Chapter

Lipoproteomics: Methodologies and Analysis of Lipoprotein-Associated Proteins along with the Drug Intervention

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Abstract

Lipoproteins are specialized particles involved in the transport and distribution of hydrophobic lipids, as cholesterol and triglycerides, throughout the body. The lipoproteins exhibit a basic spherical shape as complexes of lipids and proteins, and these latter are known as apolipoproteins. Initially, the proteins associated with lipoproteins were recognized as integral or peripheral proteins that only maintain the dynamics and metabolism of lipoproteins. However, there exist many studies on different lipoproteins evidencing that the quantity and type of apolipoproteins and lipoprotein-associated proteins are diverse and could be associated with different lipoprotein function outcomes. Here, we summarized recent processes in the determination of apolipoproteins and lipoprotein-associated proteins profiles through a proteomic approach, analyzing the major methods available and are used to achieve this. We also discuss the relevance of these lipoproteomic analyses on the human disease outcomes.

Keywords: lipoproteins, lipoproteomic, proteomic, high density lipoproteins (HDL), mass spectrometry (MS)

1. Introduction

The emerging high-throughput omics such as genomics, transcriptomics, proteomics, and metabolomics have been used in the search of new biomarkers in several diseases. Proteomic analyses or metabolomics and lipidomics as well as complementary technologies such as mass spectrometry (MS) (i.e., LC-MS-MS and MALDI-TOF/TOF), nuclear magnetic resonance spectroscopy, and other omics technologies, are being widely used in the search of new sources of markers,

candidates for vaccine, and alteration of expression patterns in response to environmental changes and signaling pathways in different diseases. The search for proteins in the dynamic system of a proteome requires various proteomics approaches and the use of proteomics is crucial for the early disease diagnosis, prognosis, and to monitor the disease development. The proteomics is essential for the understanding of complex biochemical processes, and the high-throughput proteomics increases the depth of proteome coverage.

Lipoproteins are macromolecular complex particles of lipids and proteins, which are related to the extracellular transport of lipids in many organisms [1]. Besides, lipoproteins have been also implicated as important host defense mediators and in the initiation of immune responses [2–4]. Lipid content has long been recognized as a critical factor in lipoprotein metabolism that acts as an important determinant of human health [5]. However, in last years, apolipoproteins and lipoprotein-associated proteins have been taken on relevance regarding lipoprotein metabolism since they serve as a frame for their assembly, maintain their structure, and interact with the membrane receptors and enzymes. Therefore, these proteins could be crucial in the identification of biomarkers related to diseases due to the fact that they are being studied under a global approach, denominated lipoproteome [6, 7].

2. Lipoprotein structure and metabolism

Lipoproteins are complex protein particles of an amphipathic nature, structurally are formed by an outer layer of phospholipids, free cholesterol, and apolipoproteins, and inside contain a nucleus of cholesterol esters and triglycerides [8]. When the lipoprotein complex is formed, the orientation of the hydrophilic proportions is toward the outside and the lipophilic proportions toward the interior; this structural characteristic allows the complex to have the ability to emulsify fats in extracellular fluids [8]. Based on their density defined by the protein to lipids ratios, lipoproteins are grouped into six classes: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), high density lipoproteins (HDL), and lipoprotein (a) [Lp (a)] [8, 9].

Triglycerides, derived from dietary fat absorption by the small intestine, are carried by the chylomicrons into blood. After triglyceride digestion to free fatty acids (FFA) by lipases in the peripheral tissues, the size of these particles is reduced, which leads to the formation of chylomicron remnants. These latter are cleared by liver uptake via LDL receptor-related protein (LRP). To synthesize VLDL, newly synthesized triglyceride and cholesterol by the liver are incorporated into chylomicron remnants [8, 10]. These large triglyceride-rich VLDLs are released to the circulation and travel to peripheral tissues, where the lipase digestion of triglycerides occurs resulting in the IDL formation (VLDL remnants). These IDL particles can be either cleared from the circulation by the liver in a similar manner to that described for chylomicrons remnants or can be digested by hepatic lipase to generate cholesterol-rich LDL particles, which is taken up by the peripheral tissues via LDL receptor to supply their cholesterol requirements [10].

