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# High-Density Lipoprotein Functions: Lessons from the Proteomic Approach

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## Abstract

High density lipoprotein (HDL) is the smallest and densest lipoprotein in the blood circulation. HDL forms a heterogeneous family whose members may differ according to size, density, electrophoretic mobility, composition and form. Although reverse cholesterol transport is the main feature of HDL, various other functions have been described to HDL including anti-oxidant, anti-inflammatory, anti-thrombotic, anti-infection and anti-vaso-regulator roles. In this review, we have highlighted the mutifunctionality of HDL in association with protein composition. In particular, we have reported and criticized the dozen studies that analyzed the proteomics of HDL published in the last decade, which will make it possible in the future to consider the discovery of a protein signature specific to certain physiopathological conditions. This review will address the question whether HDL proteome signature may represent a central biomarker for pathophysiology conditions.

**Keywords:** Apolipoproteins; Lipoproteins; HDL; Cholesterol metabolism; Atherosclerosis; Oxidative stress; Inflammation; Proteomics

## Introduction

High-density-lipoprotein (HDL) is a widely studied complex spherical particle renowned for its anti-atherosclerotic properties [1]. These features are likely the result of many functions fulfilled by HDL, including anti-oxidant [2-5], anti-inflammatory [6,7], anti-thrombotic [8], and vaso-regulatory [9] actions in addition to its established ability to promote reverse cholesterol transport. The multifunctionality of HDL is based on a particularly rich and heterogeneous protein composition. To date, more than a hundred HDL-associated proteins have been identified through proteomic studies [10]. The proteomic approach has made it possible to better understand the physiopathology of HDL functions. In this context, growing evidence indicates that in some pathological situations, especially during acute inflammation or oxidative stress (OxS), HDL may lose its protective properties and become a pro-oxidant and pro-inflammatory particle [11-13].

The aim of this review is to highlight the relationship between protein composition and biological functions of HDL, which will make it possible in the future to consider the discovery of a protein signature specific to certain pathologies. To this end, we will recall the characteristics, composition and functions of HDL before critically discussing the available findings of HDL proteomics.

## Brief Review of Lipoproteins

Plasma lipoproteins are spherical particles able to convey and maintain hydrophobic and amphipathic lipids in the blood circulation. The lipid moieties are thus delivered to the peripheral tissues for energy production, storage, cell membrane homeostasis, and synthesis of steroid hormones or bile salts. They consist of a hydrophobic core composed of esterified cholesterol (EC) and triglycerides (TG), and a hydrophilic shell consisting of phospholipids (PL), free cholesterol (FC) and proteins. These proteins, conventionally named apolipoproteins (Apo), regulate enzymes and receptors, which determines the metabolic fate of lipoproteins. In the bloodstream, lipoproteins undergo profound changes that modify their structure, composition and functions.

The 5 major classes of lipoproteins that are discriminated owing to their physical properties (size and density) and composition are: (1) chylomicrons (CM), the exclusive vehicles of alimentary fat, which are assembled and secreted by the small intestine in the lymph. CM are the largest (800-10,000 Å), the least dense (d=0.93 g/ml) vehicles and the richest in TG (86%). Apo B48, synthesized exclusively by the intestine, is essential for the lipoprotein assembly. Additional Apo A-I and Apo A-IV are produced by the enterocyte, whereas others are transferred by HDL, such as Apo C-II and Apo E. Apo CII enables the activation of lipoprotein lipase (LPL), located on the surface of the endothelium of extra-hepatic vessels, which exhibits TG hydrolase activity. It hydrolyzes TG and the fatty acid (FA) lipolytic products are delivered to tissues for energy production or storage. The CM remnant resulting from this catalysis is captured by the liver following the recognition of Apo E by specific receptors. CMs have a brief half-life in plasma. Their concentration increases significantly after a meal and are almost non-existent after a few hours of fasting; (2) Outside mealtimes, the TG needs of peripheral tissues are ensured by very-low-density lipoprotein (VLDL) released from the liver. They are large (300 to 700 Å), light (d=1.006 g/ml) and also very rich in TG (55%). Like CMs, VLDLs are hydrolyzed by LPL to release FAs and deliver them to peripheral tissues; (3) VLDL residues, called IDL (intermediate-density lipoprotein), can either continue their transformation into low density lipoprotein (LDL) or be recaptured by the liver via Apo E for recycling. During the passage sequentially from VLDL to IDL and then LDL, TG content decreases whereas the proportion of CE and Apo B-100 increases; (4) LDLs, considered to be pro-atherogenic, are heterogeneous in the distribution of their size, density and some of their properties. LDL size is about 220 to 272 Å and LDL density varies between 1.019 and 1.060 g/ml; and (5) HDL is among the most studied type of lipoproteins, as they are well known for their protective anti-atherosclerotic properties [1,11]. Depending on their Apos and lipid composition, each lipoprotein class has a different metabolic outcome

