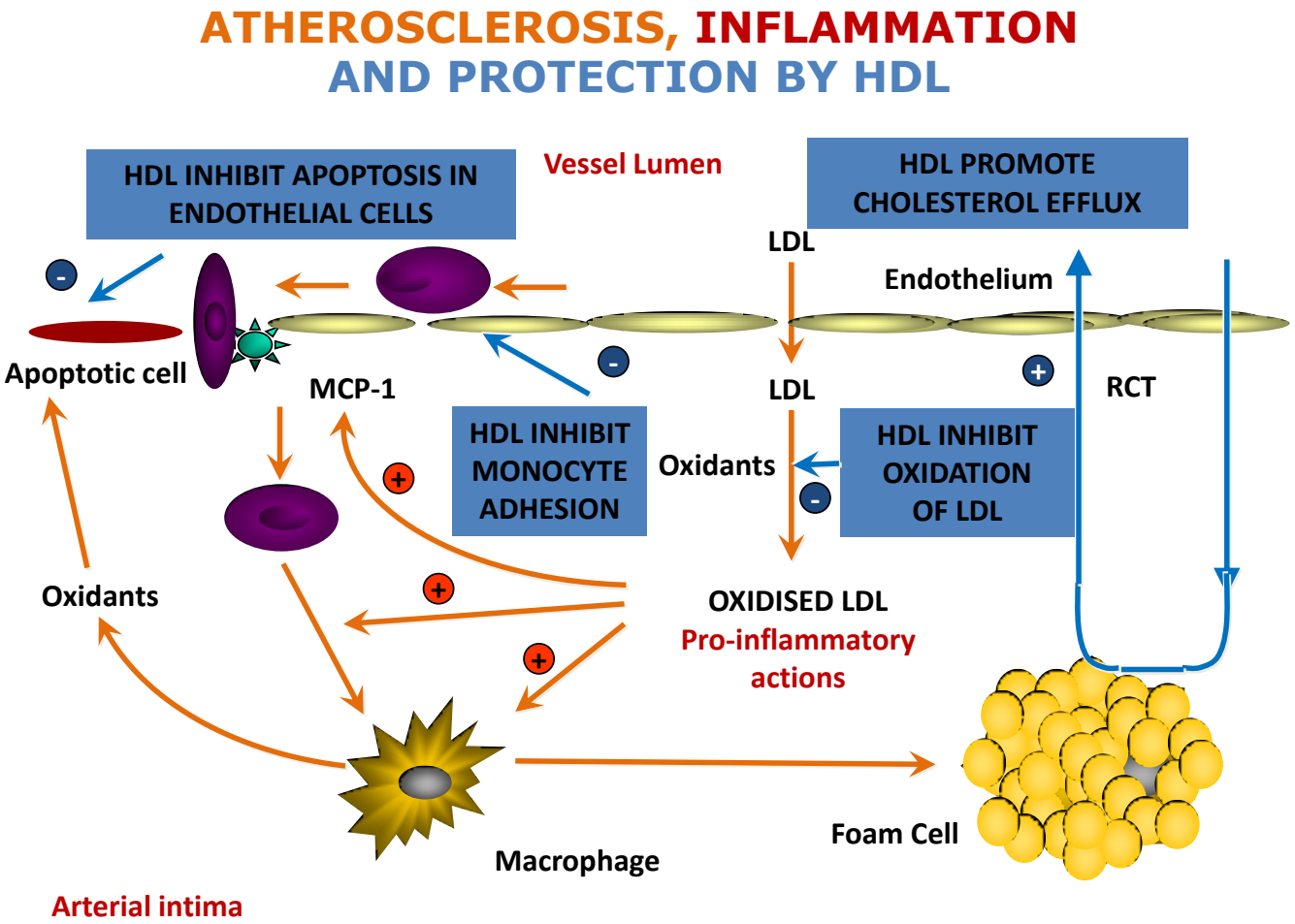

Figure 6 - Major biological activities of HDL in atherosclerosis and inflammation. (Adapted from Barter et al(98))

1.2 Chapter II) Functionally defective HDL

HDL particles can progressively lose normal biological activities and acquire altered properties as a result of alterations in HDL composition, structure and metabolism (Figure 7), which occur in the context of high cardiovascular risk, notably, dyslipidemia, insulin resistance, inflammation, infection and cardiovascular disease.(43) Such altered HDL particles have been termed 'dysfunctional HDL(99) and have been proposed to play an important role in the pathogenesis and progression of cardiovascular disease. Didactically and based on studies *in vitro* or *ex vivo* essays, HDL dysfunction is defined as complete loss of the capacity of HDL to perform normal anti-atherogenic function, while functional HDL deficiency is defined as diminished levels displayed by HDL particles of their normal anti-atherogenic functionality.

1.2.1 Altered composition

Both the proteome and the lipidome of HDL can be modified under conditions associated with accelerated atherogenesis and elevated cardiovascular risk, as characteristic of dyslipidemias, insulin-resistant states, inflammatory states and infectious diseases.

1.2.1.1 Proteome

Reduced HDL content of apoA-I is a common feature found in several disease states associated with elevated cardiovascular risk. Indeed, HDL particle content of apoA-I is

significantly decreased in hypercholesterolemic subjects.(100) In parallel, HDL content of apoE, apoC-I and apoC-III can be significantly elevated; equally, SAA tends to be concentrated in HDL of hypercholesterolemic subjects.(100) In addition, both HDL2 and HDL display elevated content of SAA and apoC-III in patients with metabolic syndrome.(101)

Patients with coronary artery disease display multiple abnormalities in the HDL proteome. Small, dense HDL3 of coronary artery disease patients is enriched in apoE and depleted of apoF, PLTP and apoJ.(102) Decreased content of apoF, a CETP inhibiting protein, suggests elevated CETP activity in coronary artery disease, consistent with low HDL-C levels. Large, light HDL2 of coronary artery disease subjects contains elevated levels of apoC-III.(103) The HDL proteome can be also significantly altered in the acute phase, during which decreased levels of apoA-I, apoA-II and apoM are observed, whereas levels of apoA-IV, apoA-V, apoE, apoJ and LBP are increased.(104-106)

The decrease in HDL apoA-I levels in inflammatory states is related both to decreased hepatic apoA-I synthesis and to replacement of apoA-I in HDL particles by SAA.(105, 107) SAA is able to replace apoA-I in small, dense HDL upon induction of the acute phase;(108, 109) as a result, plasma levels of apoA-I decrease.(110) Elevated plasma levels of SAA are accompanied by elevated levels of lipid-free apoA-I, probably due to the dissociation of apoA-I from HDL.(110)

The pro-atherogenic properties of SAA are intimately related to its biological activities as a prothrombotic and proinflammatory mediator.(111, 112) HDL enrichment in SAA enhances HDL binding to proteoglycans;(112, 113) thus, SAA might immobilise HDL particles in the arterial wall imparing their capacity to efflux cholesterol from the plaque. In addition, SAA-enriched HDL particles are rapidly cleared from the circulation;(114) SAA may then play a role in lipoprotein redistribution to the arterial wall. Furthermore, SAA increases monocyte and macrophage cytokine production, possibly at sites of atherosclerosis, thereby

contributing to the pro-inflammatory state in coronary artery disease.(115) In this pro-inflammatory milieu, SAA also upregulates tissue factor (TF) activity and TNF-alpha levels in monocytes(116) and amplifies secretory phospholipase A_2 ($sPLA_2$) secretion from smooth muscle cells.(117) Finally, SAA enrichment may impair the normal atheroprotective activities of HDL, such as cholesterol efflux capacity and anti-inflammatory function.(43, 118)

Apart from its replacement by SAA, apoA-I can undergo other modifications in the circulation. Amino acid residues in apoA-I, such as methionine, cysteine, tyrosine and lysine residues, can be selectively modified under the action of prooxidants secreted by arterial wall cells(119-121); such reactive oxygen, chlorine and nitrogen species include hypochlorite, peroxinitrite and other low-molecular-weight agents.

1.2.1.2 Lipidome

Although apolipoproteins and enzymes are major determinants of altered HDL function, such biological activities are considerably influenced by changes in the lipid content of HDL. The core enrichment in triglyceride with cholesteryl ester depletion is the most frequent abnormality of HDL lipid composition, and occurs in low HDL-C hypertriglyceridemic states associated with decreased activity of LPL and hepatic lipase, and/or decreased activity of LCAT. This pathway largely accounts for HDL depletion in cholesteryl ester and enrichment in triglyceride in the hypertriglyceridemia typical of Type 2 diabetes and metabolic syndrome,(101, 122, 123) and is accompanied by a chronic low-grade inflammatory response. Similar elevations in HDL-triglycerides, decreases in HDL-C and increase in inflammatory markers are observed in patients with coronary heart disease.(124) All of these metabolic alterations are frequently observed in the acute phase and during inflammation.(110)

Acute-phase HDL equally contain elevated levels of non-esterified fatty acids, lysophosphatidylcholine and isoprostanes as compared to normal HDL; in addition, levels of cholesteryl ester are decreased.(125) Furthermore, HDL3 from subjects with myocardial infarction are enriched in triglyceride and depleted of phospholipid.(126)

In addition to core lipids, the surface lipids of HDL can also be modified by disease. Thus, low HDL-C subjects display diminished HDL content of lysophosphatidylcholine and sphingomyelin; furthermore, triglyceride molecules are in part redistributed in the surface monolayer of such triglyceride-enriched particles.(127) The fatty acid composition of HDL can equally be affected as occurs in patients with triple vessel coronary heart disease whose HDL displays low content of polyunsaturated fatty acid (PUFAs).(124) Both an elevated sphingomyelin/phosphatidylcholine ratio and a decreased content of PUFAs can be of pathological significance as such modifications augment HDL surface rigidity and can thereby decrease both LCAT activity(128) and antioxidative capacity of HDL.(75) Finally, HDL lipids can be oxidised in vivo with a formation of biologically active pro-atherogenic compounds.(129)

1.2.2 Impaired metabolism

Alterations of HDL proteome and lipidome in cardiometabolic diseases, as shown above, are closely related to the modifications in HDL metabolism (Figure 7). Dyslipidemia, inflammation, insulin resistence, infection and cardiovascular disease all featured altered lipoprotein metabolism, including metabolism of HDL. These alterations can occur at the level of both be related to HDL formation and HDL remodeling.

As the pathways implicated in altered lipoprotein metabolism in atherogenic dyslipidemia, insulin resistance, inflammation, infection and cardiovascular disease are intimately linked, the main pathways involved can be exposed as common parts. The major common pathways of HDL formation that can be affected by the disease are: (i) reduced hepatic production of apoA-I and defective apoA-I lipidation upon interaction with ABCA1, secondary to ABCA1 and/or apoA-I deficiency, resulting in apoA-I renal catabolism and decreased levels of HDL-C; (ii) defective HDL maturation secondary to diminished cholesterol esterification by LCAT; and (iii) increased SAA production by the liver which results in apoA-I displacement with loss of this protein by renal metabolism.

The major common pathways of HDL remodeling that can be affected are: (i) elevated levels of VLDL particles due to increased hepatic production and/or deficient TG hydrolysis by LPL associated with elevated CETP activity, producing TG-enriched HDL and subnormal levels of HDL-C (ii) increased intravalscular lipolysis of TG-enriched HDL particles by hepatic lipase; and (iii) enhanced HDL remodelling by PLTP and elevated hydrolysis of HDL phospholipid by EL and $sPLA_2$.

1.2.3 Impaired biological activities

Alterations in HDL composition and metabolism, as occur under conditions associated with high cardiovascular risk, are intimately associated with impaired biological activities, including cholesterol efflux capacity as well as antioxidative, anti-inflammatory, cytoprotective, vasodilatory, antiinfectious and antithrombotic activities. In the next part, the first two functions of HDL will be discussed.

1.2.3.1 Cholesterol Efflux Capacity

Cholesterol efflux capacity can be impaired in atherogenic dyslipidemia as compared with normolipidemic controls, potentially reflecting defects in circulating levels,(130) or in the intrinsic alterations of HDL particles,(131) or both.(14) The intrinsic cholesterol efflux capacity of HDL can also be impaired during inflammation and the acute phase response, predominantly cholesterol efflux via ABCA1.(132) As cellular cholesterol efflux is largely mediated by apoA-I-containing HDL particles,(133) apoA-I replacement by SAA can significantly impact on efflux,(134) especially in HDL3 particles where SAA is predominantly present.(135) Nevertheless, SAA, in a lipid-free or lipid-poor form, may still efficiently efflux cellular cholesterol by ABCA1, functioning as an acceptor for cholesterol as well as by increasing the availability of cellular cholesterol.(136, 137) Other modifications underlying defective cholesterol efflux properties of HDL in the acute phase response involve oxidative modifications of apoA-I(138) and decreased phospholipid contents HDL particles.(139) Patients with cardiovascular disease typically present with dyslipidemia, oxidative stress and chronic inflammation, all of which may deleteriously impact on cholesterol efflux properties of HDL. As a consequence, apoB-depleted serum from patients with angiographically confirmed coronary artery disease display impaired capacity to efflux cholesterol.(14)

1.2.3.2 Antioxidative activity

HDL particles are deficient in antioxidative activity in dyslipidemic states involving low HDL-C levels, often in association with insulin resistance.(122, 123, 140) In these

studies, small, dense HDL subpopulations display a deficiency in their antioxidative activity in atherogenic dyslipidemia of metabolic syndrome(123) and well-controlled type 2 diabetes.(122) Under conditions of acute inflammatory response, diminished antioxidative activity of HDL is found, which may even turn into prooxidative action.(118) Deficiency in the antioxidative activity of HDL in established cardiovascular disease remains, however, less well studied.

The alterations in HDL particles resulting in decreased antioxidative activity of these particles involve decreases in cholesteryl ester, apoA-I, PON1 and LCAT, increases in triglyceride, serum amyloid A and CETP activity and covalent modifications of HDL by oxidation and/or glycation.(43)

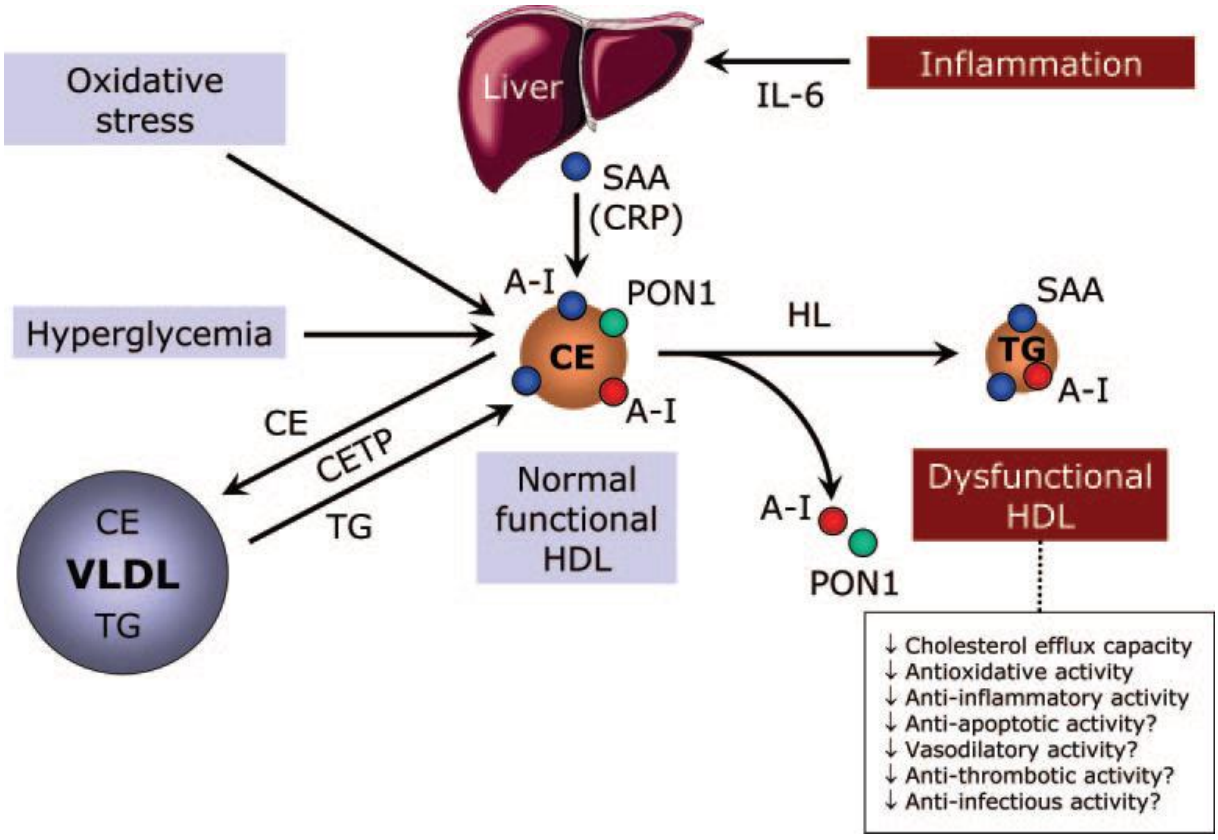

Figure 7 - Abnormal metabolism and deficient biological activities of HDL in atherogenic dyslipidemias of metabolic disease.(43)

Section II:

2. Key Questions and Objectives

2.1 Key Questions:

The multitude of biological activities expressed by HDL raises the question of the assessment of both HDL functionality ex vivo, and potential clinical benefit of HDL atheroprotective functions in vivo. Indeed, certain activities of HDL may be relevant to a specific pathophysiological context, for example, elevated oxidative stess, thrombosis or plaque progression as occurs in AMI. Identification of analytical biomarkers of multiple HDL functions that can serve as readily measurable surrogates for functional assays may therefore prove useful in a clinical setting.

Motivated by these reasons and as a function of the recent discoveries and innovations in the lipidome and proteome fields as detailed above, the following **key questions** were identified in this thesis as critical to the progress in developing more informative and effective diagnostic and therapeutic strategies in AMI patients:

(i) What are the key features of the HDL lipidome in normolipidemic subjects? How are they related to HDL functionality?

(ii) Are HDL particles dysfunctional in patients with acute myocardial infarction?

(iii) What are the key features of the HDL lipidome and proteome which underlie such functional alterations, and how are they related to defective functionality?

(iv) How does the acute inflammatory state in acute MI impact the HDL lipidome and proteome, and especially that of potently atheroprotective dense HDL3 particles?

2.2 Objectives:

Principal **objectives** of this study were therefore as follows:

(i) To identify functional clusters of lipids in HDL subpopulations and to characterise lipidomic determinants of anti-atherogenic activities of HDL subpopulations in normolipidemic subjects;

(ii) To evaluate the functionality (cholesterol efflux capacity and antioxidative activity) of HDL subpopulations in patients with MI and to compare them with their counterparts in normolipidemic healthy subjects;

(iii) To identify dynamic changes over the time course of MI in the compositional (lipidome and proteome) determinants of anti-atherogenic activities of HDL subpopulations in these patients;

(iv) To evaluate the relationship between the time-courses of functionality (cholesterol efflux capacity and antioxidative activity) of HDL subpopulations and those in systemic inflammation in MI patients, focussing on dense HDL3.

Section III:

3. Experimental

3.1 Chapter I) Materials and Methods

3.1.1 Study Population

3.1.1.1 Healthy normolipidemics

Healthy normolipidemic non-obese male volunteers were recruited at the La Pitié - Salpetrière University Hospital (Paris, France) and at the Heart Institute-InCor University of Sao Paulo Medical School Hospital (São Paulo, Brazil). All subjects were males between 32 and 67 years of age, non-smokers, and either abstainers or moderate alcohol consumers (<25 g/d). None of the subjects presented renal, hepatic, gastrointestinal, pulmonary, endocrine, or oncological disease or were receiving antioxidative vitamin supplementation or drugs known to affect lipoprotein metabolism for at least 6 weeks before the study.

3.1.1.2 Patients with MI

Sixteen male patients presenting ST Segment Elevation Myocardial Infarction (STEMI) were recruited at the Heart Institute-InCor University of Sao Paulo Medical School Hospital (São Paulo, Brazil) from April 2009 to January 2010. All patients were recruited within no later than 24 hours of clinical presentation in the Emergency Room. The diagnosis of AMI was confirmed by clinical assessment by a cardiologist, including ECG changes, troponin I elevation, and the presence of CAD on coronary angiography in accordance with the Braunwald criteria.(141) Exclusion criteria were: female gender, smoking, secondary causes of hyperlipidemia (e.g; thyroid dysfunction), presence of inflammatory or infectious diseases, AMI or stroke during last six months, use of anti-inflammatory drugs (except

aspirin) or antioxidant vitamins and use of lipid-lowering drugs during the month preceding the clinical event.