The HDL lipoproteins have the highest relative density as compared to other lipoproteins despite being the smallest in size and heterogeneous in terms of composition. These HDL particles play an important role in the transport of reverse cholesterol as a carrier in the movement of cholesterol from peripheral tissues back into the liver [11]. The liver and intestine, practically in response to the lipolysis of triglyceride-rich lipoproteins, synthesize and secrete the nascent discoid HDLs that consist primarily of phospholipids and free cholesterol. Then, these particles reach the plasma where additional exchangeable apolipoproteins are picked up and excess

free cholesterol is removed from both extrahepatic cells and other circulating lipoproteins, forming mature spherical HDL particles. Finally, these cholesterol-rich HDL particles can be delivered back to the liver [10, 12].

The protein cargo related to lipoproteins consists of apolipoproteins and lipoprotein-associated proteins. These proteins play key roles in lipoprotein metabolism such as structural component, enzyme interaction, and receptors recognition, among other functions [7]. Changes in the quantity and type of these proteins could be involved in the outcome of diseases related to lipid metabolism. Therefore, the proteomic analysis of lipoproteins is an important notion for the present revision.

3. Apolipoproteins and lipoprotein-associated proteins

The apolipoproteins can be classified as integral or peripheral, either they act as constituent components of the plasma membrane of the lipoproteins or they bind to the membrane and can be exchanged between one complex and another, respectively. Apolipoproteins are distributed throughout all lipoproteins and subfractions, with the proportion of them varying in each family. These variations in the amount of proteins present in the lipoprotein families in their membrane gives them their capacity to interact with different tissues [7, 10].

Within the apolipoprotein A (ApoA) group is the apolipoprotein A-I (ApoA-I), the main protein component of HDL, which plays multiple roles in the transport of cholesterol, in addition to having been linked to the regulation of some functions of the inflammatory and immune response [13]. ApoA-II, also found in HDL, acts as an enzymatic inhibitor of lipoproteins and liver lipases. The apolipoproteins are capable of binding to each other to modify the interaction of the lipoprotein complex, as is the case with apoA-I and apoA-II in LDL [10].

ApoA-IV is mainly synthesized in the small intestine where it is attached by enterocytes to the chylomicrons and secreted during a high-fat meal intake. ApoA-IV is associated with HDL and chylomicron remnants in circulation, but a significant portion is free [14]. On the other hand, ApoA-V participates in the regulation of triglyceride levels in plasma [15]. ApoA-V is expressed only in the liver and circulates at low concentrations. Despite this, apoA-V can be recovered in association with plasma lipoproteins [16].

The apolipoprotein B (ApoB), the main protein component of chylomicrons, LDL, VLDL, IDL, and Lp (a), is encoded by a single gene that gives rises to two isoforms: ApoB-48 and ApoB-100. ApoB-48 is produced exclusively in the intestine and is the major structural protein of chylomicrons and chylomicron remnants. ApoB-100 is expressed in the liver and is found only in VLDL, IDL, LDL, and Lp (a). ApoB-containing lipoproteins are characterized by a spherical shape and contain one single apoB-48 or apoB-100 molecule per lipoprotein [17].

Apolipoprotein C (ApoC) works as an inhibitor of certain processes and modulators of catabolism. ApoC is mainly synthesized in the liver and easily transferred between lipoprotein particles and therefore are found associated with chylomicrons, VLDL, and HDL [10]. ApoC-I is responsible for inhibiting the binding of lipoprotein to its receptor as well as the function of the esterified cholesterol transfer protein (CETP). ApoC-II is an essential cofactor of the lipoprotein lipase involved in triglyceride hydrolysis. Both apoC-III and apoC-IV inhibit triglyceride hydrolysis. In addition, apoC-III decreases the clearance of VLDL [10, 18].