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that may be a pathway of TG and cholesterol influx to tissue for CM, VLDL, IDL and LDL and reverse cholesterol transport for HDL.

## HDL Characteristics

HDL is the smallest and densest lipoprotein in the blood circulation. HDL forms a heterogeneous family whose members may differ according to size, density, electrophoretic mobility, composition and form. HDL appears in the form of spherical particles, composed of a hydrophobic core (for the most part consisting of CE and a small quantity of TG) covered by a monolayer of lipids (predominantly PL and FC) as well as various Apos, the main ones being Apo A-I (70% of total proteins) and Apo A-II (20% of total proteins) (Figure 1). After ultracentrifugation, HDL can be separated into two major sub-fractions based on their density: HDL2 (1.063<d<1.125 g/ml) and HDL3 (1.125<d<1.21 g/ml). HDL can also be separated based on its electrophoretic migration. Most HDL corresponds to the alpha-migrating, spherical HDL population that includes the HDL2 and HDL3 sub-fractions. Pre-β migrating HDL mainly represents nascent discoidal particles composed of Apo A-I associated with a few PL and FC moieties [14]. HDL, with its rich lipid/protein composition, has various functions.

Reverse cholesterol is the main function of HDL (Figure 2). This elimination pathway involves delivering excess cholesterol from peripheral tissues to the liver that represents the only organ capable of metabolizing cholesterol and removing it from the body through biliary excretion.

On the other hand, nascent HDL has a discoidal structure composed of a single layer that folds back on itself, consisting mainly of PLs, but also FC and Apo A-I [14]. The origin of discoidal HDL is mixed: on the one hand, it is secreted by the liver and small intestine, and, on the other hand, it is formed in the vascular space from excess surface material of CMs and VLDLs in response to LPL action [14]. During maturation, HDL first captures Apos C-II, C-III and E and then releases them toward TG-rich lipoproteins to gain Apos A-II and A-IV in exchange. In the bloodstream, discoidal HDL, on contact with peripheral cells, captures FC from cell membranes. This process is facilitated by the ATP-binding cassette transporter A1 transporter that removes excess cholesterol from cells and the bloodstream to transfer

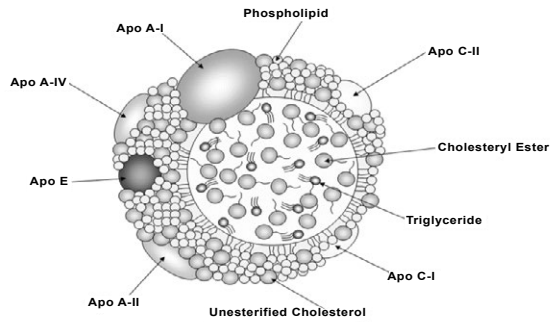


**Figure 1:** Schematic structure of HDL
HDL appears in the form of spherical particles, composed of a hydrophobic center [essentially cholesteryl ester (CE) with a small quantity of triglycerides (TG)]. The HDL is covered by a monolayer of lipids (phospholipids and unesterified cholesterol) and different apolipoproteins (Apos). The 4 main Apos in human HDLs, in order of decreasing abundance, are ApoA-I, ApoA-II, ApoA-IV, and ApoE. The association of HDL with many minor proteins and lipids influence its important functions.