Written informed consent was obtained from all study subjects and the project was approved by the Ethics Committee of InCor in accordance with local institutional guidelines conformed to the Declaration of Helsinki.

Each patient was biologically and clinically phenotyped; specifically plasma lipid profile, cardiovascular status and drug treatment status were determined. Patients with MI were treated with statins according to established clinical protocols starting from the second day after acute MI.

3.1.2 Blood samples

Blood samples were withdrawn from the cubital vein of each participant at the time of recruitment. Serum and EDTA plasma (final EDTA concentration; 1 mg/ml) were prepared from venous blood collected into sterile, evacuated tubes (Vacutainer). Plasma was immediately separated by low-speed centrifugation at 4°C. The serum was free of EDTA, which is an inhibitor of paraoxonase.(142) The serum was reserved mainly for studies of antioxidant activities of HDL while the plasma used for other assays. Serum and plasma were each mixed with sucrose (final concentration, 0.6%) as a cryoprotectant for lipoproteins,(143) aliquoted and frozen at -80°C under nitrogen; each aliquot was thawed only once directly before analyses. The addition of sucrose in serum and plasma prevented structural modification of lipoproteins during freezing. Indeed, Rumsey and colleagues(143) have shown that physica (size, integrity of apo B100) and biological (binding capacity of fibroblasts) properties of LDL were retained after freezing at -70°C, only after a solution of 10% sucrose was added to LDL before freezing. Furthermore, the addition of sucrose to

plasma at 0.6% (w/v final concentration) exerted simalary protective effects on LDL after freezing at -80°C.(144)

3.1.3 Clinical and biological parameter

Plasma levels of total cholesterol (TC), triglyceride (TG) and HDL-C were measured using commercially available enzymatic kits; LDL-C was calculated using the Friedewald formula. Plasma apoA-I and apoB were quantitated by immunoturbidimetry.(85) Systemic inflammation was assessed as the plasma level of high-sensitive C-reactive protein (hsCRP) measured by immunoassay(123) and as the plasma level of SAA, an acute phase protein, which was determined by ELISA assay (ELISA Kit KHA0011, Invitrogen, USA). In addition, interleukin (IL)-6 was quantitated in plasma using a Biochip technology (Randox, Northern Ireland).(145)

3.1.4 Determination of endogenous plasma cholesteryl ester (CE) transfer from HDL to apoB-containing lipoproteins

Determination of endogenous plasma CE transfer from HDL to apoB-containing lipoproteins was performed by modification of the method of Guérin et al.(146) as previously described.(147) CE transfer was determined after incubation of whole unlabelled individual plasmas (500µL) at 37°C or 0°C for 3h in the presence of trace amounts of radiolabelled normolipidemic HDL (<5% of the total HDL-CE mass in plasma). Iodoacetate was present at the final concentration of 1.5 mmol/L to inhibit LCAT. The radioactive content of plasma lipoproteins was quantified by liquid scintillation spectrometry with a Trilux 1450 beta counter (Perkin Elmer, USA). CE transferred from HDL was calculated using the difference between

the radioactivity counts at 37°C and 0°C, and expressed as % radioactivity recovered in plasma.

3.1.5 Isolation of lipoproteins

Taking into account the density difference between pure lipids (approximately 0.9 g/ml) and pure protein (approximately 1.35 g/ml), each lipoprotein class displays a specific density range which depends on its lipid/protein ratio. This allows lipoprotein isolation according to density, an approach developed by several laboratories, including our INSERM research unit.(148)

Plasma lipoproteins were isolated from serum and plasma by single step, isopycnic non-denaturing density gradient ultracentrifugation in a Beckman SW41 Ti rotor at 40,000 rpm for 44 hours in a Beckman XL70 ultracentrifuge at 15°C by a slight modification of the method of Chapman et al.(148) as previously described.(149)

During centrifugation, lipoproteins, whose densities range from 1.019 and 1.063 g/ml for the LDL subfractions and 1.063 and 1.21 g/ml for HDL subfractions, are subjected to the effects of gravitational forces. A sedimentation-equilibrium, or isopycnic, method separates particles on the basis of particle buoyant density. Each component in the sample moves through the gradient until it reaches an equilibrium position. The liporoteins move through th gradient until they reach the point of their respective densities. Therefore, the aim of this ultracentrifugation is to get the point of isopicnic balance of lipoproteins within a density gradient.

3.1.6 Density gradient ultracentrifugation

After thawing the samples, the density gradient was prepared using five solutions of different densities. The densities of the five solutions were: 1.006 g/ml, 1.019 g/ml, 1.063 g/ml, 1.21 g/ml and 1.24 g/ml. The solutions used for the density gradient were prepared from two stock solutions as follows:

Solution A: density 1.006 g/ml. For 1l: 8.76 g NaCl + 625 µl of gentalline (80 mg/ml to prevent bacterial contamination) adjusted to pH 7.4.

Solution B: density 1.357 g/ml. For 1l: 153 g NaCl + 354 g KBr + 625 µl of gentalline (80 mg/ml) adjusted to pH 7.4.

These solutions did not contain EDTA, for the same reason that made us choose serum to mesure for antioxidative activity of HDL, i.e. to avoid inhibition of PON1 by EDTA.

Then we applied the formula: dX (VA + VB) = (VA * dA) + (VB * dB) to prepare a solution of a desired density (dX).

We fixed a volume VA of solution A and calculated the volume of solution B (VB) required to obtain a solution of the density dX.

Densities were verified using a densimeter at 15°C to obtain solutions of a density of 1.24 g/ml 1.21 g/ml, 1.063 g/ml 1.019 g/ml and 1.006 g/ml. These solutions were stored at 4 ° C until use.

3.1.7 Preparation of samples

Plasma or serum sample was brought to a density of 1.21 g/ml with the addition of KBr (Sigma Aldrich, Saint Quentin Fallavier, France). The salt mass (m) added to a volume (V) of plasma or serum to adjust its density d1 to d2 was calculated by the formula:

$$m = V \cdot \frac{(d_2 - d_1)}{1 - v \cdot d_2},$$

where v is partial specific volume of salt at a given temperature.

Typically 0.93g KBr was added to a sample of 3ml of plasma or serum.

3.1.8 Gradient preparation

Plasma or serum mixed with KBr and solutions of different densities were carefully deposited in Ultraclear Beckman tubes, (Beckman Coulter, Roissy, France) to create a gradient. 2 ml of the solution 1.24 g/ml were deposited at first step; Subsequently, 3 ml of serum-KBr, density 1.21 g/ml, was placed very carefully with a pipette along the tube wall. Finally, other solutions of 1,063 (2ml), 1.019 (2.5 ml) and 1.006 g/ml (2.5 ml) were deposited respectively on top of each other with a Auto DensiFlow device (Buchler, FortLee, New Jersey, USA) associated with a pump (Ismatec, Zurich, Switzerland).

3.1.9 Recovery of lipoprotein subfractions

After centrifugation, each gradient was fractionated in predefined volumes from the meniscus downwards with an Eppendorf precision pipette into 11 fractions corresponding to VLDL+IDL (d<1.019 g/mL), LDL (5 subfractions, LDL1, d 1.019-1.023 g/mL; LDL2, d 1.023-1.029 g/mL ; LDL3, d 1.029-1.039 g/mL; LDL4, d 1.039-1.050 g/mL; and LDL5, d 1.050-1.063 g/mL) and HDL. Five major HDL subclasses were isolated, i.e., large, light HDL2b (d 1.063–1.087 g/ml) and HDL2a (d 1.088–1.110 g/ml), and small, dense HDL3a (d 1.110–1.129 g/ml), HDL3b (d 1.129–1.154 g/ml) and HDL3c (d 1.154–1.170 g/ml). The validity and reproducibility of this density gradient procedure, which facilitates preparative fractionation of HDL particle subspecies in a non-denaturated, native state, have been extensively documented.(34, 150)

3.1.10 Dialysis of isolated HDL subfractions

Before analyzing composition of HDL subfractions and their functional properties, it was necessary to remove the KBr and EDTA. For this purpose, lipoproteins were extensively dialysed against phosphate-buffered saline (PBS; pH 7.4) at 4°C in the dark. Subsequently, lipoproteins were stored at 4°C and used within 10 days.

3.1.11 Chemical analysis of lipoproteins

Total protein, TC, free cholesterol (FC), phospholipid (PL) and TG contents of isolated lipoprotein subfractions were determined using commercially available assays.(34, 85) CE was calculated by multiplying the difference between total and free cholesterol concentrations by 1.67.(34) Total lipoprotein mass was calculated as the sum of total protein, CE, FC, PL and TG and expressed as plasma concentrations (mg/dl). ApoA-I and apoA-II content in HDL was quantitated using commercially available kits (Diasys, France). SAA content in HDL was quantitated by ELISA assay (ELISA Kit KHA0011, Invitrogen, USA).

3.1.12 Lipidome (Figure 8)

Lipid standards. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine-N,N,N-trimethyl-d9 (PC 16:0/16:0 d9), 1-lauroyl-2-tridecanoyl-sn-glycero-3-phospho-(1'-myo-inositol) (PI 12:0/13:0), 1-dodecanoyl-2-tridecanoyl-sn-glycero-3-phosphoethanolamine (PE 12:0/13:0),

1-dodecanoyl-2-tridecanoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (PG 12:0/13:0), 1-dodecanoyl-2-tridecanoyl-sn-glycero-3-phosphate (PA 12:0/13:0), 1-dodecanoyl-2-tridecanoyl-sn-glycero-3-phospho-L-serine (PS 12:0/13:0), 1-pentadecanoyl-*sn*-glycero-3-phosphocholine (LPC 15:0/0:0), and N-heptadecanoyl-D-erythro-sphingosine (Cer d18 :1/17:0) were used as internal standards (Supplement Table 1). 1-Palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC 16:0), 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC 18:0), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (PC 14:0/14:0), 1-myristoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (PC 14:0/16:0), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (PC 16:0/16:0), 1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphocholine (PC 16:0/18:0), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC 16:0/18:1), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PC 16:0/18:2), 1,2-distearoyl-*sn*-glycero-3-phosphocholine(PC 18:0/18:0), 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC 18:0/18:1), 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PC 18:0/18:2), 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PC 18:0/20:4), 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (PC 16:0/22:6), 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (PC 18:0/22:6), 1-stearoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine (LPE 18:0), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (PE 18:0/18:0), 1-heptadecanoyl-2-(9Z-tetradecenoyl)-sn-glycero-3-phospho-(1'-myo-inositol) (PI 17:0/14:1), N-stearoyl-D-*erythro*-sphingosine (Cer d18:1/18:0), 1,2-distearoyl-*sn*-glycero-3-phosphate (PA 18:0/18:0), 1,2-distearoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (PG 18:0/18:0) and 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phospho-L-serine (PS 16:0/18:2) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). LC/MS grade solvents were used without further purification and obtained from Sigma-Aldrich (St Louis, MO, USA) or VWR (West Chester, PA, USA).

Extraction**.** HDL subpopulations were extracted according to a procedure adapted from Larijani B, et al.(151) Briefly, 30µg of total phospholipid mass determined using a commercially available assay were added to 4ml of cold $CHCl_3$/acidified CH_3OH (5:2 v/v)

containing 4µg of PC d9 32:0, 100ng of PI 25:0, 80ng of PE 25:0, 80ng of PA 25:0, 40ng of PS 25:0, 20ng of PG 25:0 and 20ng of Cer 17:0. A blank (PBS) and a control (HDL2 obtained from a reference normolipidemic plasma) sample were extracted in parallel with each batch to ensure for quality control; all samples were corrected for blank readings. K_4EDTA (200mM) solution was added (1:5 v/v) and the mixture was vortexed for 1min and centrifuged at 3600 g for 10min at 4°C. The organic phase was transferred into 5ml Chromacol glass tubes and dried under nitrogen. Lipids were reconstituted into 150µl isopropanol/hexane/water (10:5:2 v/v), transferred into LC/MS amber vials with inserts, dried under nitrogen and resuspended in 40µl of isopropanol/hexane/water (10:5:2 v/v). Molecular lipid species were analysed and quantitated by LC/MS/MS.

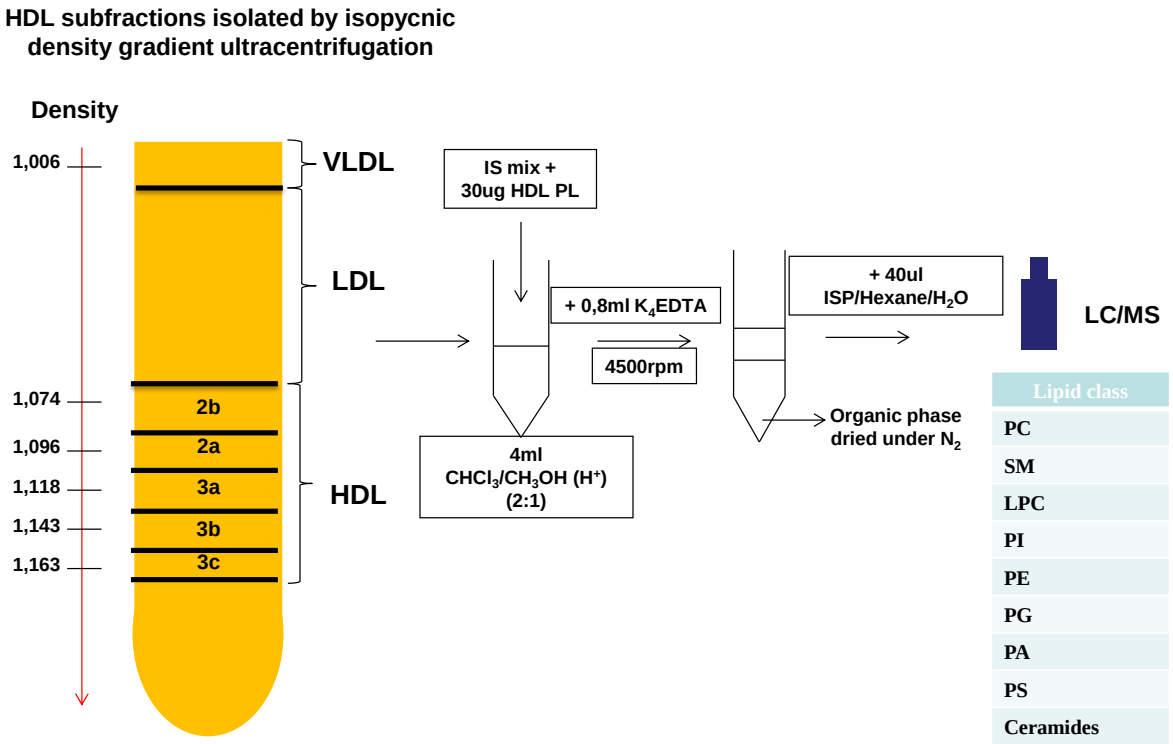

Figure 8 - Representation of lipid extraction.

LC/MS analysis**.** Seven principal PL subclasses (PC, LPC, PE, PI, PG, PS and PA) and two principal sphingolipid (SL) subclasses (SM and Cer), which together comprise >160 individual molecular lipid species and account for >95% of total plasma PL and SM,(152, 153) were assayed by LC/MS/MS (Table 2). The lipid subclasses were divided into major (those whose content was >1% of total PL+SL, i.e. PC, SM, LPC, PE and PI) and minor (those whose content was <1% of total PL+SL, i.e. PG, Cer, PS and PA).

Lipids were quantified by LC-ESI/MS/MS using a QTrap 4000 mass spectrometer (AB Sciex, Framingham, MA, USA) equipped with a turbo spray ion source (300°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 PLs and SLs was performed in positive-ion mode, except for PI species that were detected in negative-ion mode (Table 3). Sample (4µl) was injected onto a Symmetry Shield RP8 3.5µm 2.1x50mm reverse phase column (Waters Corporation, Milford, MA, USA) using a gradient from 85:15 to 91:9 (v/v) methanol/water containing 5mM ammonium formate and 0.1% formic acid at a flow rate of 0.1ml/min for 30 min. Lipid species were detected using multiple reaction monitoring reflecting the headgroup fragmentation of each lipid class. PC, LPC and SM species were detected as product ions of m/z 184, PE, PS, PG and PA as neutral losses of respectively m/z 141, 185, 189 and 115, and PI molecular species as product ions of m/z 241. Air was used as nebulising gas and N_2 as collision gas. PE, PS, PG, PI, PA and ceramide species were monitored for 18ms; PC, LPC and SM species were monitored for 30ms at a unit resolution (0.7 atomic mass unit at half peak height).

Quantification**.** Lipids were quantified using calibration curves specific for the nine individual lipid classes with up to 12 component fatty acid moieties. Twenty-three calibration curves were generated in non-diluted and 10-fold diluted matrices to correct for matrix-

induced ion suppression effects. More abundant lipid species which displayed a non-linear response in non-diluted extracts were quantified from a 10- or 100-fold diluted sample. An in-house developed Excel Macro script (Microsoft Office 2010, Redmond, WA, USA) was used to compile data from the three successive injections.

3.1.13 Cellular cholesterol efflux capacity of HDL (Figure 9)

Cholesterol efflux capacity of HDL subpopulations (HDL2b, HDL2a, HDL3a, HDL3b and HDL3c) and of total HDL were characterised in a human THP-1 monocytic cell system (ATCC, Manassas, VA, USA) at 15 µg HDL-PL/ml for HDL subpopulations and at 30 µg HDL PL/ml for total HDL.(154) HDL particles were compared on the basis of their PL concentrations because PL was shown to represent the key component determining cholesterol efflux capacity of HDL.(155) Total HDL from each donor was prepared by mixing all five HDL subfractions at their equivalent serum concentrations.

Assays of cellular cholesterol efflux were performed as previously described (156) with minor modification. In brief, THP-1 monocytes were cultured on 24-well tissue culture plates, grown in RPMI 1640 media with 10% FBS and differentiated into macrophage-like cells with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) for 48 hours and 37°C. The cells were washed and loaded for 24 hours with [3H]cholesterol-labelled acetylated LDL (acLDL, 1 µCi/mL) in serum-free RPMI 1640 culture medium supplemented with 50 mM glucose, 2 mM glutamine, 0.2% BSA, 100 µg/ml penicillin and 100 µg/ml streptomycin (further abbreviated as RGGB medium) to allow equilibration of cellular cholesterol pools. The labelling medium was removed and human macrophages were then equilibrated in RGGB for an additional 16-24 hours period. Cellular cholesterol efflux to HDL subpopulations and total HDL was assayed in serum-free medium for a 4-hour chase period. Finally culture media were

harvested and cleared of cellular debris by brief centrifugation. Cell radioactivity was determined by extraction in hexane-isopropanol (3:2), evaporation of the solvent under nitrogen and liquid scintillation counting (Wallac Trilux 1450 Microbeta, Perkin Elmer, USA). The percentage of cholesterol efflux was calculated as (medium cpm) / (medium cpm + cell cpm) x 100%. Specific cholesterol efflux was determined by subtracting non-specific cholesterol efflux occuring in the absence of cholesterol acceptors.

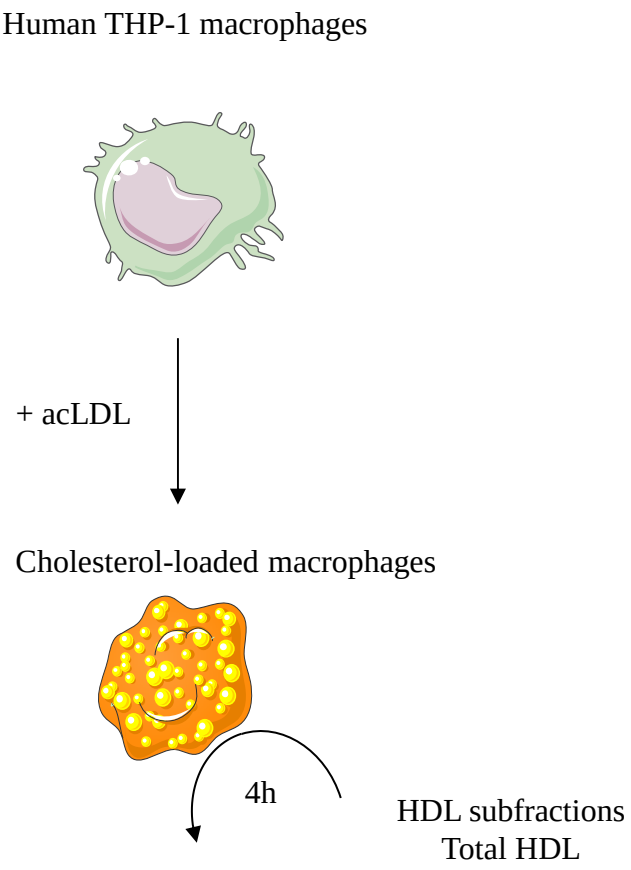

Figure 9 - Schematic representation of cellular cholesterol assay.