Several tissues synthesize apolipoprotein E (ApoE), an exchangeable apolipoprotein associated with chylomicrons, chylomicron remnants, VLDL, IDL, and a subgroup of HDL particles, but the liver and intestine are the principal producers of circulating ApoE [10]. ApoE functions as a lipoprotein ligator with hepatocytes

(clearance of apoE-containing lipoproteins) and peripheral cells related to the LDL receptor. ApoCs and apoEs are interchangeable between complexes during the conversion of VLDL to LDL. ApoEs may function as a lecithin-cholesterol acyltransferase (LCAT) activator and influence the activity of hepatic lipase and CETP [19, 20].

Proteins belonging to the lipocalins family such as apoD, apoM, the orosomucoid protein, and retinol-binding protein (RBP) interact with the surface receptors of the cells, intervening in the formation of macromolecular complexes. It has been described that apoD binds to apoA-II and apoB-100 by means of disulfide bridges; it is associated with LCAT, so it is involved in the transport of lipids in the blood system. ApoJ is involved in a wide variety of processes, acting as a chaperone protein and its main role is the inhibition of lipid transfer and is related to cell death. There are apoproteins such as apoF whose function is unknown; however, there is a theory of their role in abnormal lipid composition inhibiting CETP in the small and dense LDL particles (sdLDL) [21, 22].

In addition to apolipoproteins, lipoproteins require other proteins with specific activities to interact with the environment, which are denominated as lipoprotein-associated proteins. Between these proteins are included phospholipase A2, whose activity indicates the presence of sdLDL in plasma [23]. Serum amyloid A protein (SAA) is associated with several lipoproteins, especially in LDL [24]. Some proteins such as albumin and prenyl cysteine oxidase (PCYOX1) are responsible for protecting against oxidation by lipoproteins such as LDL and splitting the thioether bond of the prenyl cysteine generating H_2O_2, respectively. Also, proteomic studies have identified the apoL-I, PON1, and PAF-AH in small amounts linked to the complexes [25, 26].

The function of many of the proteins that interact with lipoproteins is still unknown. The proteomic analyses have shown that the lipoprotein-associated proteins are involved in cardiovascular risk, immune system, inflammatory processes, among others [7, 26]. However, there is no guide for the analysis of lipoproteins at the proteomic level basically because it depends on the biological question to be investigated, and this determines the approach and the methods or tools to be used as well as the technological platform to perform it.

4. Methodologies used for proteomic analysis of lipoproteins

Before describing the methodologies used for lipoproteomic studies, it is important to mention some general characteristics of lipoprotein-associated proteins and the importance of the research question to establish a good methodological flow chart to address this question. We will start by mentioning that must first consider the lipoprotein obtention by using methods for separation, concentration, and protein stability due to the heterogeneity of these particles.

Diverse chromatographic techniques—prior to proteomic techniques—have been used for the separation of plasma lipoproteins as well as the protein content, such as capillary electrophoresis, size exclusion, cation exchange, gel filtration, fast protein liquid, among other chromatographic techniques [7, 27–32].

The two-dimensional electrophoresis (2-DE) is one of the most usual methods that have been applied to separate the protein cargo related to lipoproteins. Although usually, the first-dimensional separation applied for the proteins in the 2-DE is on base of their charge (isoelectrofocusing), some lipoproteomic analyses have substituted this step by either one of the abovementioned methods to separate lipoproteins or electrophoresis on native gels, followed by SDS-PAGE, which could be denominated as gel-based lipoproteomics [27, 33–35].