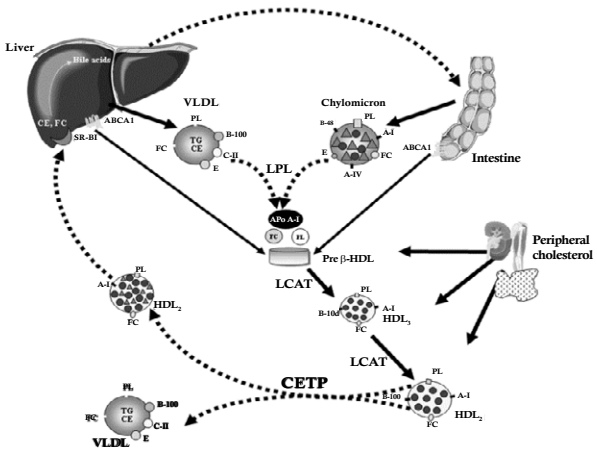


**Figure 2:** HDL biogenesis
ApoA-I is synthesized in the intestine and liver. A proportion of the ApoA-I interacts with ATP-binding cassette transporter A1 (ABCA1) to acquire phospholipids and unesterified cholesterol, generating discoidal HDLs. Free cholesterol (FC) (removed by HDL) is esterified by lecithin-cholesterol acyltransferase (LCAT) and buried in the HDL hydrophobic core, which favors a more spherical form and the formation of $HDL_3$. The persistent removal of FC and LCAT activity results in HDL2 formation. Lipid-free/lipid-poor ApoA-I can also form discoidal complexes with the phospholipids and cholesterol that dissociate from the surface of triglyceride-rich lipoproteins (chylomicrons and VLDLs) that are undergoing lipolysis by lipoprotein lipase (LPL). This pathway has the potential to make a significant contribution to the total HDL pool.

them to HDL [15-17]. As soon as FC is removed by HDL, it is esterified by lecithin-cholesterol acyltransferase (LCAT) and buried in the HDL hydrophobic core, which favors a more spherical form and the formation of $HDL_3$. To esterify cholesterol, LCAT uses acyl groups at position Sn2 on PLs as substrates. HDL metabolism therefore implies a continuous transfer of PLs. They mainly originate from VLDL and IDL particles. A part of the CE is transferred to CMs and VLDLs (in exchange for TGs) in response to cholesteryl ester transfer protein (CETP) activity. Thus, under the combined action of LCAT and CETP, $HDL_3$ changes into larger TG-enriched $HDL_2$. $HDL_2$ then returns to the liver, adheres to the hepatocyte via the scavenger receptor BI, following Apo A-I recognition, transfers cholesterol and undergoes hepatic lipase hydrolysis. Cholesterol returns in this way to the liver and is directly eliminated in the bile or transformed into biliary salts (Figure 2).

## HDL as an Anti-Atherosclerotic Agent

Atherosclerosis or atheroma is defined by the World Health Organization as a variable combination of changes of the intima of arteries, consisting of the focal accumulation of lipids, complex carbohydrates, blood products, fibrous tissue and calcium deposits, in association with medial changes. It is a progressive phenomenon with three successive stages: fatty streaks, simple arterial plaque and then complicated plaque.

During the first step, LDL accumulates in the intima. It is a passive phenomenon that is all the more important due to high LDL concentrations in the bloodstream [18]. This lipid infiltration phase is followed by LDL oxidative modification, of which the first mechanism is the oxidation of a lysine molecule in Apo B [19]. LDL is oxidized mainly through the interaction of its lipid components (PL and CE) with reactive oxygen radicals ($O_2^-$ and $OH^-$) present in small quantities in the bloodstream. Lipid hydroperoxides formed in this way catalyze

the non-enzymatic formation of other oxidized PLs. This step occurs in the intima via lipoxygenase and myeloperoxidase pathways that favor the addition of hyroperoxide lipids to LDL [20]. When the level of oxidized lipids within the LDL is sufficient, LDL becomes oxidized and pro-inflammatory [20]. They then trigger atherosclerotic phenomena by initiating endothelial activation within the intima, an indispensable step at the beginning of the second stage.

The second stage begins with the recruitment of circulating monocytes, their transformation into macrophages and then into foam cells [21]. Endothelial activation leads to the expression at the endothelial surface of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) or E-selectin that will allow the adhesion of circulating monocytes [22] In the bloodstream, the integrins expressed by the monocytes are characterized by weak ligand activity. Their activation by pro-inflammatory molecules makes it possible to increase this affinity [23,24]. After adhesion, circulating monocytes penetrate the sub-endothelial space with the help of monocyte chemotactic protein-1 (MCP-1) where they change into macrophages under the influence of monocyte-colony stimulating factor produced by the activated endothelium. Some of these macrophages change into foam cells [21] by capturing oxidized LDL (oxLDL) via scavenger receptors, whereas others induce a local chronic inflammatory reaction by secreting pro-inflammatory cytokines. The activation of the inflammatory process is initiated by toll-like receptors expressed on the surface of the macrophages, activated by binding with their various ligands such as heat-shock protein 60, lipopolysaccharide and oxLDL. The activation of the macrophages by the toll-like receptor leads to the production of pro-inflammatory cytokines [25], protease like matrix metallo protease, vasoactive molecules such as nitric oxide (NO) and procoagulant factors [12]. Pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α or interleukin (IL)-1, maintain endothelial activation, increasing the adhesion of new circulating monocytes.