3.1.14 Antioxidative activity of HDL (Figure 10)

Antioxidative activities of serum-derived HDL3b and 3c subpopulations (final concentration of each, 10 mg total mass/dl), and of total HDL (final concentration, 40 mg total mass/dl), were assessed at a physiological HDL to LDL ratio of 5-15 mol/mol towards reference LDL (d 1.019-1.063 g/ml; final concentration, 10 mg TC/dl) isolated from one healthy normolipidemic control subject.(122, 123, 140) HDL particles were compared on the basis of their total mass concentrations because both protein and lipid components were shown to contribute to the capacity of HDL to inhibit LDL oxidation.(75),(74) Total HDL from each donor was prepared by mixing all five HDL subfractions at their equivalent serum concentrations.

HDL subfractions and total HDL were added to LDL directly before oxidation. Lipoprotein oxidation was induced by an azo-initiator 2,2′-azo-bis-(2-amidinopropane) hydrochloride (AAPH; final concentration 1 mmol/l)(85) as a model of mild oxidation induced by free radicals in the arterial intima.(157) Serum was used as a source of HDL for this assay to ensure intact paraoxonase activity, which is inhibited by EDTA.(158) Thereby this assay employs mild oxidative conditions and integrates the antioxidative activities of several HDL components, i.e. apoA-I, antioxidative enzymes and lipophilic low-molecular-weight antioxidants.(74) Accumulation of conjugated dienes was measured as the increment in absorbance at 234 nm.(85, 122, 123, 140) Absorbance kinetics were corrected for the absorbance of AAPH itself run in parallel as a blank. The kinetics of diene accumulation revealed two characteristic phases, the lag and propagation phases (Figure 10). For each curve, the duration of each phase, average oxidation rates within each phase and amount of

dienes formed at the end of the propagation phase (maximal amount of dienes) were calculated.(85, 122, 123, 140)

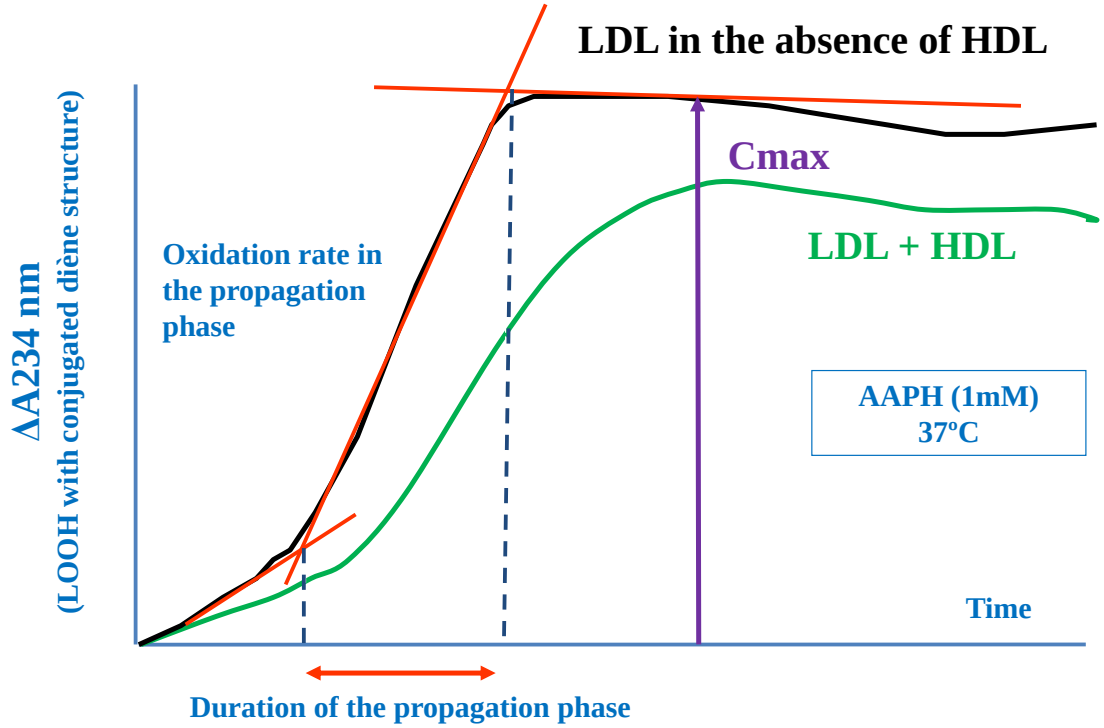

Figure 10 - Analysis of oxidation kinetics measured as absorbance increase at 234 nm.

3.1.15 Statistical analysis

Distributions of all variables were analysed for normality using the Kolmogorov-Smirnov test. Normally-distributed variables are expressed as means ±SD unless otherwise indicated; non-Gaussian distributed variables are expressed as median (minimum, maximum). Between-group differences in normally-distributed variables were analyzed using the Student's t-test. For non-Gaussian distributed variables, the Mann-Whitney U-test was used, or such variables were log transformed to ensure normality before statistical analysis.

Differences in dichotomous variables were analyzed by Fisher's exact test. Spearman's correlation coefficients were calculated to evaluate relationships between variables.

3.2 Chapter II) HDL composition and function in healthy normolipidemic subjects

3.2.1 Clinical and biological parameters

All fourteen subjects were males between 32 and 67 years of age, non-smokers, and either abstainers or moderate alcohol consumers (<25 g/d). None of the subjects presented renal, hepatic, gastrointestinal, pulmonary, endocrine, or oncological disease or were receiving antioxidative vitamin supplementation or drugs known to affect lipoprotein metabolism for at least 6 weeks before the study. Plasma lipid profiles were assessed, with normal HDL-C (57±16 mg/dL) and apoA-I (140±17 mg/dL) levels and normal TG (86±24 mg/dL) and apoC-III (8.6±1.7 mg/dL) concentrations. Total cholesterol (190±25, mg/dL), LDL-C (121±28 mg/dL), apoB100 (98±23 mg/dL) and PL (191±61 mg/dL) concentrations were also measured. They did not have an inflammatory state with normal levels of hsCRP (0.8 (0.2-4.8), mg/L) and SAA (4.3 (2.3-61.0), mg/L).

3.2.2 Plasma levels and chemical composition of HDL particle subpopulations

Consistent with published data,(19, 85) HDL content of major lipid classes (PL, FC, CE, TG) calculated on the basis of total HDL mass showed a distinct trend to decrease with increment in total protein content and particle density across the HDL particle spectrum (Table 4). Plasma concentrations of HDL subfractions obtained from normolipidemic controls and their content of lipids and protein (wt/wt) of are shown in table 4.

3.2.3 Lipidome of HDL particle subpopulations in healthy normolipidemic subjects

An original LC-MS/MS methodology for PL and sphingolipid (SL) profiling involving reverse-phase LC separation was applied to the analysis of human plasma HDL subpopulations isolated by isopycnic density gradient ultracentrifugation. This approach features separation of analytes and internal standards as a key step and allows matrix effects and varying ionization efficiencies to be accurately taken into account. For quantification, a set of non-naturally occurring internal standards were added prior to lipid extraction.

Using this methodology, 162 individual molecular lipid species were identified in five normolipidemic HDL subpopulations across the nine lipid subclasses, including 23 PC, 22 SM, 9 LPC, 25 PE, 17 PI, 11 PG, 24 ceramide (Cer), 18 PS and 13 PA species. PC species clearly predominated, accounting together for 73 to 77% of total PL+SL, followed by SM (15-21%), lysoPC (2.4-3.9%), PE (1.5-2.2%), PI (1.7-2.4%), PG (0.27-0.37%), Cer (0.09-0.16%), PS (0.03-0.53%) and PA (0.02-0.05%) species.

A high level of heterogeneity in lipid content was found across HDL subpopulations. While absolute levels of the majority of lipid subclasses in HDL followed circulating concentrations of HDL particles shown in Table 4, SM and Cer tended to be enriched in large, light HDL, whereas PS preferentially associated with small, dense particles (Figure 11). As a result, when expressed as a wt% of PL+SL, PS, but also PC, lysoPC, and PA, showed a marked tendency to increase progressively in parallel with increase in hydrated density and reduction in size from HDL2b to HDL3c. Indeed, small, dense HDL were enriched in PC, PS and PA relative to large, light HDL ($p=0.01$, $p=0.003$ and $p<0.001$ for

trend, respectively; Figure 12). Furthermore, HDL3c was enriched in lysoPC (+48%, p=0.05) and PS (18-fold, p<0.01) relative to HDL2b (Figure 12). Similarly, PE, PI and PG tended to concentrate in small, dense HDL; these trends did not however attain significance (Figure 12). Interestingly, lipid species of PS were localized almost exclusively in the densest HDL3c subfractions; PS content varied from 0.03% of total PL+SL in HDL2b to 0.53% in HDL3c. As a consequence, the percentage of negatively charged PLs PI, PS, PG and PA increased with HDL density from 2.0% of total PL+SL in HDL2b to 3.3% in HDL3c.

Concomitant with such enrichment in PLs, the proportion of SM and Cer decreased progressively in parallel with HDL density from 20% and 0.16% of total PL+SL in HDL2b to 15% and 0.10% in HDL3c, respectively (Figure 12). As a result, the SM/PC ratio decreased from 0.28 in HDL2b to 0.18 in HDL3c, consistent with earlier data.(12, 75) Similar relationships between HDL content of lipid subclasses and density were also observed when they were expressed on the basis of total HDL lipids (Figure 13).

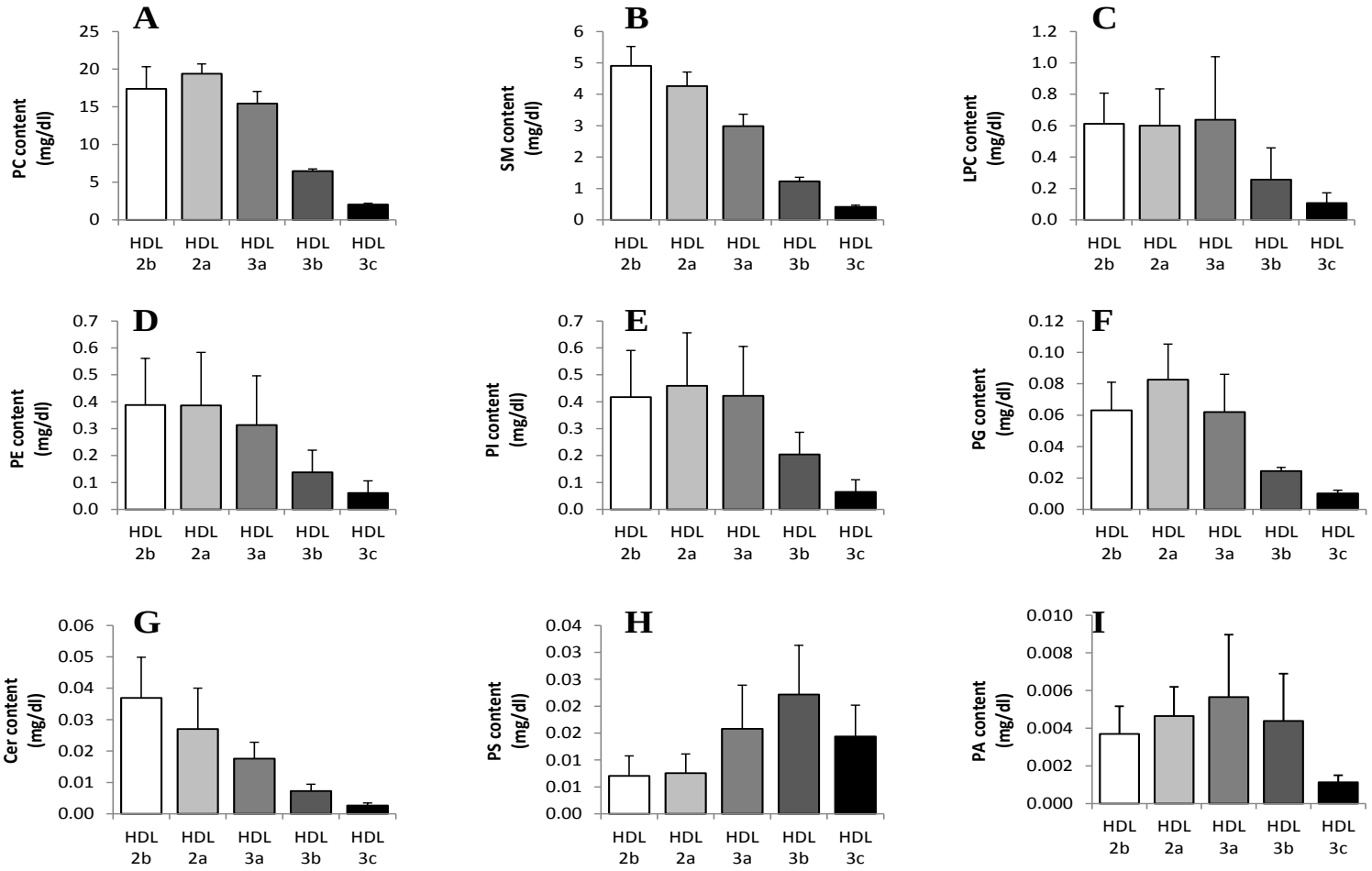

Figure 11 - Lipidome of HDL subpopulations in healthy normolipidemic subjects. Absolute levels of PC, SM, LPC, PE, PI, PG, Cer, PS, PA, CE, FC and TG in each HDL subpopulation, expressed as mg/dl, are shown.

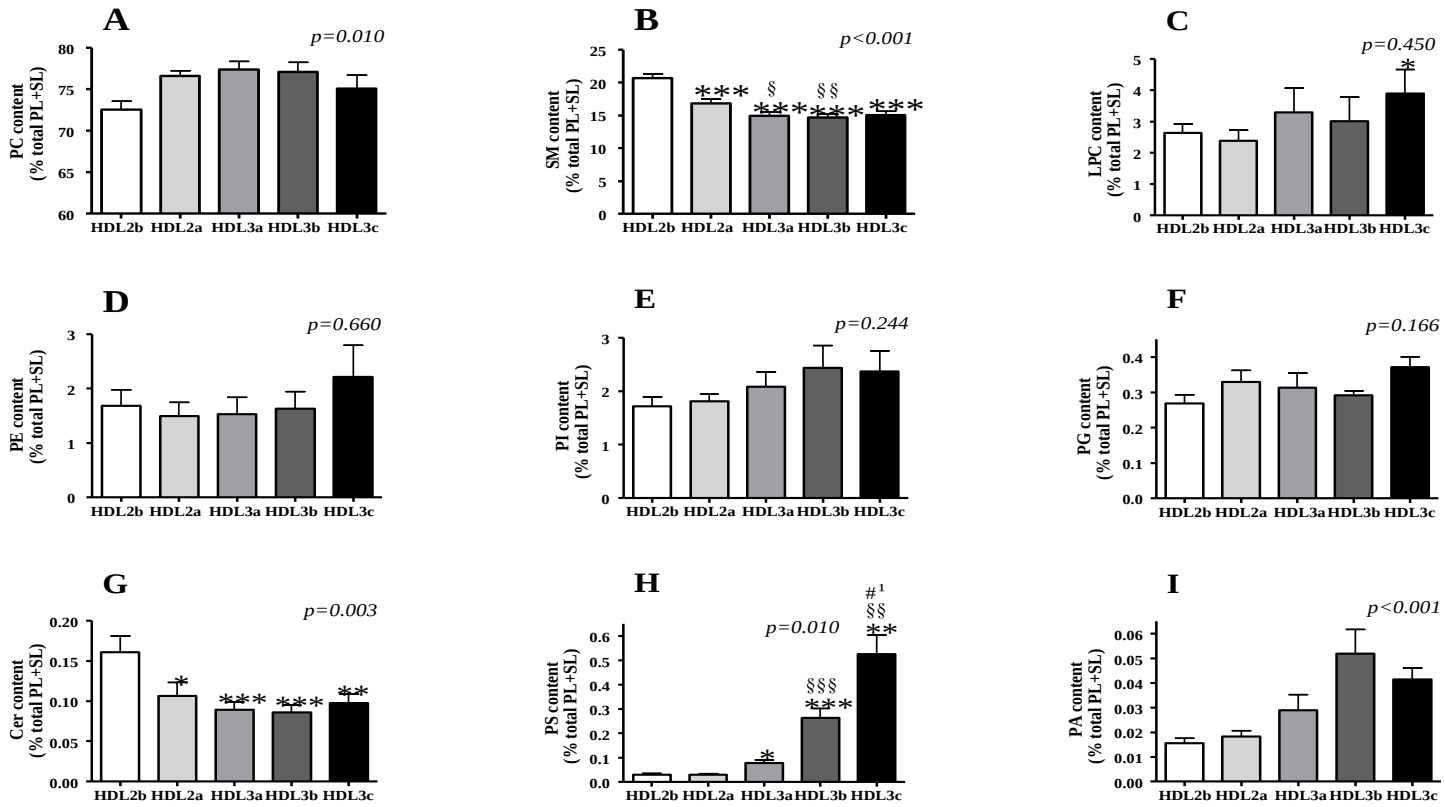

Figure 12 - Phosphosphingolipidome of HDL subpopulations in healthy normolipidemic subjects. HDL contents of PC (A), SM (B), LPC (C), PE (D), PI (E), PG (F), Cer (G), PS (H) and PA (I), expressed as wt % of total phosphosphingolipidome in each HDL subclass, are shown in the order of decreasing abundance; *p<0.05, **p<0.01, ***p<0.001 vs. HDL2b; §p<0.05, §§p<0.01, §§§p<0.001 vs. HDL2a; #p<0.05 vs. HDL3a; ≠p<0.05 vs. HDL3b. In the upper right corner, p-values for the trend between HDL subpopulations are shown.

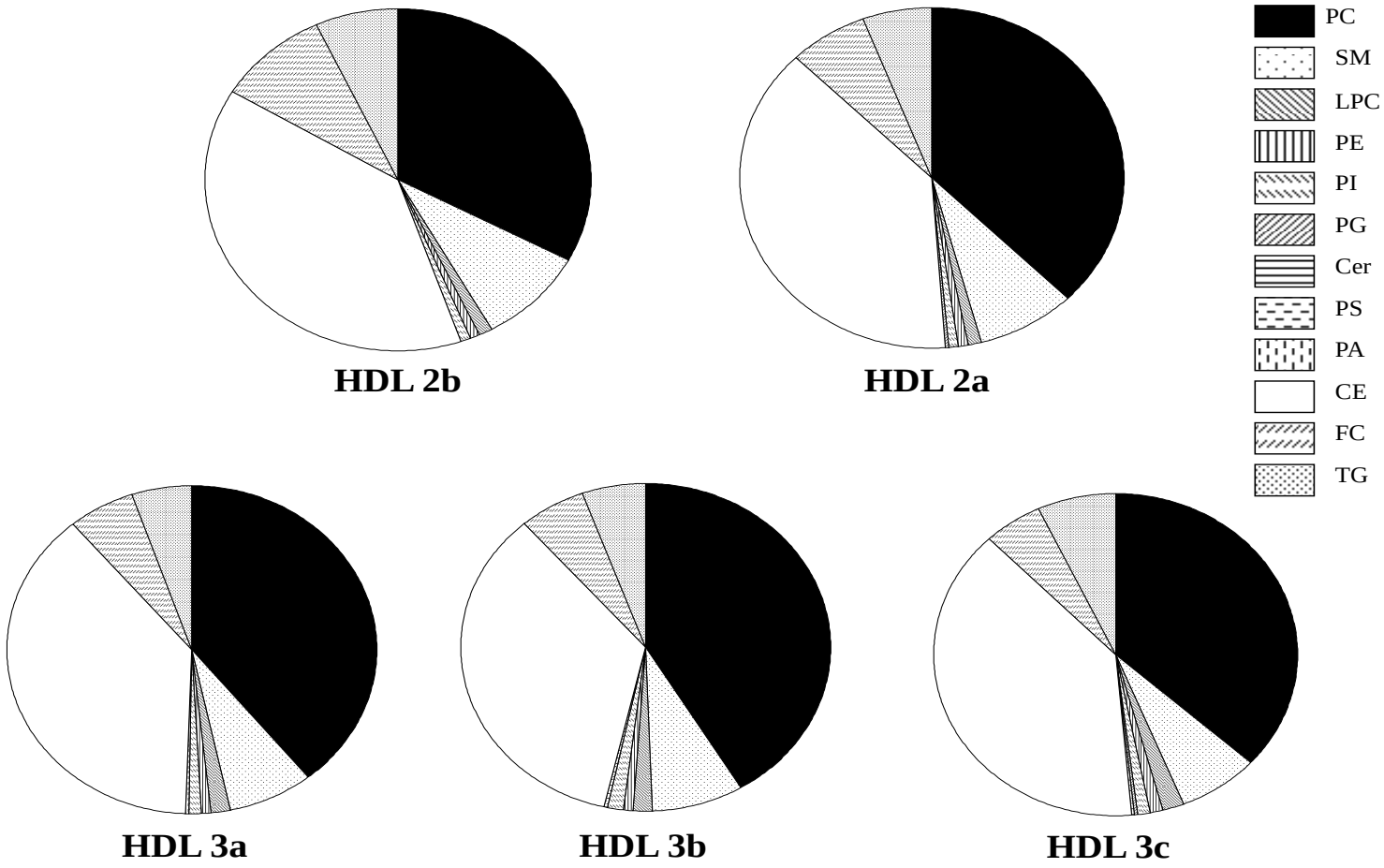

Figure 13 - Lipidome of HDL subpopulations in healthy normolipidemic subjects. HDL contents of PC, SM, LPC, PE, PI, PG, Cer, PS, PA, CE, FC and TG, expressed as wt % of total lipid in each HDL subpopulation, are shown.