The third step is the formation of adult plaque. The accumulation of plaque lipids forms a cluster called a lipid core, which is isolated by a fibro-muscular cap. The cap is composed of smooth muscle cells and proteins from the extracellular matrix (collagen, elastase) that stabilize the plaque.

Then, over the years, the development of the plaque becomes complicated (rupture, thrombosis and/or aneurism), thereby generating clinical manifestations of cardiovascular diseases such as myocardial infarction and cerebrovascular accidents. However, the anti-oxidant, anti-inflammatory and anti-thrombotic properties of the HDL prevent the anti-atherosclerotic lesions.

## Anti-Oxidant Properties of HDL

HDL has the capacity to prevent LDL oxidation, a key step in atherosclerosis initiation and progression [1] A number of mechanisms come into play:

(i) The first is the transfer of LDL-derived oxidized PLs to HDLs. Parthasarathy et al. showed for the first time that LDL oxidation is inhibited in the presence of native HDL [26] They formulated two hypotheses: either HDL-derived PLs compete with LDL-derived PLs or LDL-derived oxidized PLs are transferred from oxLDL to HDLs. The anti-oxidant functions of HDLs are mediated by withdrawal and then transport of these oxidant molecules in animal models of atherosclerosis [19] and in humans [2]. The withdrawal of lipid hydroperoxides from oxLDL is done with the help of Apo A-I, the main HDL protein [20]. The anti-oxidant activity of Apo A-I is independent of the degrading enzymes of the oxidized lipids, particularly paraoxonase (PON) [3].

(ii) The second mechanism includes the destruction of these oxidant molecules. HDL transports enzymes capable of destroying oxidized lipids. In fact, paraoxonase (PON) and platelet-activating factor acetylhydrolase (PAF-AH) associated with HDL allow the hydrolysis of the oxidized PLs of the oxLDL. PON-1 [27] and PON-3 [28] are glycoproteins capable of hydrolyzing oxidized long-chain FAs located at the Sn-2 position of the PLs. In this way, they make HDL a protective agent in LDL peroxidation. Shish et al. [5] demonstrated this by showing that transgenic mouse HDL that does not express these enzymes loses its protective power over LDL oxidation. The second enzyme that protects LDL from oxidation is PAF-AH [29], which is capable of hydrolyzing not only PAF, but also short-chain FAs that esterify PLs at the Sn-2 position of glycerol, and are products formed from lipoprotein oxidation. Oxidized PLs from oxLDL transferred to HDL are in this way inactivated through hydrolysis. Gluthatione phospholipid peroxidase has also been identified as a participant in the anti-oxidant functions of HDL [3]. $HDL_3$ (richer in PON-1), Apo A-I, LCAT and PAF-AH are recognized as being better anti-oxidant particles than $HDL_2$ [30]. HDL, therefore, plays a decisive role in the elimination of pro-oxidant molecules.

## Anti-Inflammatory Properties of HDL

Pro-inflammatory cytokines (IL1, IL6, IL8 and TNFα) favor the expression of adhesion molecules such as VCAM-1 and ICAM-1 by endothelial cells that allow the recruitment of circulating leukocytes and their penetration into the arterial wall [31]. A number of studies have demonstrated the capacity of HDL to reduce the expression of these adhesion molecules *in vitro* [6,7]. Xia et al. show that this phenomenon occurs through the inhibition of sphingosine kinase present in endothelial cells [32]. This enzyme serves to catalyze key steps during which TNFα stimulates the expression of adhesion molecules. Similarly, it has been demonstrated that $HDL_3$, which is richer in Apo A-I and sphingosine1-phosphate or sphingosylphosphorylcholine, more effectively inhibits the expression of adhesion molecules than does $HDL_2$ [33]. Moreover, HDL makes it possible to reduce the monocyte chemotactism of their transmigration by inhibiting the expression of MCP-1 [34] in response to LDL oxidation [4,35]. Two protein components of HDL, PON-1 and PAF-AH, are also implicated in anti-inflammatory phenomena by participating in the inhibition of monocyte transmigration. In the acute inflammatory phase, it has been shown that PON-1 [36] and PAF-AH [37] activity is lower.

## Anti-Thrombotic Properties of HDL

HDL possesses certain endothelial-protective properties that are expressed by three mechanisms: (1) vasoregulatory action by stimulating nitric NO; (2) apoptosis inhibition of endothelial cells; and (3) anticoagulant action by reducing platelet aggregation.

First of all, HDL favors the endothelial production of NO, a powerful vasodilator, by stimulating NO synthase. HDL regulates the intra cellular distribution of NO synthase, which is an enzyme located in the plasma membrane and exists in balance with adjacent membrane lipids. By depleting membrane FC, oxLDL can disrupt this homeostasis and attenuate enzyme function [38,39]. However, HDL ensures the conservation of the local lipid environment of the NO synthase as well as its efficacy. Furthermore, Yuhanna et al. have shown that HDL-Apo A-I binding to the scavenger receptor class B-I receptor causes an intracellular enzyme cascade mediated by kinase pathways that stimulate NO synthase activity [40].

On the other hand, HDL is indirectly capable of reducing endothelial cell apoptosis, thereby slowing down the pathogenesis of atherosclerosis. Certain pro-atherogenic factors stimulate endothelial apoptotic phenomena such as oxLDL or TNFα [41]. The anti-apoptotic action is the result of a limitation in the increase in intercellular calcium usually induced by oxLDL [41], a phenomenon counteracted by HDL. In addition, HDL can curtail the action of TNFα through caspase-3 inhibition [42]. HDL also favors the proliferation and migration of endothelial cells, a mechanism that is essential for neovascularization. Cell proliferation induced by HDL is calcium dependent as well [43], whereas cell migration is secondary to the activation of the kinase pathway [36].

Finally, the anti-coagulant action of HDL is expressed through decreases in thrombin generation and platelet activation. HDL favors the production not only of NO, but also of prostacyclin via the cyclooxygenase pathway [44,45]. NO and prostacyclin act synergistically to inhibit platelet activation and the synthesis of platelet growth factors [46], which is indispensable for the generation of thrombin. Naqvi et al. have shown that platelet aggregation is inversely correlated with circulating HDL levels [47]. In rats, the injection of Apo A-I Milano inhibits platelet aggregation, which suggests the same mechanism *in vivo* [8]. In addition, HDL decreases platelet activation by decreasing the production of thromboxane A2 [48]. By blocking the initial stages of thrombus formation, i.e., platelet activation and aggregation, HDL retards the development of atherosclerotic lesions (Figure 3).

## Anti-Infectious Properties of HDL

HDL is also implicated in many other functions such as the regulation of the complement system or the immune system [3]. Its protective role in the innate immune system has been demonstrated in work done on *trypanosome brucei brucei,* a parasite responsible for sleeping sickness transmitted by the tsé tsé fly in tropical and subtropical Africa [49]. *trypanosome* lytic factor-1 is defined as an assemblage of lipids and a minor sub-fraction of Apos on the surface of HDL, composed of haptoglobin-related protein, Apo L-1 and Apo A-1. Shiflett et al. have shown that these proteins, to be synergistic in cytotoxic phenomena, had to be assembled together on native HDL [50]. Cytotoxic activity requires complex recognition by a receptor on the surface of *trypanosome*, its endocytosis and localization in the lysosome. The acidity present in the latter activates *trypanosome* lytic factor-1, which then activates the process of cell death. All of the anti-atherosclerotic properties of HDL that associate anti-oxidant, anti-inflammatory, anti-thrombotic functions or cholesterol efflux are summarized in Figure 4.

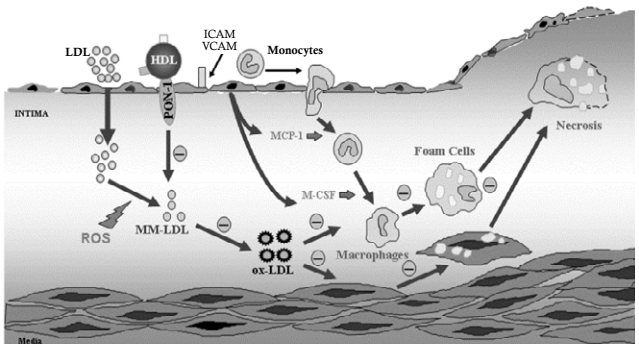


**Figure 3:** HDL Properties
The inflammation occurs in the subendothelial matrix layer of blood vessel walls as LDL particles become oxidized. However, HDL intervenes at the different steps and inhibits the processes leading to atherosclerosis as illustrated by the diagram.