3.2.4 Biological activities of HDL particle subpopulations in healthy normolipidemic subjects

3.2.4.1 Cholesterol efflux capacity of HDL subpopulations in THP-1 macrophages

The capacity of individual HDL subpopulations to mediate cellular efflux of free cholesterol was evaluated in macrophage-like human THP-1 cells which efflux cholesterol predominantly via the ABCA1-dependent pathway.(154) On the basis of unit PL mass content, both small, dense HDL3b and 3c particles displayed greater efficacy in removing cellular cholesterol as compared to other HDL subpopulations ($p<0.001$; Figure 14, A). Thus, the cholesterol efflux capacity varied from 4.5% for HDL2b to 8.0% for HDL3c. Moreover, HDL3c exhibited higher cholesterol efflux capacity than HDL3b ($p<0.001$).

3.2.4.2 Antioxidative activity of HDL subpopulations

Antioxidative activity of HDL particles was assessed as inhibition of free radical-induced LDL oxidation. Consistent with previous data,(85) the inhibitory effects of small, dense HDL3b and HDL3c on LDL oxidation were superior relative to those of large, light HDL2b on the basis of total mass, with respect to both reduction in LDL oxidation rate in the propagation phase (3.8-fold, $p<0.05$, and 5.2-fold, $p<0.01$, for HDL3b and 3c respectively;

Figure 14, B) and increases in the duration of this phase (7.2-fold, p<0.01, and 8.0-fold, for HDL3b and 3c p<0.01, respectively; Figure 14, C).

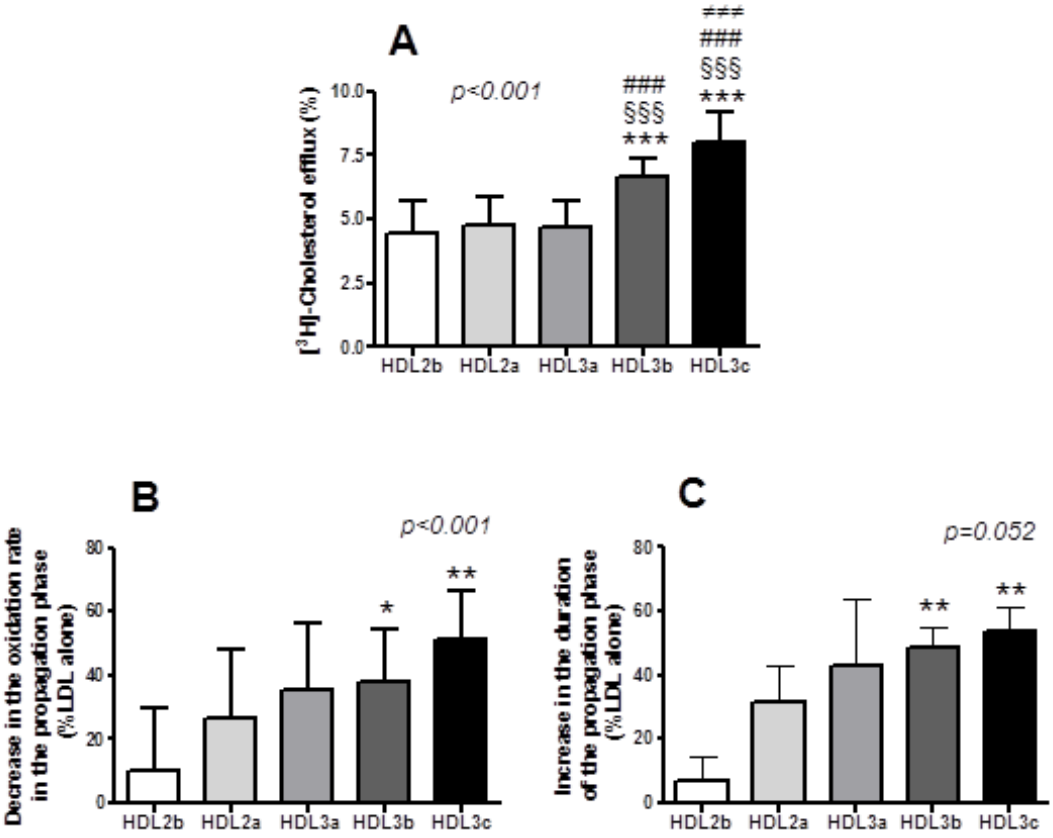

Figure 14 - Biological activities of HDL subpopulations in healthy normolipidemic subjects. Cholesterol efflux capacity in THP-1 cells expressed as % [3H]-cholesterol efflux to HDL subpopulations compared on the basis of unit PL mass content (A). Antioxidative activity of HDL subpopulations compared on the basis of total mass content, towards LDL oxidation, expressed as a decrease in the LDL oxidation rate in the propagation phase (B) and an increase in the duration of this phase (C). *p<0.05, **p<0.01, ***p<0.001 vs. HDL2b; §p<0.05, §§§p<0.001 vs. HDL2a; ###p<0.001, #p<0.05 vs. HDL3a; ≠≠≠p<0.001, ≠p<0.05 vs. HDL3b. In the upper part, p-values for the trend between HDL subpopulations are shown.

3.2.5 Interrelationships between components of the HDL lipidome and HDL functionality

3.2.5.1 Correlations of components within the lipidome of HDL particle subpopulations

Correlational analysis revealed multiple significant positive correlations within the HDL lipidome (Figure 15, A). Thus, HDL contents of SM, CE and FC, which were enriched in large, light particles, were intercorrelated; in addition, SM and CE were positively correlated with Cer, which was itself predominantly associated with large HDL.

Furthermore, intercorrelations were observed between HDL contents of negatively charged PI, PS and PA, which were enriched in small, dense particles (Figure 15, A). In addition, PS and PA were intercorrelated with LPC, which was also enriched in small, dense HDL. Next, PA was positively, albeit weaker, correlated with PE and PG, while LPC was positively associated with PE, PG and Cer. Finally, the contents of PG and Cer were positively correlated with that of TG. A series of negative correlations between lipid subclasses were largely consistent with the positive associations described above (Figure 15, A). In particular, HDL PC was negatively associated with SM, LPC, PE and Cer.

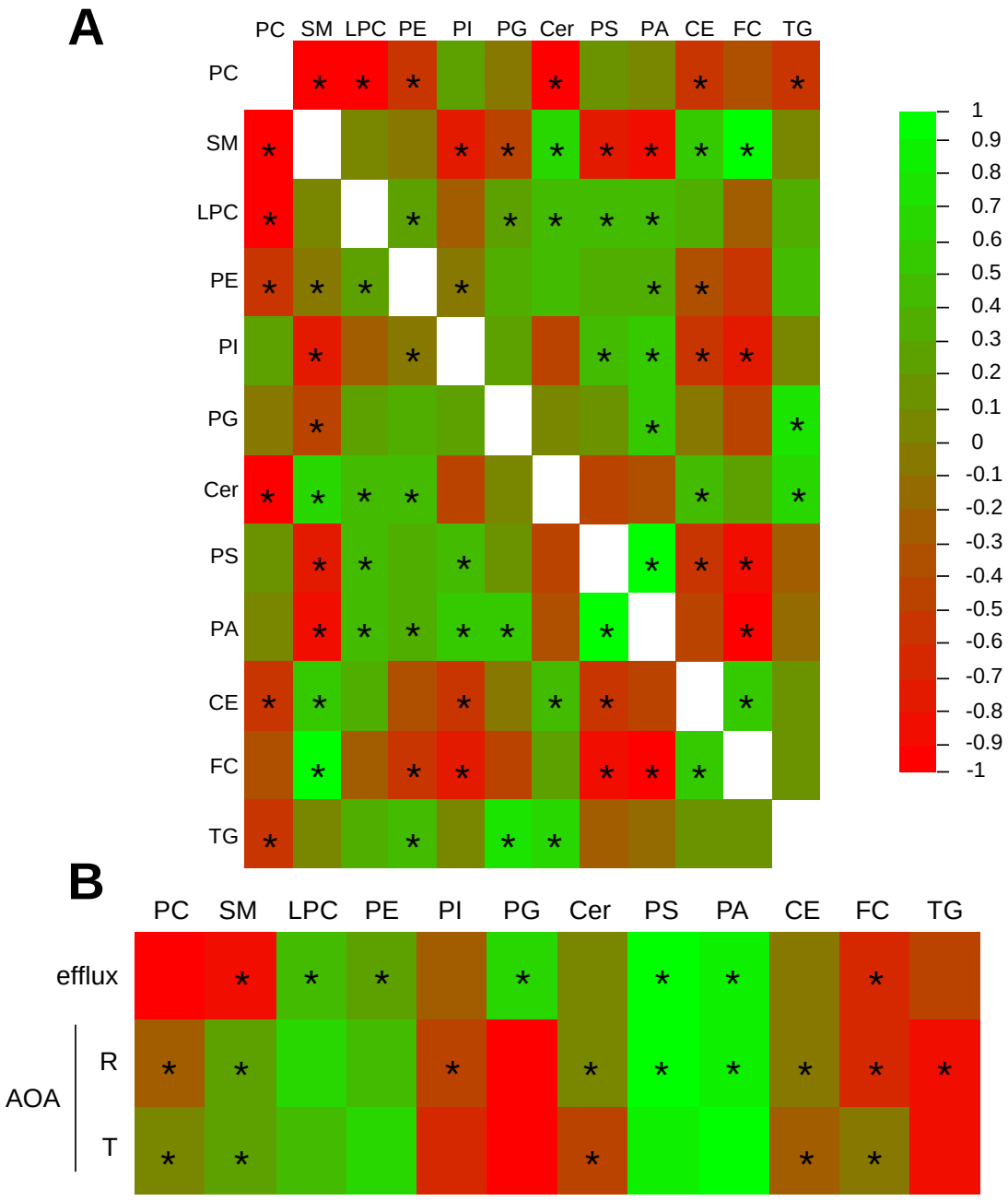

Figure 15 - Correlations between the content of lipid classes and subclasses in HDL subpopulations from healthy normolipidemic subjects (A). Correlations between the content of lipid classes and subclasses vs. biological activities of HDL subpopulations in healthy normolipidemic subjects (B). Positive and negative correlations are labelled with green and red, respectively; r-values are colour-coded as indicated in the scale. Significant correlations ($p<0.05$) are labelled with asterisks. Efflux, cholesterol efflux capacity in THP-1 cells; AOA, antioxidative activity towards LDL calculated on the basis of LDL oxidation rate in the propagation phase (R) or duration of this phase (T).

3.2.5.2 Correlations of lipidome components with biological activities in HDL particle subpopulations

All biological activities of HDL subpopulations evaluated were strongly intercorrelated, reflecting their preferential association with small, dense, protein-rich HDL3. Such biological activities were significantly correlated with the components of the HDL phosphosphingolipidome (Figure 15, B). Indeed, HDL capacity to efflux cellular cholesterol from THP-1 cells was positively associated with the contents of several lipid subclasses, including LPC, PE, PG, PS and PA, and negatively associated with those of SM and FC. Metrics of antioxidative activity of HDL towards LDL were positively correlated with the contents of SM, PS and PA, and negatively correlated with those of PC, PI, Cer, CE, FC and TG. Strikingly, HDL content of negatively charged PS revealed essentially positive correlations with all metrics of HDL functionality, reflecting enrichment of PS in small, dense HDL3.

3.3 Chapter III) HDL composition and function in patients with acute MI

3.3.1 Clinical and biological parameters

Ten Brazilian male volunteers, healthy, non-smoking, normolipidemic, age-matched subjects were recruited as controls. All STEMI patients (males recruited within 24h of presentation in the Emergency Room; n=16; mean age, 54 years) presented elevated levels of the key biomarkers for myocardial necrosis, ie. CKMB (222 [2.3-500] ng/ml) and troponin (56 [0.2-100] ng/ml). No significant differences were observed in body mass index (BMI) and blood pressure between STEMI patients and male, healthy, non-smoking, normolipidemic, age-matched controls (n=10; Table 5). The STEMI group included both hypertensive (53%) and diabetic (19%) individuals; 21% of patients had a history of previous MI. Patients and controls were non-smokers.

The STEMI group displayed elevated plasma concentrations of fasting glucose ($p<0.01$) and creatinine ($p<0.05$). Furthermore, STEMI patients displayed markedly increased plasma levels of hsCRP (10.0-fold; $p<0.001$), SAA (19.3-fold; $p<0.001$) and interleukin-6 (IL-6; 13.6-fold; $p<0.001$) paralleled by elevated leucocyte count (3.2-fold; $p<0.01$; Table 5), clearly documenting an acute systemic inflammatory state. Three patients presented with a highly elevated inflammatory response as documented by individual levels of hsCRP >20 mg/l, SAA >800 mg/l and IL-6 >30 ng/l. Exclusion of these patients from the study population did not impact our major conclusions regarding alterations in the HDL lipidome and functionality in acute MI (see below); therefore, these patients were included in all analyses.

Plasma lipid profiles of STEMI patients were characteristic of atherogenic dyslipidemia, with subnormal HDL-C (-31%, $p<0.001$) and apoA-I (-17%, $p<0.001$) levels and

elevated TG (+73%, $p<0.01$) and apoC-III (+52%, $p<0.01$) concentrations relative to controls (Table 5). Consistent with the presence of moderate hypertriglyceridemia, endogenous plasma CE transfer protein (CETP) activity was significantly increased (+35%, $p<0.01$) in STEMI patients vs. controls (Figure 16). By contrast, no differences in total cholesterol, LDL-C, non-HDL-C, apoB100 and PL concentrations were observed between the groups.

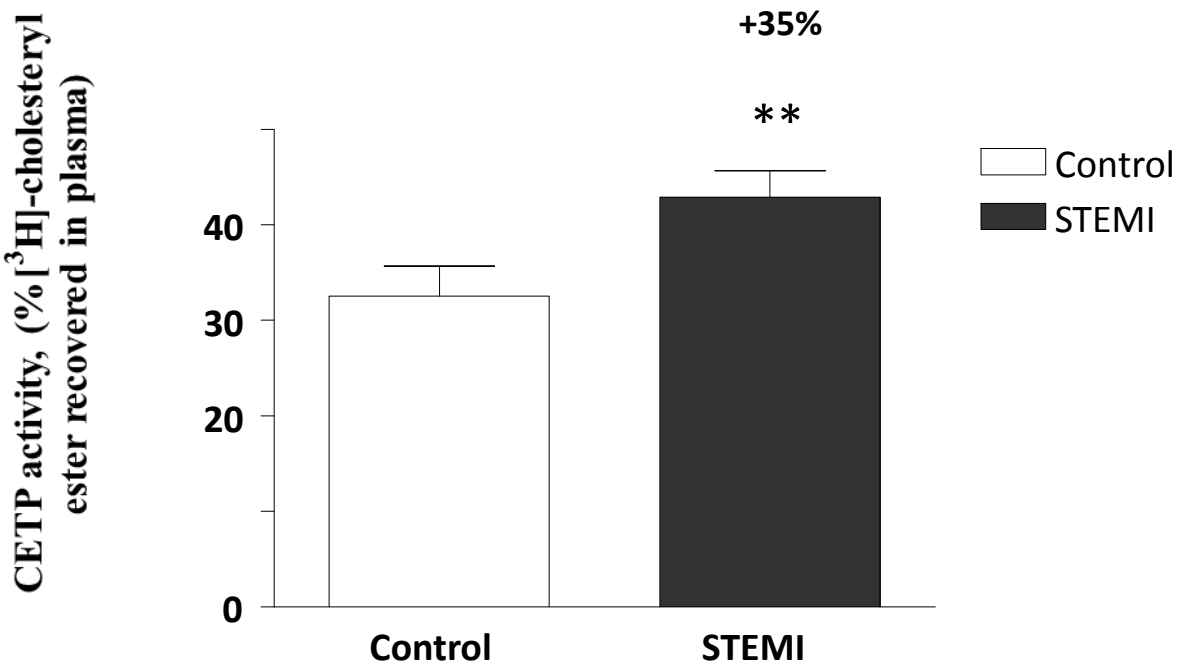

Figure 16 – Plasma CETP activity in STEMI patients and control subjects. [3H]-Cholesteryl ester-labeled HDL was incubated with unlabeled plasma for 3h and CETP activity was determined as radioactivity recovered in plasma. The number denotes % increase in CETP activity in the STEMI vs control group; ** $p < 0.01$ vs. controls.

3.3.2 Plasma levels and chemical composition of HDL particle subpopulations

Plasma concentrations of HDL subpopulations did not differ between STEMI patients and the control group, although levels of CE-rich HDL2b tended to be lower in the MI group (Table 6). By contrast, neutral lipids of the hydrophobic HDL core were markedly altered in STEMI patients. Indeed, CE content was significantly depleted in all STEMI HDL subpopulations (up to -23%, $p<0.01$) vs. control group (Table 6; except HDL3b). Concomitantly, STEMI HDLs were enriched in TG (up to +50%, $p<0.05$, in HDL2b). As a result, the CE/TG ratio was significantly lower in HDL2b (3.7±2.4 vs. 6.0±2.7, $p<0.05$) and tended to be lower in other HDL subpopulations (HDL2a, 5.2±3.0 vs. 7.4±3.2, $p=0.14$; HDL3a, 5.7±2.4 vs. 7.5±2.8, $p=0.25$; HDL3b, 5.5±2.7 vs. 7.2±2.8, $p=0.21$; HDL3c, 4.3±2.2 vs. 6.3±2.8, $p=0.08$) in STEMI patients as compared to controls, consistent with enhanced CETP-mediated transfer of TG to HDL. In contrast, total polar lipids of the HDL surface monolayer were less affected by STEMI. Indeed, only small increments in wt% PL content in HDL2a and HDL3a (up to +17%, $p<0.01$; Table 6) were found in STEMI patients relative to controls.

Plasma concentrations of total HDL were unchanged in STEMI patients vs. controls, while CE were depleted (-23%, $p<0.001$) and PL enriched (+15%, $p<0.01$; Table 6), consistent with the observations made in HDL subpopulations.

3.3.3 Protein composition of HDL subpopulations

As compared to controls, plasma levels of HDL apoA-I tended to be diminished in HDL subpopulations (-47%, p=0.06, in HDL2b) in STEMI (Figure 17, A); levels of apoA-II were unaffected. Furthermore, the content of apoA-I, when expressed either as % of total HDL protein, (Figure 17, B) or relative to total HDL lipid and protein mass, was significantly reduced (up to -23%, $p<0.01$) in HDL2b, HDL3b and HDL3c subpopulations from STEMI patients vs. their counterparts from controls. By contrast, no differences in the proportion of total protein (Table 6) and of apoA-II in HDL subpopulations were found between the groups.

Absolute levels of HDL-associated SAA were significantly elevated in HDL2a, 3a, 3b and 3c subpopulations (up to +11.8-fold, $p<0.01$; Figure 17, C) in the STEMI group. HDL enrichment in SAA was equally evident in STEMI patients when expressed as % of apoA-I (Figure 17, D); such enrichment increased with increase in density from HDL2 to HDL3 subpopulations.

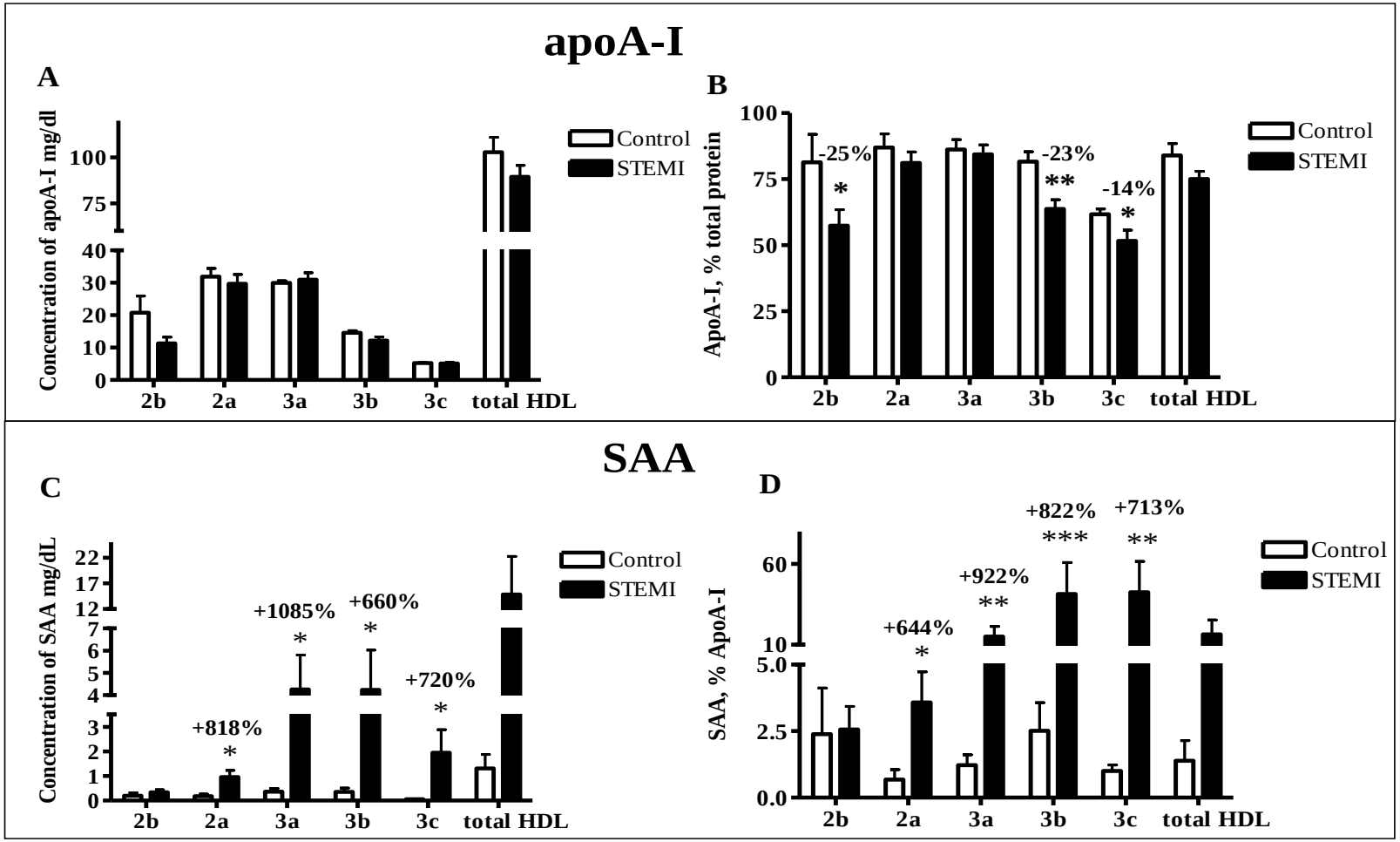

Figure 17 - Levels (A, C) and content (B, D) of apoA-I (A, B) and SAA (C, D) in HDL subpopulations and in total HDL, expressed as mg/dL (A, C), % of total protein (B) and % apoA-I (D), in STEMI patients and control subjects; ** p<0.01, * p<0.05 vs. controls.

3.3.4 Lipidome of HDL subpopulations

Nine PL and SL subclasses were quantitatively measured in HDL particles, in the order of decreasing abundance, PC, SM, LPC, PE, PI, PG, Cer, PS and PA. In STEMI HDLs, plasma levels of the most abundant PL subclass, PC, were unchanged (Figure 18). When expressed as % of total PL+SL, a small reduction (-9%; $p<0.05$) in the PC content of STEMI HDL3c was observed relative to control HDL3c (Figure 19). Plasma levels of another key stuctural PL, SM, were diminished in the HDL2b subfraction in STEMI (-35%, $p<0.05$; Figure 18). However, when SM was expressed as % of total PL+SL, only a small reduction (up to -11%) was found in STEMI HDL3b, 3c and total HDL (Figure 19).

Inversely, HDL content of LPC, a primary product of PC lipolysis, was elevated in HDL2a, 3b and 3c subpopulations from STEMI patients (up to 3.7-fold, $p<0.01$; Figure 20, A); similarly, the % content of LPC was increased in STEMI HDL2b, 2a, 3b, 3c and in total HDL (up to 3.0-fold vs. corresponding control HDL, $p<0.01$; Fig. 20, B). Absolute levels of PE and PI were unchanged in STEMI HDL (Figure 18); when expressed as % of total PL+SL, an increase in PE of +53% and an increase in PI of +27% was evidenced in total HDL and HDL3a from the STEMI group, respectively, relative to controls (Figure 19).

By contrast, STEMI HDLs tended to display diminished levels of ceramide species relative to controls; these differences reached significance in HDL2b and in total HDL (up to -42%, $p<0.05$; Figure 21, B). When expressed as % of total PL+SL, HDL content of Cer also showed a tendency to be lower in STEMI, with a significant effect observed in HDL3b (Figure 22, B). Phosphatidylserine, a negatively charged minor PL, was specifically depleted in STEMI HDL3b on a % PL basis (Figure 22, B).

Finally, both total HDL and all HDL subpopulations from STEMI patients were enriched in PA (up to 8.3-fold, $p<0.001$; Figure 20, C), a product of PL hydrolysis and a key cellular

signalling molecule. When expressed as % of total PL+SL, the enrichment of PA in STEMI HDL was similarly pronounced and increased with HDL density from 5.0-fold ($p<0.001$) in large HDL2b to 8.4-fold ($p<0.01$) in small HDL3c (Figure 20, D).

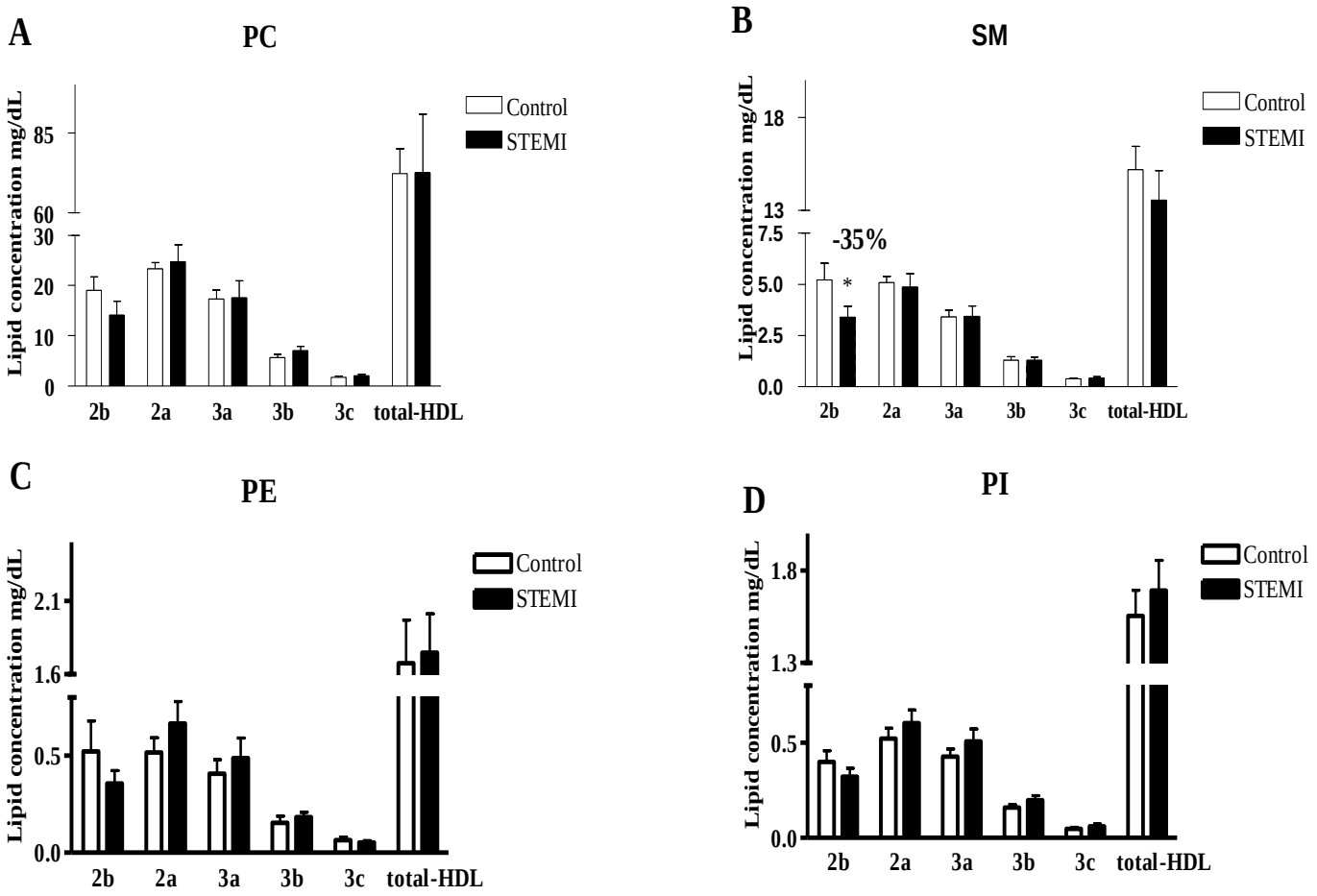

Figure 18 - Levels of major lipids, expressed as mg/dl, in HDL subpopulations and in total HDL from STEMI patients (n=16) and control subjects (n=10). (A) PC; (B) SM; (C) PE; (D) PI; * $p<0.05$ vs. controls.

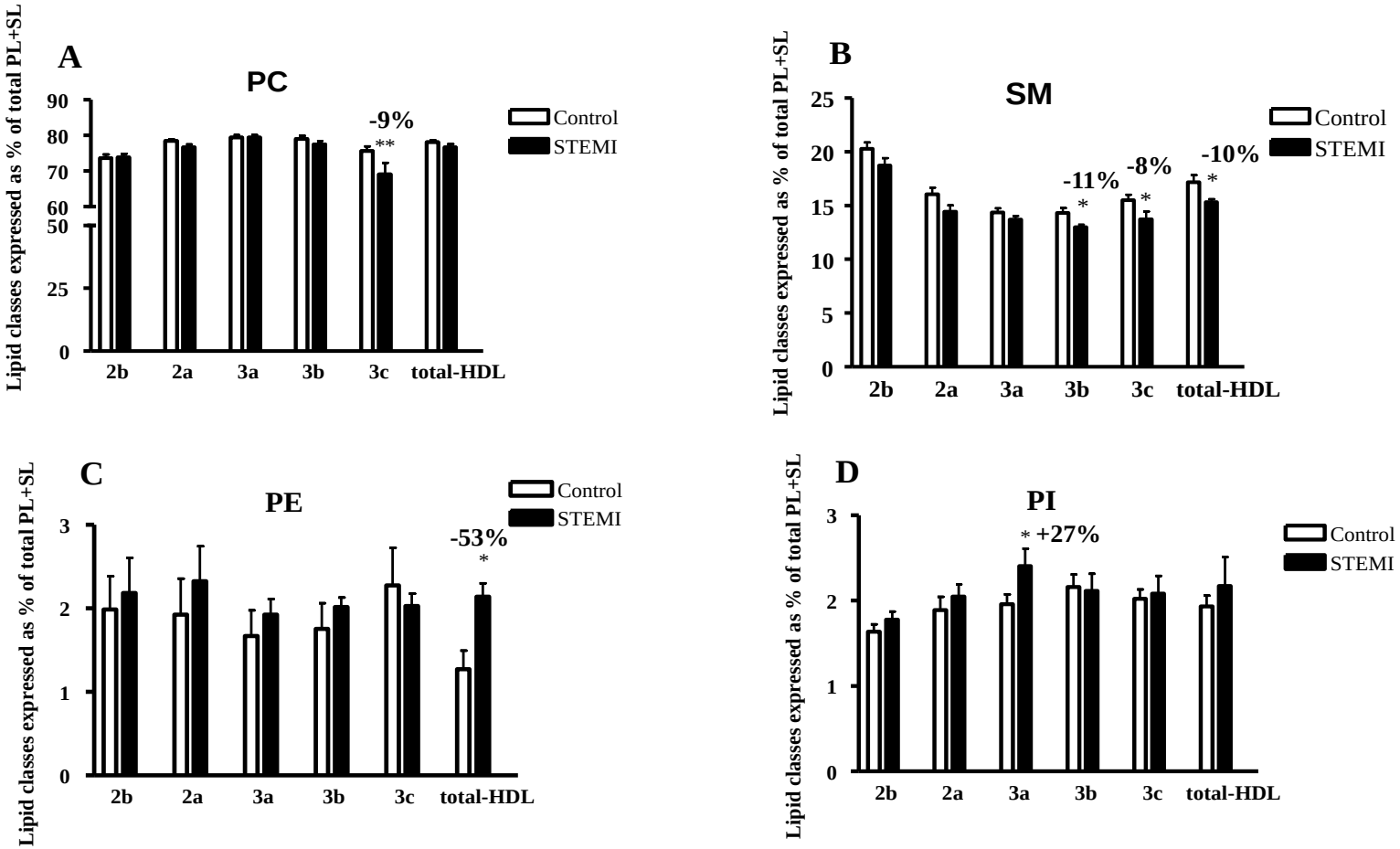

Figure 19 - Contents of major lipids, expressed as % of total PL+SL, in HDL subpopulations and in total HDL from STEMI patients (n=16) and control subjects (n=10). (A) PC; (B) SM; (C) PE; (D) PI; ** p<0.01, * p<0.05 vs. controls.

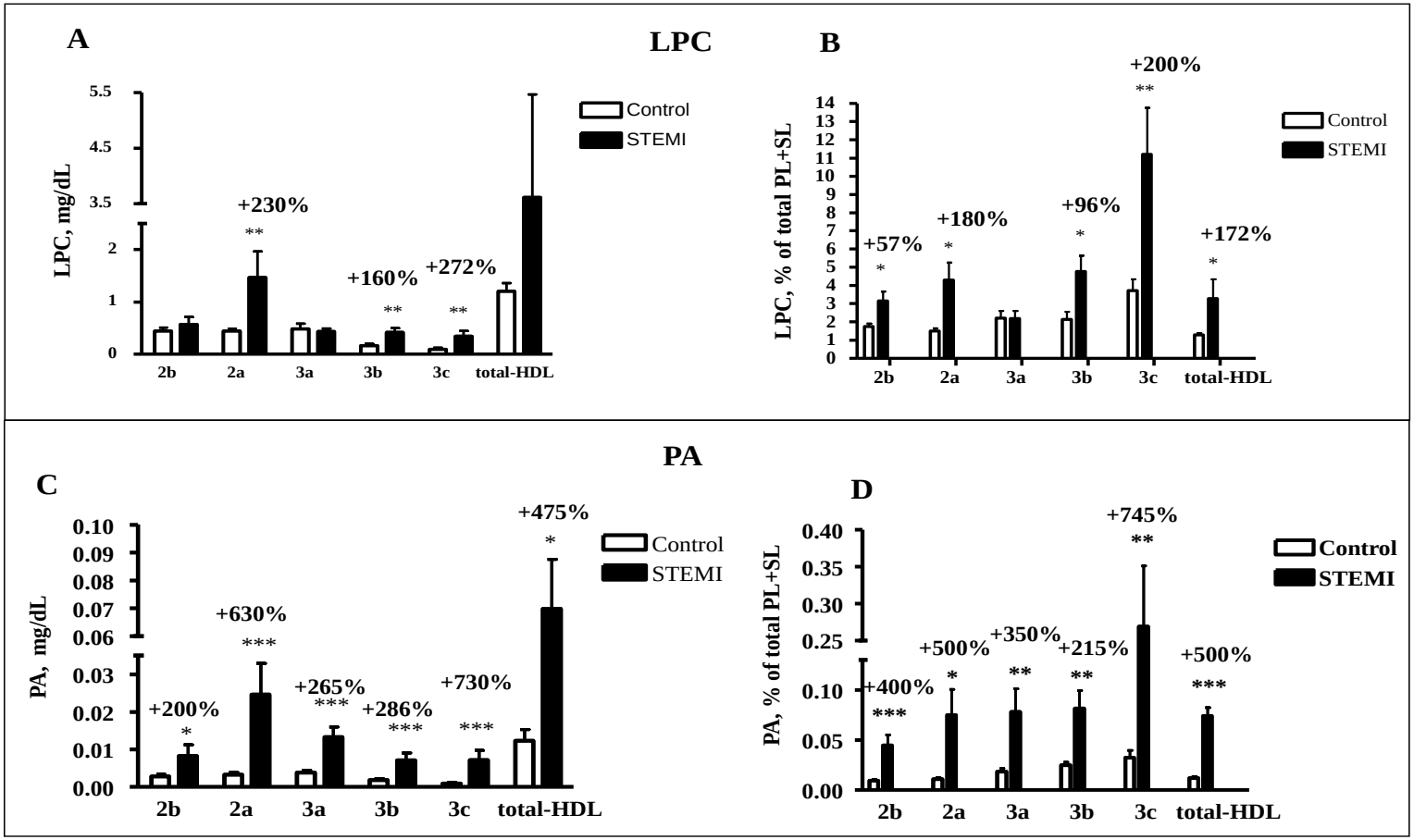

Figure 20 - Levels (A, C) and content (B, D) of LPC (A, B) and PA (C, D) in HDL subpopulations and in total HDL, expressed as mg/dL (A, C) and % of total PL+SL (B, D), in STEMI patients and control subjects; ** p<0.01, * p<0.05 vs. controls. ; *** p<0.001, ** p<0.01, * p<0.05 vs. controls.