## HDL Lipid and Protein Composition

The multiple facets of HDL action are the result of its particularly rich and heterogeneous lipid and protein composition. Lipidomics studies of HDL have identified more than 200 different lipid molecules [51]. In order of abundance, the four main classes of lipids that make up HDL are: (1) PLs, mainly phosphatidylcholine; (2) sphingolipids: sphingomyelin, ceramide and lysosphingolipid; (3) FC or EC; and finally (4) TGs. Lipids play a key role in the anti-atherogenic functions of HDL. For instance, PLs play an important role in the mechanisms of cholesterol efflux by facilitating membrane lipid fluidity [52]. The latter also allows the transfer of hydroperoxidized lipids from oxLDL to HDL [53]. Lysosphingolipids participate in the anti-inflammatory effect of HDL by reducing the expression of MCP-1 [34]. Finally, S1P mediates anti-thrombotic and vasoprotective effects [9].

Often acting in synergy with lipids, the proteins associated with HDL also seem to have an essential part in the metabolic outcome of HDL [32]. Novel approaches that study the proteome have so far identified more than a hundred proteins associated with HDL [10]. Any modification of this protein composition contributes toward impairing the biological functions of HDL. In particular, it has been shown that inflammation induces many changes in HDL composition [54]. In fact, in the event of OxS generated during the acute inflammatory phase, HDL can be converted into a pro-oxidative and pro-inflammatory molecule, incapable of preventing LDL oxidation and promoting reverse cholesterol transport [11,13]. The inflammatory properties of HDL can be determined by an inflammation score described by Navab [55]. It defines the pro- or anti-inflammatory nature of HDL by measuring with fluorescence techniques the quantity of free radicals produced using 2',7' dichlorofluorescein-diacetate. HDL with an inflammation index>1 is considered to be pro-inflammatory, whereas a score <1 describes it as being anti-inflammatory. Several authors use this score to study whether, in the precise framework of a pathology, HDL metabolism is disturbed as, for example, in systemic lupus erythematosus [56,57], articular rheumatisms [58,59], Crohn's disease [60,61] or HIV [62]. In fact, Watanabe et al. in this way identified the pro-inflammatory HDL of patients suffering from articular rheumatisms following their proteomic study. The study showed that there is a significant increase in haptoglobin, SAA, clusterin and fibrinogen in pro-inflammatory HDL composition [63]. Similarly, Van Leuven et al. also noted an increase in SAA in the HDL of patients with active Crohn's disease [64]. Yet SAA can substitute for Apo A-I and thereby be responsible for a decrease in cholesterol efflux capacities, contributing to the loss of the protective effects of HDL [54,65]. The SAA increase in HDL in an inflammatory context is influenced by inflammatory cytokines such as TNF-α and IL-6. Similarly, ceruloplasmin and Apo J are also increased in HDL that has become pro-inflammatory [66]. In a similar way, it has been shown that the PON-1 [36] and PAF-AH [37] activities of HDL diminish during the acute inflammatory phase. HDL then loses its protective functions and paradoxically hastens atherosclerosis [67-69], thereby increasing cardiovascular risk in these chronic ailments. A quantitative measure of HDL-cholesterol levels alone is no longer adequate for a prediction of the functionality and anti-inflammatory properties of HDL. Proteomic analysis of HDL has become an indispensable tool toward a better understanding of HDL functions through the nature of the transported proteins.