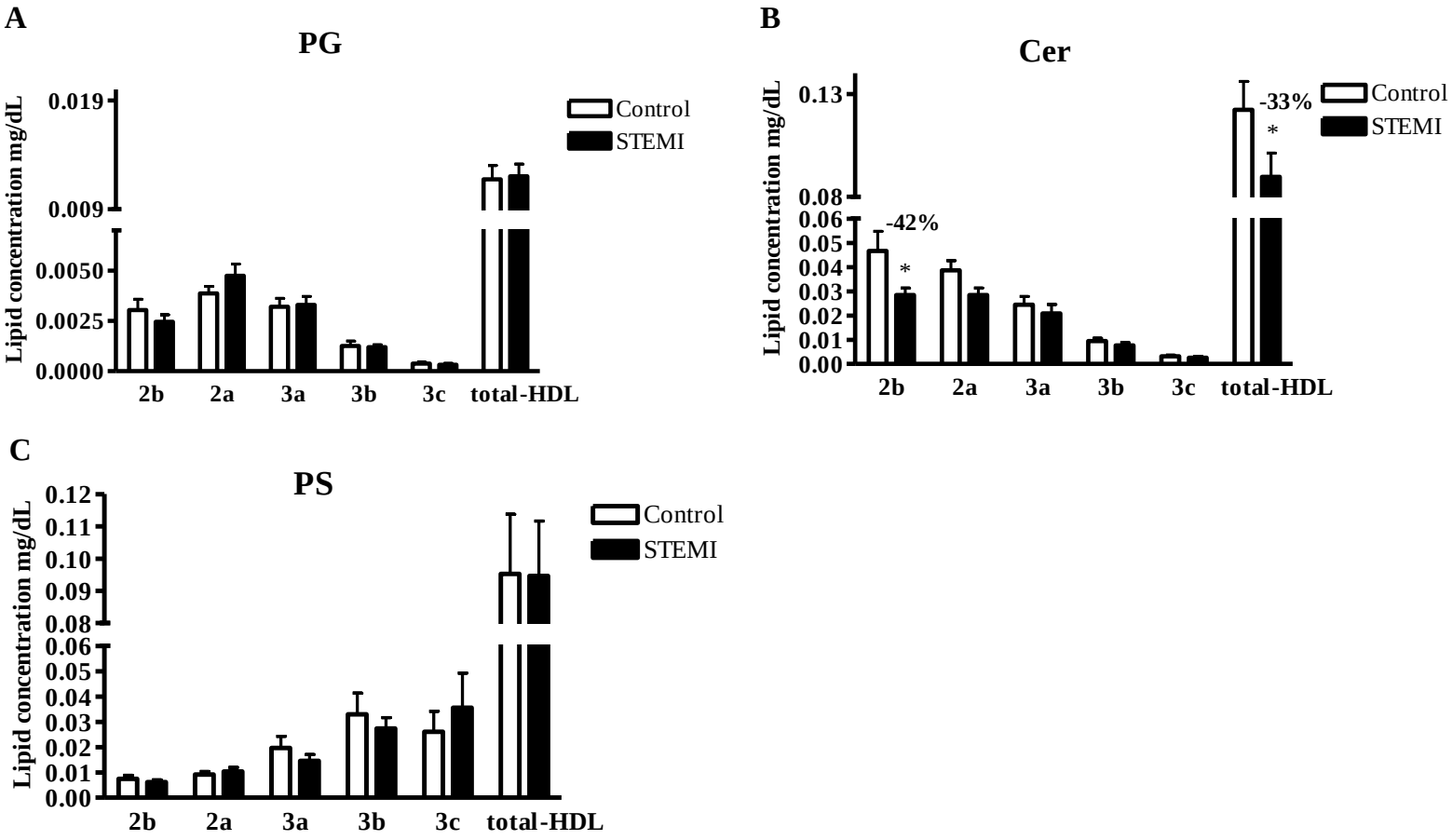

Figure 21- Levels of minor lipids, expressed as mg/dl, in HDL subpopulations and in total HDL from STEMI patients (n=16) and control subjects (n=10). (A) PG; (B) Cer; (C) PS; * $p<0.05$ vs. controls.

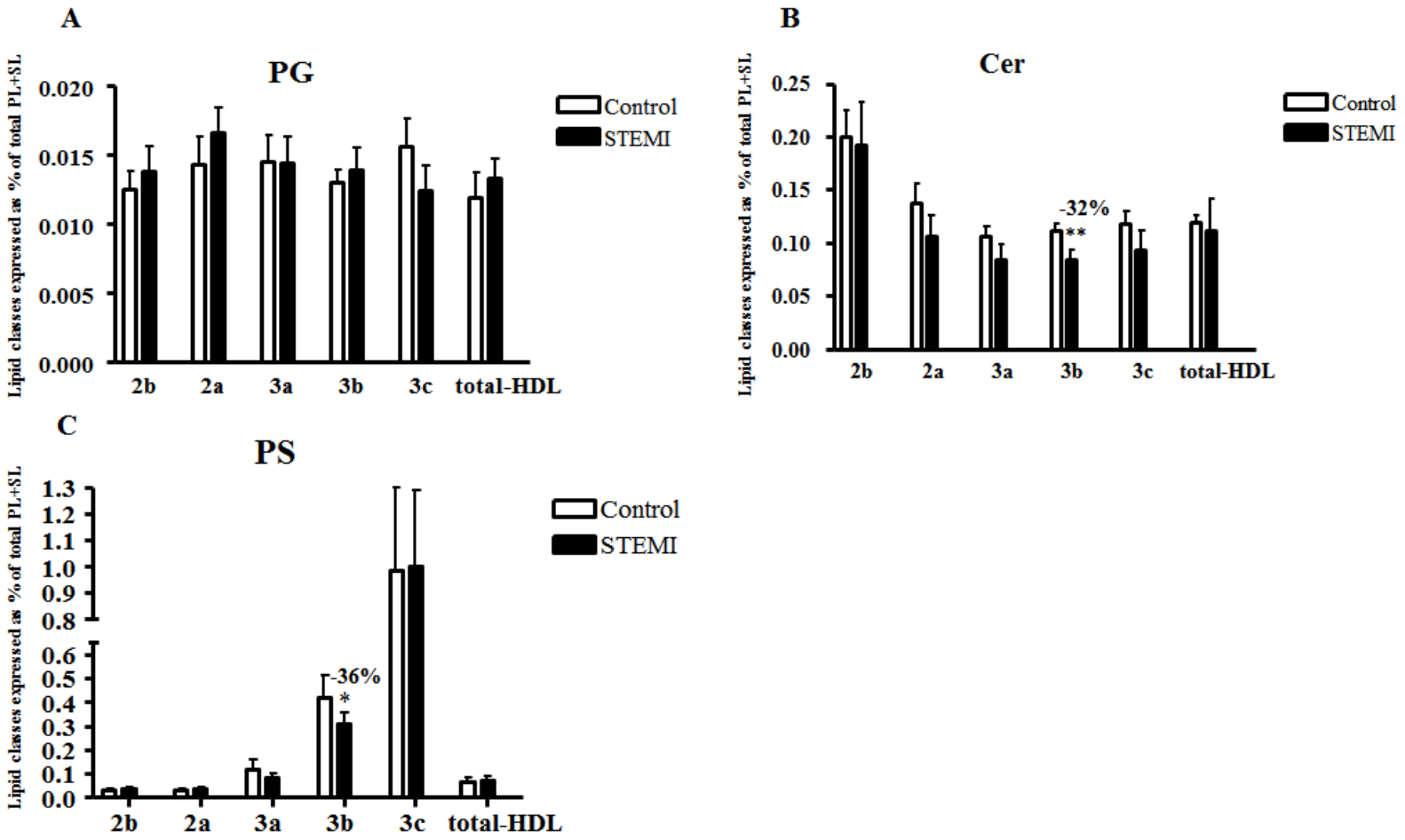

Figure 22- Contents of minor lipids, expressed as % of total PL+SL, in HDL subpopulations and in total HDL from STEMI patients (n=16) and control subjects (n=10). (A) PG; (B) Cer; (C) PS; ** p<0.01, * p<0.05 vs. controls.

3.3.5 Biological activities of HDL particle subpopulations in STEMI patients vs normolipidemic subjects

3.3.5.1 Cholesterol efflux capacity of HDL subpopulations in THP-1 macrophages

The capacity of all STEMI HDL subpopulations (assessed at 10 µg total PL/ml), with the exception of HDL3c, to promote cholesterol efflux from lipid-laden THP-1 macrophages was significantly reduced relative to their counterparts from normolipidemic controls (HDL2b, -23%, $p<0.05$; HDL2a, -30%, $p<0.001$; HDL3a, -25%, $p<0.01$; HDL3b, -32%, $p<0.001$; Figure 23). In parallel, cholesterol efflux capacity of total HDL tended to be reduced in STEMI (Figure 23).

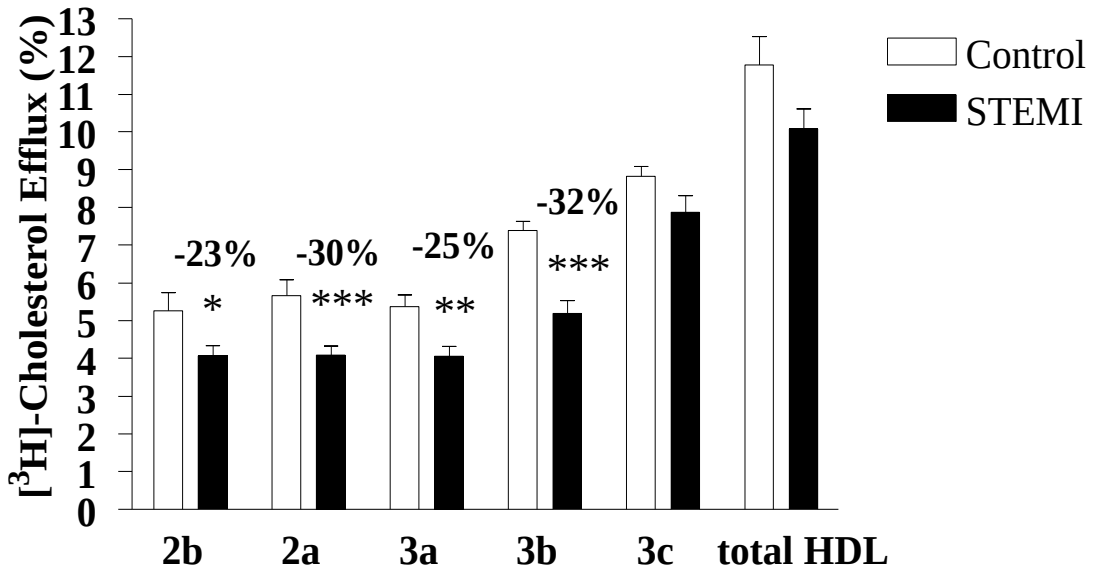

Figure 23 - Cholesterol efflux capacity of HDL subpopulations (10 µg total PL/ml) and of total HDL (30 µg PL/ml) in STEMI patients (n=16) and normolipidemic controls (n=10);*** p<0.001,** p<0.01, *p<0.05 vs. corresponding HDL type from controls.

3.3.5.2 Antioxidative activity of HDL subpopulations

Addition of HDL subfractions from controls or STEMI patients to reference LDL in the presence of AAPH at physiological HDL/LDL ratios of about 2 to 6 mol/mol for HDL3b and 3c, and about 10 to 15 for total HDL, led to significant delay in rates of LDL oxidation (Figure 24). Such antioxidative activity of HDL was markedly impaired in STEMI patients. The inhibitory effects of small, dense HDL3b and 3c, and of total HDL, on LDL oxidation were significantly lower in STEMI patients relative to their counterparts from controls (impairment in the HDL-mediated prolongation of the propagation phase of LDL oxidation of -65%, $p<0.01$, -53%, $p<0.05$, and -68%, $p<0.05$, in HDL3b, HDL3c and total HDL, respectively; Figure 24, B). Similarly, HDL-mediated decreases in the propagation rate of LDL oxidation tended to be impaired in STEMI patients (HDL3b, -44%, $p=0.13$; HDL3c, -30%, $p=0.11$; total HDL, -16%, $p=0.30$; Figure 24, A).

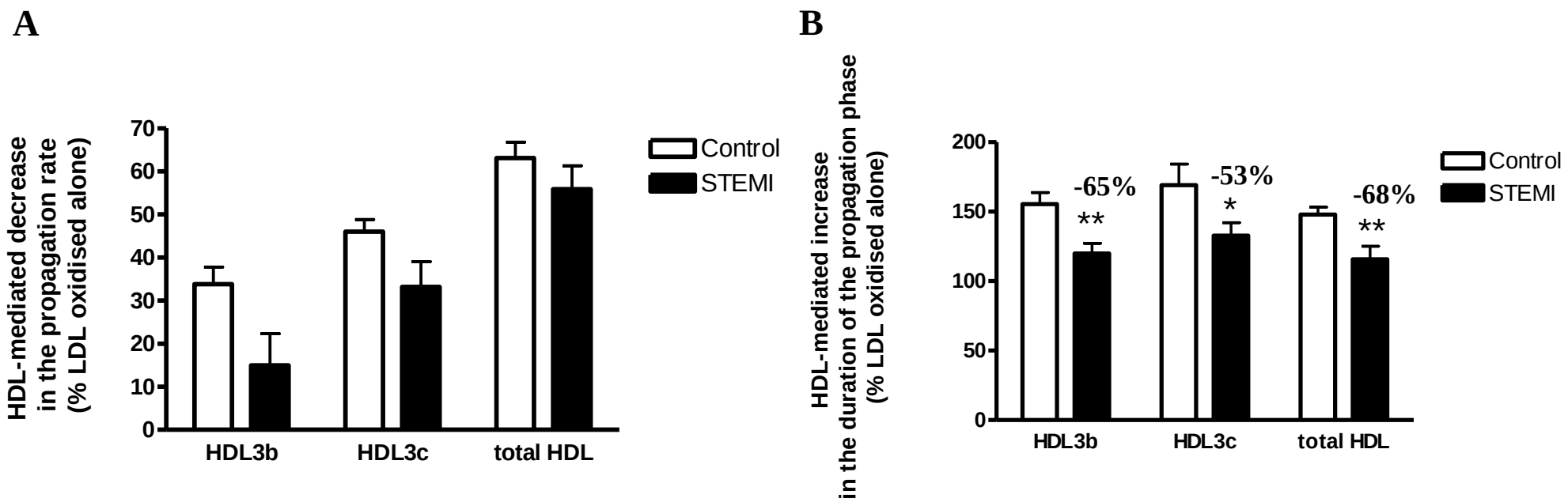

Figure 24 - Influence of small, dense HDL3b (10 mg total mass/dl) and HDL3c (10 mg total mass/dl) subpopulations and of total HDL (30 mg total mass/dl) on AAPH-induced oxidation of reference LDL (LDL, 10 mg TC/dl; AAPH, 1 mM) in STEMI patients (n=16) and normolipidemic controls (n=10). Influence of HDL particles on the oxidation rate in the propagation phase of LDL oxidation (A) and on the duration of this phase (B) are shown. LDL was oxidised in PBS at 37oC and conjugated diene formation was measured by absorbance increment at 234nm; ** $p<0.01$, * $p<0.05$ vs. corresponding HDL type from controls.

3.3.6 Interrelationships between plasma biomarkers, components of the HDL lipidome and proteome, and HDL functionality

Across the whole study population, plasma levels of hsCRP were negatively correlated with biomarkers of HDL metabolism (HDL-C, apoA-I and PL concentrations; Table 7). CETP activity was positively correlated with hsCRP, IL-6, TG and apoB100 levels, but negatively correlated with HDL-C and apoA-I levels.

Globally, levels of PC, SM, PE, PI, PG, PS and Cer, but not LPC and PA, were positively correlated with HDL-C levels in all HDL subpopulations, with the most systematic relationships found for PC, SM, PS and Cer (Table 8). HDL contents of PC, SM, PE, Cer and PS were positively related to plasma PL abundance. By contrast, plasma levels of apoC-III were negatively correlated with HDL content of PC, SM, PI and PS (Table 8), but positive correlated with HDL LPC and PA abundance. Plasma CRP was negatively associated with PC and SM in HDL2b, whereas SAA was systematically and positively correlated with HDL PA.

Strong intercorrelations were observed between cholesterol efflux capacities for all five HDL subpopulations; furthermore, the cholesterol efflux capacity of total HDL was correlated with those of HDL subpopulations (Table 9). Strong intercorrelations were equally observed between antioxidative activities of HDL3b vs. HDL3c vs. total HDL. In addition, positive correlations were found between cholesterol efflux capacity and antioxidative activities of HDL3 and total HDL.

The cholesterol efflux capacity of individual HDL subpopulations was negatively correlated with biomarkers of inflammation (Table 10). In addition, the cholesterol efflux properties of HDL particles were positively associated with HDL-C and PL levels and negatively with CETP activity, whereas those of total HDL were positively associated with HDL-C. Similarly,

antioxidative activity of HDL3b, 3c and total HDL was negatively associated with biomarkers of inflammation (Table 10).

The cholesterol efflux capacity of HDL subpopulations was positively related to their contents of FC, CE, total protein and apoA-I (Table 11). Inversely, HDL contents of TG and SAA were negatively related to the capacity of HDL particles to efflux cellular cholesterol. The antioxidative activity of small, dense HDL3c was negatively correlated with its content of FC, while that of total HDL was positively associated with contents of CE (Table 12). Both apoA-I and apoA-II content in total HDL were positively correlated with antioxidative activity. By contrast, SAA content in both HDL3c and total HDL correlated negatively with their antioxidative activities (Table 12). Remarkably, SAA content displayed stronger correlations with antioxidative activities than with the cholesterol efflux capacities of HDL.

Finally, systematic positive correlations were found between circulating levels of PC, SM, PE, Cer and PS, and cholesterol efflux capacity in both individual HDL subpopulations and total HDL (Table 13). When expressed as % of total PL+SL, such positive correlations persisted only for SM, PE and Cer. In clear contrast, the lipidome of HDL subpopulations did not reveal significant correlations with their respective antioxidative activities.

Section IV:

4. Discussion, Conclusions and Perspectives

4.1 Discussion

Lipidomic analyses obtained by earlier data by several techniques revealed that each lipoprotein class displays specific pattern of major lipid classes. Our structure-function analysis of HDL particles in normolipidemic subjects performed in the first part of our studies, revealed, for the first time, pronounced heterogeneity in the phosphosphingolipidome across human plasma HDL subpopulations. Employing a novel LC/MS/MS approach, the abundance of PC, LPC, PS and PA was shown to be elevated in small, dense relative to large, light HDL, while the inverse occurred for SM and Cer. Furthermore, after correlational analysis of the data, multiple components of the HDL phosphosphingolipidome were strongly correlated with multiple biological activities of HDL, notably cholesterol efflux capacity from THP-1 cells and antioxidative activity towards LDL.

PC is the key structural phospholipid of cell membranes and lipoproteins, and represents the principal plasma phospholipid that accounts for 33-45 wt% of total lipid in HDL.(30) The enrichment of PC in small vs. large HDL reflect predominance of surface components in small vs. large particles.(159) LPC is the product of PC hydrolysis in the LCAT reaction, an important step of HDL maturation from small pre-beta to large alpha-particles and is also a bioactive lipid possessing major surface active properties in cell membranes and lipoproteins.(160) The LPC enrichment in small, dense HDL can be explained by its enrichment of PC and the preferential association of LCAT with this fraction.(12)

PS, a negatively charged minor PL, has been reported to be enriched in small discoid pre-beta HDL relative to large spherical alpha-particles.(161) Mechanistically, this observation may derive from ABCA1-mediated translocation of PS through the plasma membrane to participate in the formation of nascent HDL by apoA-I.(162) PA is another negatively-charged bioactive lipid enriched in small, dense vs. large, light

HDL. PA is a lipid second messenger with multiple binding partners that can be produced in platelets by the action of phospholipase D(163) and is implicated as a cellular signalling molecule in proinflammatory pathways.(164) The preferential association of PA with small, dense HDLs may reflect their enrichment in apoL-I,(11) a lipid-binding protein with high affinity for PA.(165) Intriguingly, PI, another negatively charged PL, tended to be enriched in small, dense HDL3. All three negatively-charged PL subclasses, i.e. PS, PA and PI were therefore predominantly present in HDL3, consistent with the elevated surface electronegativity of this HDL subclass.(166)

By contrast, SM and Cer, two SL subclasses present in HDL at 3-6 and 0.02-0.05 wt% respectively, were enriched in large, light HDL, in parallel to FC. These data are consistent with earlier findings from our laboratory.(12) As Cer are primarily derived from the hydrolysis of SMs, the observed distributions may reflect common metabolic pathways for these lipid subclasses. Apparently, both SM and Cer pools are not in equilibrium across HDL subpopulations, consistent with the slow rate of transfer of SM through the aqueous phase.(167) The low SM content of small HDL may in part result from the depletion of SM in nascent HDL derived from the exofacial leaflet of the plasma membrane as shown in cell culture experiments.(168) These data suggest that marked heterogeneity in the phosphosphingolipidome of individual HDL subpopulations may reflect their distinct and complex metabolic origins.

Our structure-function analysis demonstrated that the heterogeneity in the HDL lipidome was linked to HDL function. The small, dense, protein-rich HDL3 was superior to large, light, lipid-rich HDL in terms of the two biological activities which were evaluated in this study, i.e cholesterol efflux capacity from THP-1 cells and protection of LDL from free-radical-induced oxidation. Such superior functional properties of small vs. large particles were previously reported for cholesterol efflux via ABCA1(65) and for protection of LDL from oxidation.(85) Strong inter-correlation of these activities is

striking, potentially reflecting a distinct proteome(169) and lipidome(12) of small, dense HDL3. Indeed, small, dense HDL3 is not only quantitatively enriched in protein but equally contains a much higher number of distinct, functional proteins as compared to large, light HDL2.(169) Importantly, the HDL content of some of these proteins correlates with HDL function.(169)

In the present study, the both evaluated metrics of HDL functionality exhibited significant correlations with the phosphosphingolipidome of HDL. Several phospho- and sphingolipids can directly impact biological activities of HDL. The capacity of HDL to efflux cellular cholesterol via SR-BI is proportional to the HDL content of PLs, (63) presumably acting via increasing lipid surface of HDL. Not only quantity but equally quality, i.e. molecular composition and physical properties, of HDL PL influence HDL functionality. Thus, both LPC and PI, (62, 170, 171) two PL subclasses regulating intracellular signalling cascades, were shown to enhance cholesterol efflux capacity of HDL via ABCA1. Similarly, PS appears to activate ABCA1-dependent cellular cholesterol efflux as suggested by experiments with apoptotic cells (172) and with PS-phospholipase A_1-overexpressing mice.(173) In addition, PA is a downstream mediator of protein kinase C-stimulated cholesterol efflux from fibroblasts.(164) The potent cholesterol efflux capacity of small, dense HDLs may therefore reflect their elevated content of PC, LPC, PS and PA.

Physical state of PLs represents another important determinant of the ability of HDL to accept cellular cholesterol.(64) Indeed, high SM content decreases both the fluidity of surrounding liquid-crystal lipids and cellular cholesterol efflux to HDL containing such lipids.(174) Similarly, free cholesterol reduces fluidity of surface PLs in the liquid-crystal phase. The low abundance of SM and FC in small, dense HDL may therefore result in elevated fluidity of surface lipids in this subpopulation, potentially enhancing functionality.(12) In a similar fashion, HDL surface lipids can markedly

influence antioxidative effects of HDL, acting in part via modulation of physical properties.(75) Accelerated efflux of cell-derived proinflammatory lipids and LDL-derived prooxidative lipids to more fluid HDL particles may underlie such relationships.

Our structure-function analysis of normolipidemic human HDL particles has therefore identified negatively-charged PS and PA together with LPC, SM and Cer as potential contributors to HDL functionality in a normolipidemic subjects.

Upon completion of the studies normolipidemic human HDL particles, we set out to study a clinical setting, acute myocardial infarction, in which HDL-C levels represent a strong negative biomarker of recurrent cardiovascular events.(18) We focused on the impact of acute myocardial infarction on the potent atheroprotective properties of HDL particles, especially those of small, dense HDL3. Both quality and quantity of these particles are not reflected by routine clinical measurements of HDL-C and could represent a more informative biomarker of CV as compared to circulating HDL-C levels.

Upon comparison with age- and sex-matched normolipidemic control subjects, the STEMI cohort was distinguished by low levels of HDL-C and apoA-I, moderate hypertriglyceridemia associated with both elevated apoC-III concentrations and endogenous CETP activity, hyperglycemia and acute systemic inflammation as indicated by high levels of hsCRP, SAA, IL-6 and leucocytes. On this metabolic background, the capacity of individual STEMI HDL subpopulations to promote cholesterol efflux from human THP-1 macrophages was significantly attenuated relative to their counterparts in controls. Moreover, the capacity of STEMI small, dense HDL3b and 3c to delay free radical-induced LDL oxidation, an integral measure of the anti-oxidative activity of HDL particles, was significantly reduced. Importantly, such functional deficiencies of HDL were observed in assays employing identical concentrations either of HDL PL (cholesterol efflux), or of total HDL mass (antioxidative

activity), thereby revealing defective intrinsic activities of HDL particles independently of their plasma abundance.

It is noteworthy that despite low levels of HDL-C, total plasma mass concentrations of HDL particles were unaffected by STEMI. Low circulating HDL-C levels are therefore clearly an imperfect biomarker of functional deficiency of HDL in STEMI. This finding is consistent with that of Khera et al,(14) in which use of HDL-C as a metric to estimate HDL cellular cholesterol efflux activity in patients with incident coronary disease was inadequate and inferior to cholesterol efflux capacity of apoB-depleted plasma.

The significant negative correlations between plasma CRP, SAA and IL-6 levels and leucocyte count on the one hand and those of HDL-C and apoA-I concentrations on the other attest to acute systemic inflammation as the major driver of alterations in HDL metabolism, and in turn, of molecular composition and function, in STEMI. Mechanistically, these alterations can primarily be mediated by enhanced hepatic production of TG-rich lipoproteins and SAA,(126, 175, 176) reduced production of apoA-I(177) and elevated CETP activity; the latter may be driven primarily by increased plasma levels of TG-rich lipoprotein acceptors for CETP and results in the enrichment of HDL in triglyceride at the expense of CE.(178) Moreover, elevated apoC-III levels exert an inhibitory effect on lipoprotein lipase activity, thereby favouring a hypertriglyceridemic phenotype.(179) In addition, the contents of SAA and TG in STEMI HDL subpopulations were systematically elevated relative to their counterparts from controls, whereas the contents of apoA-I and CE were significantly reduced, consistent with partial replacement of apoA-I by SAA and CE by TG as is known to occur under acute-phase conditions.(105) The importance of this pathway is consistent with positive correlations of CETP activity with CRP, IL-6, TG and apoB100 levels and negative correlations of CETP activity with HDL-C and apoA-I levels. Enrichment of the

HDL core in TG diminishes their structural stability and enhances their propensity to lipid hydrolysis by hepatic lipase, thereby decreasing HDL particle size and accelerating HDL/apoAI catabolism by the kidneys.(180)

The physicochemical modifications induced in STEMI HDL by acute phase inflammation translated into deficient functionality. Indeed, our structure-function analysis revealed that not only apoA-I, but also several lipid classes (FC, CE, SM, PE and Cer) contributed positively to the HDL functions assayed, while enhanced SAA and TG contents exerted negative impact. Remarkably, altered protein composition was associated both with defective cholesterol efflux capacity and antioxidative activity of HDL; by contrast, components of the altered phosphosphingolipidome primarily correlated with defective cholesterol efflux, consistent with earlier reports on the functional roles of HDL proteins and PLs.(75, 181, 182)

ApoA-I plays a key role in both the cholesterol efflux and antioxidative activities of HDL, via its interaction with cell membrane proteins, primarily ABCA1,(181) and via inactivation of lipid hydroperoxides,(75) respectively; equally apoA-I may undergo covalent modification upon interaction with both one- and two-electron oxidants.(183) ApoA-I replacement by SAA primarily impairs the antioxidative/antiinflammatory activity of HDL, whereas cholesterol efflux remains less affected.(136, 137) Mechanistically, these interrelationships result from the absence of antioxidative properties of SAA that on the other hand efficiently effluxes cellular cholesterol.(136, 137) Consistent with the above, SAA abundance was strongly associated with impaired antioxidative activity of HDL3 in this study. In addition, replacement of CE by TG in the lipid core of HDL may alter the conformation of apoA-I, with a potential to further impair atheroprotective properties of HDL.(123, 184). By contrast, SM can beneficially impact cellular cholesterol efflux to HDL as a result of its

specific affinity to cholesterol.(182) It cannot be excluded that such modifications may act synergistically to impair HDL functionality.

Interestingly, we observed strong intercorrelations between cholesterol efflux capacities and antioxidative activities of individual HDL subpopulations, which equally correlated with those of the total HDL fraction. Clearly then, the antiatherogenic actions of all HDL subpopulations were affected by the uniquely inflammatory milieu of STEMI. Importantly, individual HDL subpopulations were differentially impacted by STEMI. Indeed, while apoA-I depletion was predominantly associated with large HDL2b and small HDL3b particles, SAA was most strongly enriched in small HDL3b and 3c. Furthermore, HDL content of 6 out of 9 classes of PL and SL analysed (PC, SM, LPC, Cer, PS, PA) was altered in the small, dense HDL3b subpopulation. Arguably a direct consequence of such pronounced alterations in the proteome and lipidome, the cholesterol efflux activity of STEMI HDL3b was most strongly attenuated as compared to the other HDL subpopulations. In parallel, the capacity of HDL3b to protect LDL from oxidative stress was also markedly decreased, identifying this subpopulation as the preferential target of STEMI-mediated inflammation and metabolic changes across the plasma HDL pool. The preferential association of SAA with the HDL3b subclass(109, 110) calls for further structure-function investigations of these particles in STEMI.

In parallel to modifications of HDL core lipid composition, alterations in HDL metabolism in STEMI resulted in distinct changes in HDL surface lipids. Thus, the phospho- and sphingolipidome of all major STEMI HDL subpopulations (and of total HDL) featured elevated contents of LPC and PA, two lipolytic products of PL metabolism. Interestingly, LPC and PA contents were most markedly elevated in the small, dense HDL3 subclass. This finding may be of special relevance to the biological activities of dense HDL3 in STEMI, as LPC is a bioactive lipid possessing major surface-active properties in cell membranes and lipoproteins,(160) as discussed

above, whereas PA, a negatively-charged bioactive lipid, is implicated as a cellular signalling molecule in proinflammatory pathways.(164) Formation of LPC and PA can result from hydrolysis of polar HDL surface PLs by phospholipases under acute-phase conditions; secretory phospholipase A_2, an acute-phase protein whose levels and activity are markedly elevated in acute coronary syndromes, is implicated in such effects.(185, 186)

In contrast to LPC and PA, the % contents of PC, SM, Cer and PS were predominantly reduced in dense HDL3. Circulating levels of the latter four lipid subclasses in HDLs positively correlated with plasma HDL-C, apoA-I and/or PL concentrations, suggesting that their reductions reflect global alterations of intravascular HDL remodelling. Negative correlations of inflammatory biomarkers with HDL PC and SM and positive correlations with HDL PA support this contention. ApoC-III may equally contribute to depletion of specific PL species in HDL, acting via delayed lipolysis of VLDL-derived TG and diminished flux of VLDL surface fragments to the HDL pool. This pathway is consistent with negative correlations between circulating levels of apoC-III and major HDL PL and SL subclasses.

A major limitation of the both normolipidemic and MI studies is that largely the conclusions are derived from correlational data and should be considered with caution. Direct data, obtained using HDL enriched and/or depleted in the lipids of interest, are needed; such studies are presently ongoing in our laboratory to address this point. Another important challenge to be fully addressed represents handling of the vast amount of data produced by lipidomic analyses and interpretation of the results, such as metabolic pathway investigation, molecular dynamic simulation and statistical analysis.

Another limitation of the MI study lies in the fact that HDL properties were not studied in patients with stable coronary artery disease, in addition to acute MI. The

results obtained in STEMI patients may reflect complex alterations in the circulating levels, proteome and lipidome cargo and functionality of HDL which either precede the coronary event, or which occur during the acute phase, or both. Thus, results of such complex analysis of HDL in others disease states are eagerly awaited.

4.2 Conclusions and perspectives

The present studies have revealed for the first time a marked heterogeneity in the phosphosphingolipidome across human plasma HDL subpopulations in two different metabolic conditions. Furthermore, impact of the heterogeneity of the HDL lipidome on key atheroprotective HDL functions, notably cholesterol efflux capacity from THP-1 cells and antioxidative activity towards LDL, was characterised.

Our major findings are as follows: (i) negatively-charged PS and PA together with LPC, SM and Cer represent potential contributors to HDL functionality in normolipidemic subjects, (ii) complex changes occur in the molecular composition and functionality of HDL particle subpopulations in the acute phase of STEMI, and (iii) alteration in the both HDL lipidome and proteome, notably elevated content of SAA, LPC and PA at the expense of other proteins and lipids, can contribute to functional deficiencies of HDL in cholesterol efflux and antioxidative actions.

These studies, using a complex structure-function analysis of the HDL particles, provide new, original insights into molecular mechanisms implicated in normal and deficient antiatherogenic activities of HDL particles, primarily small, dense HDL3b and 3c, in two different metabolic conditions. They bear the potential, then, to identify clinically relevant, atheroprotective HDL components. Using this original LC-MS/MS approach, HDL lipidomics can equally contribute to the identification of biomarkers of both normal and deficient HDL functionality, which may in turn prove useful as biomarkers of cardiovascular risk superior to HDL-C levels.

Furthermore, HDL-based therapies specifically designed to target beneficial subspecies of circulating HDL pool can be developed. Other approaches to therapeutically correct HDL deficiency observed in acute phase of MI can embrace targeted treatment with specially designed agents, e.g. inhibitors of CETP,(187) agents upregulating synthesis of apoA-I, apoA-II or apoA-IV, apoA-I mimetic peptides(188) or specific peptide inhibitors for

secretory phospholipases A_2.(189) Induction of selective increase in the circulating concentration of small, dense HDL subfractions possessing elevated anti-atherosclerotic activity may thus become a new therapeutic approach in the patients with acute MI.

These studies are highly original and will undoubtedly contribute to expand our knowledge of cardiovascular and metabolic diseases and potentially impact beneficially on cardiovascular morbidity and mortality in the near future.

Table 2 - Individual molecular lipid species assayed in HDL

Name	Retention time, min
SM (d18:1/14:0-d16:1/16:0)*	7.80
SM (d18:2/14:0)*	6.42
SM (d18:0/16:0)*	11.40
SM (d18:1/16:0-d16:1/18:0)*	10.10
SM (d18:2/16:0)*	8.51
SM (d18:1/17:0-d17:1/18:0-d19:1/16:0)*	11.60
SM (d18:1/18:0)*	13.00
SM (d18:2/18:0-d18:1/18:1)*	11.00
SM (d18:1/19:0)*	16.50
SM (d18:1/20:0-d16:1/22:0)*	16.70
SM (d18:2/20:0)*	14.10
SM (d17:1/22:0-d16:1/23:0)*	18.60
SM (d18:1/22:0-d16:1/24:0)*	20.60
SM (d18:2/22:0-d18:1/22:1-d16:1/24:1)*	17.90
SM (d18:1/23:0-d17:1/24:0)*	22.60
SM (d18:2/23:0-d18:1/23:1-d17:1/24:1)*	19.90
SM (d18:1/24:0)*	24.80

SM (d18:2/24:0-d18:1/24:1)*	21.80
SM (d18:2/24:1-d18:1/24:2)*	18.90
SM (d18:2/24:2)*	16.50
SM (d19:1/24:0)*	26.40
SM (d19:1/24:1)*	23.00
Cer(d18:0-22:0)	26.80
Cer(d18:0-24:0)	29.30
Cer(d18:1-14:0)	9.82
Cer(d18:1-16:0)	13.00
Cer(d18:1-18:0)	16.70
Cer(d18:1-19:0)	18.70
Cer(d18:1-20:0)	20.70
Cer(d18:1-22:0)	25.20
Cer(d18:1-23:0)	27.30
Cer(d18:1-24:0)	28.60
Cer(d18:1-24:1)	26.10
Cer(d18:1-25:0)	29.40
Cer(d18:1-26:0)	30.10
Cer(d18:1-26:1)	29.10

Cer(d18:2-14:0)	8.14
Cer(d18:2-16:0)	10.80
Cer(d18:2-18:0)	14.20
Cer(d18:2-20:0)	18.00
Cer(d18:2-21:0)	20.20
Cer(d18:2-22:0)	22.30
Cer(d18:2-23:0)	24.50
Cer(d18:2-24:0)	26.70
Cer(d18:2-24:1)	23.30
Cer(d18:2-24:2)	20.60
LPC(16:0)	2.53
LPC(16:1)	2.27
LPC(18:0)	3.31
LPC(18:1)	2.74
LPC(18:2)	2.38
LPC(20:3)	2.54
LPC(20:4)	2.37
LPC(22:5)	2.48
LPC(22:6)	2.35

LPE(18:0)	3.23
LPE(18:1)	2.60
PA(32:0)	21.20
PA(32:1)	18.30
PA(34:0)	25.00
PA(34:1)	22.30
PA(34:2)	19.60
PA(36:1)	26.60
PA(36:2)	24.00
PA(36:3)	20.90
PA(36:4)	19.80
PA(38:3)	25.60
PA(38:4)	24.30
PA(38:5)	21.60
PA(38:6)	19.80
PC(32:0)	12.90
PC(32:1)	11.10
PC(32:2)	9.36
PC(34:0)	16.50

PC(34:1)	14.00
PC(34:2)	12.00
PC(34:3)	10.30
PC(36:1)	17.70
PC(36:2)	15.40
PC(36:3)	13.20
PC(36:4)	12.20
PC(36:5)	10.60
PC(38:3)	16.90
PC(38:4)	15.60
PC(38:5)	13.20
PC(38:6)	12.00
PC(40:2)	0.00
PC(40:3)	21.00
PC(40:4)	18.60
PC(40:5)	16.60
PC(40:6)	15.50
PC(40:7)	13.10
PC(40:8)	11.30

PE(32:1)	11.10
PE(34:0)	16.60
PE(34:1)	14.30
PE(34:2)	12.20
PE(34:3)	10.50
PE(36:1)	17.90
PE(36:2)	15.60
PE(36:3)	13.30
PE(36:4)	12.35
PE(36:5)	10.70
PE(36:6)	9.41
PE(38:2)	19.40
PE(38:3)	17.10
PE(38:4)	15.90
PE(38:5)	13.30
PE(38:6)	12.30
PE(40:4)	18.90
PE(40:5)	16.80
PE(40:6)	15.80

PE(40:7)	13.40
PE(42:5)	19.90
PE(42:6)	17.50
PE(42:7)	16.80
PG(34:1)	20.50
PG(34:2)	17.80
PG(36:1)	24.80
PG(36:2)	22.40
PG(36:3)	19.70
PG(36:4)	18.50
PG(38:2)	26.30
PG(38:3)	24.20
PG(38:4)	22.80
PG(38:5)	19.70
PG(38:6)	17.10
PI(32:0)	17.40
PI(32:1)	15.10
PI(34:0)	21.30
PI(34:1)	18.70

PI(34:2)	16.30
PI(36:1)	22.90
PI(36:2)	20.20
PI(36:3)	17.70
PI(36:4)	16.60
PI(36:5)	14.40
PI(38:3)	22.00
PI(38:4)	20.60
PI(38:5)	18.00
PI(38:6)	16.50
PI(40:4)	23.90
PI(40:5)	21.60
PI(40:6)	20.40
PS(34:1)	18.50
PS(34:2)	16.10
PS(36:1)	22.60
PS(36:2)	19.90
PS(36:3)	17.40
PS(36:4)	16.40

PS(38:1)	26.90
PS(38:2)	24.20
PS(38:3)	21.60
PS(38:4)	20.30
PS(38:5)	17.70
PS(38:6)	16.20
PS(40:3)	25.80
PS(40:4)	23.60
PS(40:5)	21.30
PS(40:6)	20.10
PS(40:7)	17.60

*SM species were measured as the sum of the isomers displayed in brackets

Table 3 - Internal standards and mass spectrometrical conditions employed for each PL and SL subclass

Lipid Class	No of assayed species	ISTD		Parent Ion	MS Experiment	DP	EP	CE	CXP
		type	amount						
Phosphatidylcholine (PC)	23	PCd9 16:0/16:0	4000	$[M+H]^+$	PIS +184m/z	116	10	45	12
Lysophosphatidylcholine (LPC)	9	PCd9 16:0/16:0	400	$[M+H]^+$	PIS +184m/z	126	10	37	12
Phosphatidylethanolamine (PE)	23	PE 12:0/13:0	80	$[M+H]^+$	NL 141m/z	114	10	44	15
Lysophosphatidylethanolamine (LPE)	2	PE 12:0/13:0	80	$[M+H]^+$	NL 141m/z	114	10	44	15
Phosphatidylinositol (PI)	17	PI 12:0/13:0	100	$[M-H]^-$	PIS -241m/z	-175	-10	-56	-15
Phosphatidylserine (PS)	18	PS 12:0/13:0	40	$[M+H]^+$	NL 185m/z	111	10	29	20
Phosphatidylglycerol (PG)	11	PG 12:0/13:0	20	$[M+NH_4]^+$	NL 189m/z	66	10	25	10
Phosphatidic acid (PA)	13	PA 12:0/13:0	80	$[M+NH_4]^+$	NL 115m/z	96	10	21	22

Sphingomyelin (SM)									
• Sphinganine base (d18:0)	1								
• Sphingosine base (d18:1)	14	PCd9 16:0/16:0	4000	$[M+H]^+$	PIS +184m/z	139	10	39	12
• Other bases: (d16:1), (d17:1),(d18:2), (d19:1)	13								
Ceramide (Cer)									
• Sphinganine base (d18:0)	2				PIS +266m/z				
		Cer d18:1/17:0	20	$[M+H]^+$		70	10	40	20
• Sphingosine base (d18:1)	12				PIS +264m/z				
• Other base (d18:2)	10				PIS +262m/z				

ISTD: internal standard, MS: mass spectrometry, PIS: product ion scan, NL: neutral loss, DP: declustering potential, EP: entrance potential, CE: collision energy, CXP: cell exit potential

Table 4 - Total mass (mg/dl) and % chemical composition of lipids and protein (wt/wt) of HDL subfractions from normolipidemic controls.