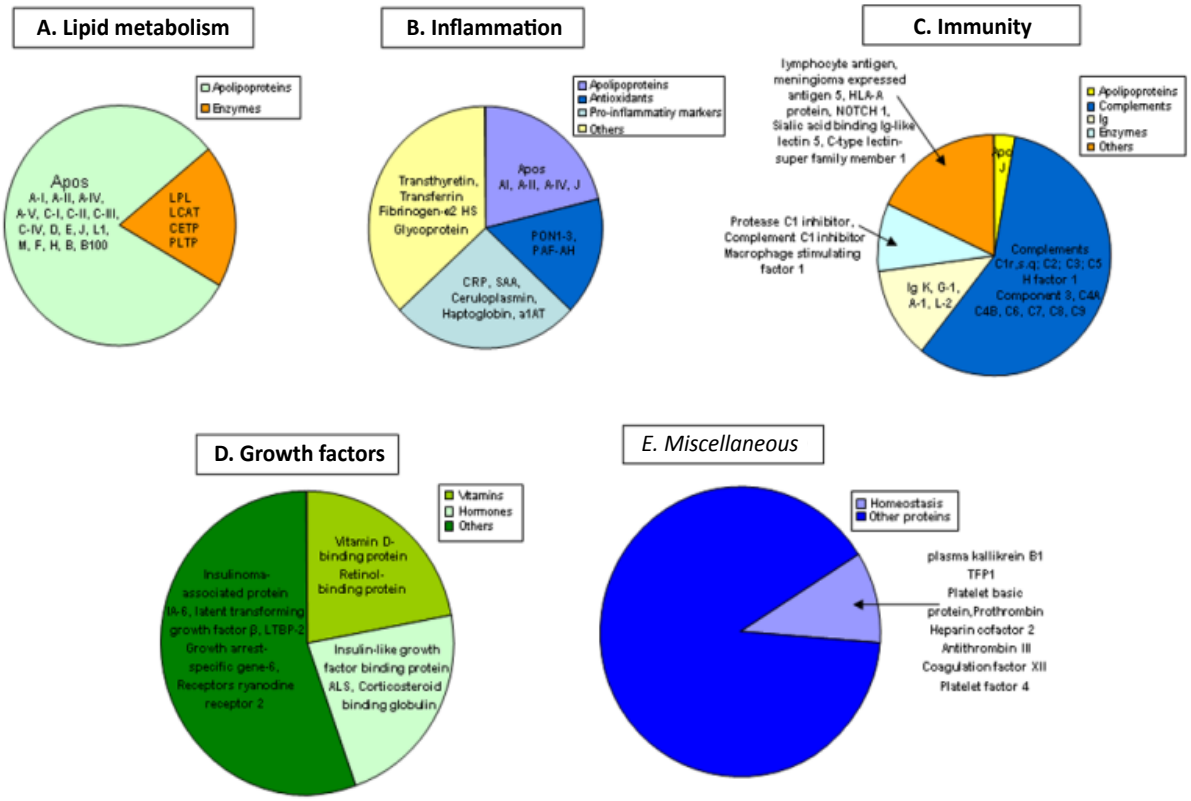


**Figure 4:** HDL proteomics and functions of proteins
The proteins of HDL, identified in the different studies by the technique of proteomics, were assembled according to specific functions: (A) Lipid metabolism; (B) Inflammation; (C) Immunity; (D) growth factors activity; and (E) miscellaneous.

## Methodological Considerations of HDL Proteomic Studies

A review of the literature allowed us to extract a dozen studies that analyzed the proteomics of HDL published during the last decade [70-78]: five studies performed a proteomic analysis of HDL from healthy subjects only. On the other hand, another five compared the proteome of HDL from healthy and unhealthy subjects. The possible pathologies were: cardiovascular disease [70,76], articular rheumatism [63], terminal kidney failure with hemodialysis [77] or psoriasis [78]. Proteomic studies of HDL in a pathological context are a recent phenomenon, as the vast majority of these studies were published in 2012.

(i) **Population and methodology:** All the studies used human models based on blood samples. They involved adult subjects exclusively. There were no pediatric studies. The first investigations, before 2012, were heterogeneous and often lacked details on the selected population and methodology employed. The number of subjects included varied and was often low, from 4 individuals [72,73] to a maximum of 33 [70] with a median of 13 subjects. They were healthy subjects, participation was voluntary [71-74] and, depending on the study, they were isolated or compared with a group of patients. The latter either presented cardiovascular diseases [70,76] or chronic inflammatory diseases such as articular rheumatism, psoriasis or terminal kidney failure with hemodialysis [63,77]. The inclusion criteria were poor or absent, particularly with regard to age, body mass index, personal or family histories (especially ones that were dyslipidemic and cardiovascular), alcohol consumption or smoking. Yet tobacco plays a significant role in aggravating the atherosclematous process by modifying endothelial and anti-thrombotic functions as well as increasing local chronic inflammation. Mezzetti et al. showed that the various compounds in cigarette smoke induce significant OxS that leads to lipid peroxidation [79]. In addition, obesity and abusive alcohol consumption can also disturb lipid metabolism by impairing hepatic functions after the development of hepatic steatosis or fibrosis. Furthermore, in two studies, the sex ratio of the subjects was unknown [73,74], whereas most studies used an exclusively male population [72-75], especially those that only focused on subjects in good health. With regard to the studies that included unhealthy subjects, the sex ratio varied: 95% were women in the study on articular rheumatism, 43% and 60% males in the terminal kidney failure under hemodialysis and psoriasis studies, respectively. Yet lipid metabolism is extremely different between a woman who has reached menopause and a young man in his thirties, since their respective hormonal status cannot be compared. For example, the activity of hepatic triglyceride lipase depends on this status. It is an enzyme that is implicated in the formation of discoidal nascent HDL as well as the uptake of cholesterol from HDL and CM remnants by the liver. It has been demonstrated that its cellular expression depends among other things on sexual steroids: the activity of triglyceride lipase is inhibited by estrogens and stimulated by progestins [80,81]. Hormonal influence is critical in HDL activity. Similarly, in the studies [73,74], the subjects were described as “healthy donors” without any clinical or biological references. The

study subjects [71,72] were defined as being normal lipidic with details on the expected values of the lipid profile in a single case [71,72]. On the other hand, the studies involving unhealthy subjects had a more rigorous methodology. Physical activity and nutrition should be ''left unspecified''. Yet these parameters also influence OxS and, consequently, the qualitative and/or quantitative nature of the HDL [75]. None of the authors precisely noted the dietary habits of the subjects tested. It is, however, acknowledged that the diet of subjects who follow a Mediterranean diet rich in fruits, vegetables, vegetable oil, fish and grains typically includes a much higher anti-oxidant intake of β carotene, vitamins E and C, zinc, selenium or even ω3 than does that of an individual whose diet is rich in saturated fats. A recent study showed that supplementation with ω3-type polyunsaturated fatty acids over five consecutive weeks has led to modifications in a dozen proteins of the HDL proteome [75]. All these parameters (nutrition, smoking, BMI, hormonal status) therefore influence the composition and thus potentially the properties of HDL. It is therefore essential that subjects be recruited using extremely precise inclusion criteria in order to be able to analyze the results of HDL proteomics without any interpretative bias.

## Protein Classification and Distribution According to Functional Groups

The studies as a whole isolated more than 130 different proteins associated with HDL. The number of proteins discovered varied from 28 [71] to 122 [77]. Depending on the technique used, the number and type of proteins changed. These proteins can be distributed according to their biological properties as summarized in the Figure 4.

The distribution of the proteins according to the defined properties is for illustrative purposes only and does not constitute an exhaustive list. Even today, no roles have been assigned to more than 70 identified proteins. The discovery of novel HDL functions can probably be envisaged in the near future.

## Conclusion and Perspectives

Given the multitude of HDL-mediated biological actions, HDLs are fascinating particles. Proteomic analyses that have been in development over the last few years have made it possible to better understand the physiopathology of these properties. Already heavily investigated for their role in cardiovascular diseases, HDLs now are the focus of research carried out on chronic inflammatory diseases. In fact, their transformation into pro-inflammatory and pro-oxidant molecules under the influence of OxS may aggravate and maintain an existing inflammatory state and favor the early onset of cardiovascular diseases. The future objective would be to find a protein signature associated with the inflammatory disease under study in order to refine its diagnostic and therapeutic management. Proteome analyses have the potential to identify marker proteins of importance for a given disease state. In particular, HDL proteins of low mass range which usually escape detection by 2D gel electrophoresis can be interrogated by affinity-based MALDI-TOF MS as a screening tool. The MALDI-MS profiling approach combined with an affinity-enrichment procedure appears fully appropriate to pinpoint enrichment or impoverishment of distinct protein cargos in HDL according to disease conditions, including transport disturbances, OxS, inflammation, infection, thrombosis and others. Thus a proteome signature will be obtained by differentiating HDL samples of pathologic groups from those of control groups with high confidence. Undoubtedly, proteomic fingerprinting will help predict HDL dysfunctions and identify subjects at risk of diseases.

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