	HDL2b	HDL2a	HDL3a	HDL3b	HDL3c
Total mass (mg/dl)	76.3±28.4	88.0±12.9***	72.8±16.9*§§	32.4±8.0**$^{\S\S\S\#\#\#}$	13.8±3.8***$^{\S\S\S\#\#\#\neq\neq\neq}$
PL	31.6±4.4	29.2±3.8*	27.5±3.0***§	25.1±3.1***$^{\S\S\#\#}$	19.6±2.9***$^{\S\S\S\#\#\#\neq\neq\neq}$
CE	27.0±4.4	22.6±3.2***	20.9±2.9***§§§	16.9±3.2***$^{\S\S\S\#\#\#}$	15.9±3.6***$^{\S\S\S\#\#\#}$
FC	6.6±0.6	4.0±0.5***	3.0±0.3***§§§	2.7±0.6***§§§	2.1±0.5***$^{\S\S\S\#\#\#\neq\neq}$
TG	4.9±2.1	3.4±1.3***	2.9±1.3***§§	2.7±1.5***§§	2.8±2.0***
Total protein	30.0±4.6	40.8±4.8***	45.6±4.3***§§§	52.6±6.2***$^{\S\S\S\#\#\#}$	59.6±6.6***$^{\S\S\S\#\#\#\neq\neq\neq}$

*p<0.05, **p<0.01, ***p<0.001 vs. HDL2b; §p<0.05, §§p<0.01, §§§p<0.001 vs. HDL2a; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. HDL3a; $^{\neq}$p<0.05, $^{\neq\neq}$p<0.01, $^{\neq\neq\neq}$p<0.001 vs. HDL3b.

Table 5 - Clinical and biological characteristics of STEMI patients (n=16) and control subjects (n=10)

	Controls	STEMI patients
Age, y	54±11	57±9
Current smokers, %	0	0
Hypertension, %	0	53***
Diabetes, %	0	19***
Previous myocardial infarction, %	0	21***
BMI, kg/m^2	24.4±3.1	26.8±2.9
Systolic blood pressure, mm Hg	113±14	121±35
Diastolic blood pressure, mm Hg	72±11	67 ±20
Plasma parameters:		
Fasting glucose, mg/dL	90 (78-106)	120 (88-377)**
Creatinine, mg/dL	0.90 (0.70-1.10)	1.10 (0.85-1.44)*
hsCRP, mg/L	0.6 (0.2-4.5)	6.0 (0.6-47.6)***
SAA, mg/L	4.3 (2.3-61.0)	83.0 (4.7-1093.0)***
IL-6, ng/L	0.74 (0.39-1.97)	10.1 (1.56-78.9)***
Lecocytes count, $10^3 cells/mm^3$	13.0±4	4±0.4**
Total cholesterol, mg/dL	186±23	182±50

LDL-C, mg/dL	117±25	115±41
HDL-C, mg/dL	52±11	36±11***
Non-HDL-C, mg/dL	134±28	146±54
Triglycerides, mg/dL	87±28	152±91*
PLs, mg/dL	191±61	223±29
ApoB100, mg/dL	102±24	103±34
ApoA-I, mg/dL	137±14	113±21***
ApoC-III, mg/dL	8.6±1.7	13.0±6.3**

***p<0.001, **p<0.01, *p<0.05; vs. control subjects.

Table 6 - Total mass (as mg/dl plasma) and weight % lipid and protein composition of HDL subpopulations from STEMI patients and control subjects

	Group	HDL2b	HDL2a	HDL3a	HDL3b	HDL3c	Total HDL
Total mass	Control	81.3±34.1	94.0±12.9	79.7±15.0	35.9±7.3	14.2±2.7	306.1±48.4
	STEMI	62.1±23.0	92.0±24.0	82.6±18.9	38.8±10.3	15.4±5.5	290.9±59.4
CE	Control	28.5±5.0	23.6±3.7	22.2±4.1	18.8±3.2	16.6±2.4	23.6±3.6
	STEMI	22.9±4.6**	18.3±3.5**	17.1±3.2**	16.0±3.7	12.9±3.4**	18.3±3.2***
TG	Control	5.6±2.3	3.8±1.5	3.4±1.3	3.1±1.7	3.4±1.9	4.0±1.6
	STEMI	8.4±3.8*	4.9±2.3	4.3±2.0	4.0±1.8	4.8±2.7	5.2±2.3
PL	Control	31.3±5.1	29.2±4.5	28.1±3.5	26.5±2.6	20.2±2.6	28.0±8.0
	STEMI	33.2±4.6	34.1±2.7***	32.5±3.2**	28.2±2.7	21.0±5.3	32.2±2.6**
FC	Control	6.5±0.7	3.9±±0.5	2.9±0.3	2.5±0.3	2.0±0.4	4.0±0.5

	STEMI	5.9±1.0	3.6±0.5	2.9±0.7	2.8±1.3	2.3±0.8	3.7±0.6
Total protein	Control	28.1±4.6	39.5±5.2	43.5±4.5	49.1±5.6	57.9±4.8	39.5±4.0
	STEMI	29.8±2.3	38.9±2.8	43.7±3.4	49.9±2.6	59.6±5.0	40.0±2.2

*p<0.05, **p<0.01, ***p<0.001 vs. controls;

Table 7 - Correlations between clinical and biological parameters in STEMI patients (n=16) and normolipidemic controls (n=10)

	hsCRP	CETP activity
Age	0.12	0.06
BMI	**0.40**	0.31
Fasting glucose	0.28	-0.05
Creatinine	0.12	0.35
LDL-C	0.24	0.31
HDL-C	**-0.40**	**-0.70**
Triglycerides	0.25	**0.63**
ApoB100	0.24	**0.47**
ApoA-I	**-0.39**	**-0.39**
ApoC-III	-0.07	0.22
Phospholipids	**-0.42**	-0.30
SAA	**0.92**	0.41
hsCRP	1.0	**0.42**
IL-6	**0.81**	**0.41**
Leucocyte count	**0.60**	0.34

Significant correlations ($p<0.05$) are highlighted in red.

Table 8 - Correlations between clinical and biological parameters vs. lipidome of HDL particles in the whole study population

		HDL-C	TG	ApoA-I	ApoC-III	PL	CETP activity	hsCRP	SAA
Lipid class	HDL type								
PC	HDL2b	**0.60**	**-0.56**	0.19	**-0.52**	0.27	**-0.52**	**-0.42**	-0.01
	HDL2a	**0.50**	-0.24	0.18	**-0.43**	**0.45**	-0.12	-0.03	**0.42**
	HDL3a	**0.47**	0.36	0.23	-0.13	**0.67**	-0.05	0.41	0.47
	HDL3b	0.26	0.38	0.31	0.24	0.38	-0.01	0.38	0.36
	HDL3c	0.14	**0.43**	**0.47**	-0.02	0.11	-0.16	0.16	-0.01
	HDLtot	**0.77**	-0.16	**0.63**	**-0.73**	0.61	-0.25	-0.18	0.42
SM	HDL2b	**0.68**	**-0.60**	0.16	**-0.47**	**0.40**	**-0.52**	**-0.45**	-0.03
	HDL2a	**0.45**	-0.37	0.15	-0.32	0.34	-0.35	-0.07	0.30

	HDL3a	**0.39**	0.03	0.20	0.21	**0.42**	-0.38	-0.01	-0.01
	HDL3b	**0.54**	-0.18	0.05	-0.21	0.36	-0.37	-0.14	-0.32
	HDL3c	0.25	0.23	**0.51**	0.21	0.07	-0.25	-0.12	-0.19
	HDLtot	**0.68**	**-0.49**	0.29	-0.32	**0.49**	**-0.57**	-0.27	0.06
LPC	HDL2b	0.42	-0.32	0.04	-0.08	0.29	-0.23	-0.48	-0.49
	HDL2a	-0.19	0.21	-0.20	-0.02	-0.27	0.18	-0.07	0.20
	HDL3a	-0.16	**0.50**	-0.25	0.48	0.07	-0.14	0.14	-0.10
	HDL3b	-0.21	0.23	0.06	**0.58**	-0.15	0.18	-0.10	-0.14
	HDL3c	-0.07	0.40	0.33	**0.44**	0.06	0.13	0.12	0.04
	HDLtot	-0.21	0.54	-0.35	0.48	-0.11	0.47	0.10	0.35
PE	HDL2b	**0.60**	-0.11	0.04	-0.34	**0.40**	-0.22	-0.21	-0.37
	HDL2a	0.30	-0.04	0.06	-0.27	**0.44**	0.26	0.26	0.23

	HDL3a	0.26	0.35	-0.06	0.02	**0.45**	0.15	0.31	0.05
	HDL3b	0.20	0.28	0.09	0.08	**0.45**	0.22	0.09	-0.12
	HDL3c	0.32	0.14	0.12	-0.06	0.33	-0.09	-0.26	**-0.48**
	HDLtot	**0.57**	-0.13	0.04	-0.16	**0.47**	0.02	-0.04	-0.14
PI	HDL2b	**0.57**	-0.39	0.32	**-0.45**	0.31	**-0.55**	-0.24	0.06
	HDL2a	0.23	-0.17	0.21	-0.28	0.27	-0.07	0.28	**0.58**
	HDL3a	0.20	0.27	0.23	0.02	0.36	-0.10	0.35	0.36
	HDL3b	0.44	0.06	**0.64**	-0.22	0.18	0.19	0.15	0.29
	HDL3c	0.12	0.33	**0.71**	-0.15	-0.05	-0.13	0.11	0.02
	HDLtot	**0.55**	-0.22	0.33	-0.48	0.37	**-0.66**	-0.06	0.42
PG	HDL2b	**0.43**	-0.29	0.13	-0.26	0.29	-0.28	-0.28	-0.31
	HDL2a	0.07	-0.04	0.05	0.04	0.12	0.22	0.22	0.16

	HDL3a	0.16	0.39	0.08	0.23	0.31	0.28	0.33	-0.09
	HDL3b	0.46	-0.1	-0.21	-0.14	0.23	-0.25	0.11	-0.27
	HDL3c	0.06	0.33	0.12	-0.04	0.10	0.17	0.11	-0.36
	HDLtot	0.35	0.14	0.05	-0.02	0.2	-0.22	-0.04	0.06
PA	HDL2b	0.16	**-0.45**	-0.24	-0.32	-0.19	-0.13	0.20	**0.41**
	HDL2a	-0.17	0.12	-0.18	**0.45**	-0.14	0.20	-0.16	-0.05
	HDL3a	-0.07	-0.34	-0.34	-0.14	-0.24	0.14	0.41	**0.54**
	HDL3b	-0.01	0.16	-0.02	**0.41**	-0.21	-0.09	0.13	0.06
	HDL3c	0.01	0.28	0.06	0.38	0.23	0.20	0.24	**0.53**
	HDLtot	-0.2	0.09	-0.12	0.06	-0.04	0.34	-0.1	0.28
PS	HDL2b	**0.83**	**-0.54**	**0.59**	**-0.48**	**0.54**	**-0.54**	-0.27	-0.09
	HDL2a	**0.61**	-0.19	**0.42**	-0.31	**0.55**	-0.09	0.18	0.42

	HDL3a	0.26	-0.02	**0.47**	-0.18	0.37	-0.07	-0.02	0.34
	HDL3b	0.32	-005	**0.60**	-0.11	0.26	-0.17	-0.07	-0.08
	HDL3c	0.20	0.32	-0.05	0.14	-0.04	-0.04	0.18	0.07
	HDLtot	0.35	0.34	0.19	0.18	0.1	0.03	0.11	0.14
Ceramide	HDL2b	**0.68**	-0.38	0.08	-0.2	**0.45**	**-0.44**	-0.39	-0.38
	HDL2a	**0.42**	-0.26	0.07	0.07	0.34	-0.29	-0.14	-0.23
	HDL3a	0.27	-0.13	0.10	0.17	0.26	-0.14	-0.04	-0.14
	HDL3b	0.34	0.12	**0.47**	0.09	0.20	-0.32	-0.04	-0.07
	HDL3c	0.26	0.19	-0.09	-0.01	0.36	-0.35	-0.22	**-0.51**
	HDLtot	**0.63**	-0.22	0.12	-0.10	**0.47**	**-0.45**	-0.29	-0.37

Significant correlations ($p<0.05$) are higlighted in red.

Table 9 - Correlations between cholesterol efflux capacity of HDL particles measured in the THP-1 culture cell in the whole study population.

	[3H]-Cholesterol efflux (%) to:					
	HDL2b	HDL2a	HDL3a	HDL3b	HDL3c	total HDL
[3H]-Cholesterol efflux (%) to:						
HDL2b	1	**0.84**	**0.75**	**0.47**	**0.63**	**0.58**
HDL2a		1	**0.85**	**0.65**	**0.61**	**0.63**
HDL3a			1	**0.63**	**0.67**	**0.60**
HDL3b				1	**0.58**	**0.44**
HDL3c					1	**0.58**
total HDL						1

Significant correlations ($p<0.05$) are highlighted in red.

Table 10 - Correlations between cholesterol efflux and antioxidative activity of HDL particles vs. clinical parameters in the whole study population

	AOX of HDL3c		AOX of HDL3b		AOX of total HDL		[3H]-Cholesterol efflux (%) to:					
	Based on R	Based on T	Based on R	Based on T	Based on R	Based on T	HDL2b	HDL2a	HDL3a	HDL3b	HDL3c	total HDL
Fasting glucose	-0.08	0.02	-0.28	-0.23	-0.04	**-0.50**	-0.16	-0.27	-0.17	-0.27	-0.35	-0.17
Leucocyte count	-0.34	-0.28	**-0.40**	**-0.53**	-0.38	**-0.55**	-0.30	**-0.45**	-0.35	**-0.40**	-0.17	-0.05
BMI	-0.27	-0.21	-0.25	-0.33	-0.39	**-0.54**	-0.22	**-0.50**	**-0.40**	**-0.52**	-0.36	**-0.68**
HDL-C	-0.06	-0.08	0.06	0.07	0.08	0.22	0.29	**0.51**	**0.47**	**0.55**	0.23	**0.40**
LDL-C	-0.03	0.14	0.17	0.15	-0.10	0.00	0.20	0.05	-0.09	-0.12	0.02	-0.18

Triglycerides	-0.15	-0.04	-0.13	-0.17	-0.01	-0.23	-0.07	-0.30	-0.36	-0.37	-0.14	-0.31
ApoA-I	0.24	0.09	0.02	-0.13	0.12	0.01	-0.04	0.06	0.05	0.30	0.07	-0.17
ApoC-III	0.10	0.18	-0.02	-0.01	**0.44**	0.14	0.01	-0.19	-0.23	-0.29	-0.30	-0.33
ApoB100	-0.19	-0.02	0.03	0.04	-0.07	-0.03	0.21	0.00	-0.12	-0.29	0.00	-0.11
Phospholipids	-0.09	-0.09	0.32	0.17	0.11	0.12	**0.50**	0.38	0.39	0.29	0.25	0.17
hsCRP	**-0.63**	**-0.54**	**-0.45**	**-0.58**	**-0.59**	**-0.55**	-0.39	-0.37	-0.37	**-0.51**	-0.12	-0.14
IL-6	**-0.55**	**-0.53**	-0.34	**-0.56**	**-0.40**	**-0.50**	-0.24	**-0.43**	-0.37	-0.63	-0.39	-0.31
CETP activity	-0.15	-0.05	-0.12	-0.12	-0.29	-0.26	-0.18	-0.36	**-0.49**	**-0.50**	-0.13	-0.11
SAA	**-0.69**	**-0.62**	-0.35	**-0.63**	**-0.64**	**-0.67**	-0.39	**-0.54**	-0.38	**-0.50**	0.00	-0.08

AOX , antioxidative activity ; R, oxidation rate in the propagation phase; T, duration of the propagation phase. Significant correlations ($p<0.05$) are highlighted in red.

Table 11 - Correlations between total lipid and protein chemical composition vs. cholesterol efflux of HDL subpopulations and of total HDL in the whole study population

		% cholesterol efflux to corresponding HDL subpopulation	% cholesterol efflux to total HDL
% of total PL+SL	HDL type		
FC	HDL2b	**0.77**	0.41
	HDL2a	**0.48**	0.38
	HDL3a	0.43	0.48
	HDL3b	0.05	0.03
	HDL3c	-0.40	-0.27
TG	HDL2b	0.05	-0.21
	HDL2a	-0.23	-0.33
	HDL3a	-0.31	-0.28
	HDL3b	**-0.45**	-0.24
	HDL3c	0.06	-0.24
PT	HDL2b	**0.58**	**0.44**
	HDL2a	**0.41**	**0.52**
	HDL3a	0.21	**0.51**
	HDL3b	-0.06	0.22

	HDL3c	-0.16	-0.12
CE	HDL2b	**0.66**	**0.60**
	HDL2a	**0.54**	**0.60**
	HDL3a	**0.52**	**0.52**
	HDL3b	**0.50**	**0.47**
	HDL3c	**0.47**	0.31
ApoA-I	HDL2b	0.37	0.36
	HDL2a	0.32	0.42
	HDL3a	0.32	**0.46**
	HDL3b	**0.42**	0.30
	HDL3c	-0.01	0.04
ApoA-II	HDL2b	-0.14	0.03
	HDL2a	0.36	0.39
	HDL3a	0.35	0.48
	HDL3b	0.02	0.05
	HDL3c	-0.12	-0.03
SAA	HDL2b	-0.02	-0.17
	HDL2a	-0.22	-0.1

HDL3a	-0.42	-0.25
HDL3b	**-0.56**	-0.18
HDL3c	-0.22	-0.42

Significant correlations ($p<0.05$) are higlighted in red.

Table 12 - Correlations between total lipid and protein chemical composition vs. antioxidative activity of the HDL3c subfraction and of total HDL in the whole study population

	AOX of HDL3c		AOX of total HDL	
	Based on R	Based on T	Based on R	Based on T
FC, wt %	**-0.47**	-0.31	-0.19	-0.18
CE, wt%	0.06	0.11	0.19	**0.52**
ApoA-I, % total protein	0.35	0.30	**0.36**	**0.52**
ApoA-II, % total protein	0.25	0.20	0.45	**0.46**
ApoA-I, , wt%	0.41	0.27	**0.50**	**0.41**
ApoA-II, wt%	0.32	0.43	**0.40**	0.20
SAA, % apoA-I	**-0.78**	**-0.67**	**-0.93**	**-0.65**

AOX , antioxidative activity ; R, oxidation rate in the propagation phase; T, duration of the propagation phase. Significant correlations ($p<0.05$) are highlighted in red.

Table 13 - Correlations between lipidome and cholesterol efflux capacity of HDL particles in the whole study population

		% cholesterol efflux to corresponding HDL subpopulation	% cholesterol efflux to total HDL
Lipid class	HDL type		
PC	HDL2b	**0.56**	0.30
	HDL2a	**0.42**	0.19
	HDL3a	0.40	-0.04
	HDL3b	**0.40**	0.24
	HDL3c	0.20	-0.16
SM	HDL2b	**0.66**	**0.42**
	HDL2a	**0.53**	0.30
	HDL3a	**0.46**	0.30
	HDL3b	**0.47**	0.27
	HDL3c	0.06	0.14
LPC	HDL2b	0.37	0.32
	HDL2a	-0.12	0.25
	HDL3a	**0.69**	**0.59**
	HDL3b	0.36	0.20

	HDL3c	0.03	0.09
PE	HDL2b	**0.70**	**0.72**
	HDL2a	**0.45**	**0.73**
	HDL3a	**0.45**	**0.65**
	HDL3b	0.15	**0.68**
	HDL3c	**0.54**	**0.68**
PI	HDL2b	0.13	-0.03
	HDL2a	-0.05	-0.07
	HDL3a	0.27	0.06
	HDL3b	0.20	-0.25
	HDL3c	0.10	-0.14
PG	HDL2b	**0.70**	**0.59**
	HDL2a	0.31	**0.57**
	HDL3a	0.30	**0.50**
	HDL3b	0.20	**0.54**
	HDL3c	0.33	0.36
PA	HDL2b	-0.31	-0.17
	HDL2a	-0.24	-0.08

	HDL3a	-0.15	0.25
	HDL3b	-0.23	0.23
	HDL3c	0.06	0.19
PS	HDL2b	**0.55**	**0.61**
	HDL2a	**0.48**	**0.58**
	HDL3a	0.29	0.07
	HDL3b	**0.38**	0.73
	HDL3c	0.12	-0.13
Ceramide	HDL2b	**0.75**	**0.59**
	HDL2a	**0.75**	**0.72**
	HDL3a	**0.62**	**0.57**
	HDL3b	0.34	**0.44**
	HDL3c	0.17	**0.49**

Significant correlations ($p<0.05$) are highlighted in red.

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