Pre-analysis		**Chromatography**	**Mass spectrometry**	**Analysis**	**Post-analysis**	
Source	**Methods**	**chromatographic technique = Instrumentation (method)**	**Mass analyzer = Instrumentation (method)**	**Database search**	**Number of lipoproteins and apoproteins**	**References**
Mice C57BL/6 J: plasma	Gel filtration/size exclusion chromatography, phospholipid-containing particles using CSH	LC-ESI = C_{18} reverse phase column (GRACE; 150 × 0.500 mm)	Quadrupole/TOF = 4800 scans, mass range: 300–1800 *m/z,* charge states:2–5, excluded target ions: 300 s	Swiss-Prot protein knowledge base for *Mus musculus*, PeptideProphet algorithm	VLDL/LDL: 32, HDL: 104; lipid poor lipoproteins: 55	[37]
Human serum (HDL)	Trypsin, delipidation, gold nanoparticles and LDI-MS, EDX, SPE, FT-IR		MALDI-MS = nr	Swiss-Prot, Lipidmaps database	HDL (delipidation): 10 (prior), 6 (after). HDL (anion exchangers): 23	[38]
Mice C57BL/6 J: apoA-I KO and apoA-II KO, apoA-I KO and WT: plasma	Particles of CSH. Plasma separation by size exclusion chromatography	LC-ESI = column (C18 reverse phase (150 × 0.500 mm))	Quadrupole/TOF = MS/MS. tolerance were set to ±35 PPM, and up to 3 missed tryptic cleavage sites were allowed	UniProtKB/Swiss-Prot Protein Knowledgebase, Peptide Prophet algorithm	HDL: WT:25 vs. ApoA-I KO: 21; ApoA-II KO: 11 vs. WT: 14; and ApoA-IV: 6 KO vs. WT: 7	[28]
17 Subjects (exposure to organic pollutants): plasma (HDL)	Ultracentrifugation (290,000 g, 15°C, 4 h)	nLC: Column C18, ESI	Linear ion trap-Orbitrap: CID. ms/ms: 0.6 Da	MaxQuant v1.5.0, human Uniprot/Swiss-prot database	LCMS permit identified the pathway of interaction between HDL-proteins	[39]
34 Men (2 dietary: weight loss/high car): plasma (LDL, ApoC-III)	ELISA assay		MALDI-TOF: 500 laser shots mass spectra		Detection and concentrations values of 12 apoC-III glycoforms	[40]
Mice (female, LDLr−/−, 8 week old): plasma (VLDL, LDL, HDL)	FPLC, ELISA assay	LC	Orbitrap: mass tolerance: 0.8 Da	Swiss-Prot database	HDL: 91 LDL: 49 VLDL: 39	[41]
1000 Children (6–8 years, Nepal rural zone): plasma	Cation exchange chromatography	LC	Orbitrap	Refseq 40 database	Apo-AI, Apo-AII, Apo-CIII	[42]

Pre-analysis		Chromatography	Mass spectrometry	Analysis	Post-analysis	
Source	**Methods**	**chromatographic technique = Instrumentation (method)**	**Mass analyzer = Instrumentation (method)**	**Database search**	**Number of lipoproteins and apoproteins**	**References**
458 Children (6–11 years, exposure to environmental tobacco smoke): serum (HDL, LDL)		SPE-HPLC-TIS-MS/MS			LC detected various polyfluoroalkyl substances in serum A negative association PFOS and non-HDL	[43]
57 Males (exposed to arsenicum): plasma, urinary (LDL, HDL, Lp(a), Apo-A1, Apo-B)	Centrifuged at 3500 rpm for 10 min		ICP-MS: extraction voltage −100 V, Rf power 1400 W, focus voltage 12 V, and nebulizer gas flow rate (using a Burgener Miramist nebulizer) 0.83 L/min. Dwell times were 50 ms for 75As and 10 ms for internal standard (72 Ge)		ICP-MS: Potential risk of the arsenic on lipoproteins and apolipoproteins	[44]
Human healthy, normolipidemic males: plasma (LDL)	Gel filtration chromatography, ultracentrifugation.	nLC: column (IntePepMap 100, C18, particle size 3 um), flow rate = 300 nL/min ESI: 2.5 kV, 150°C	Triple quadrupole TOF: mass tolerance: 50 mDa, 350–1800 *m/z* window, MSscan type: 0.25 s	UniProtKB/Swiss-Prot Protein Knowledgebase, Peptide Prophet algorithm	LC-MS permit the abundance protein as well as antioxidant activity	[30]
110 Samples (purchased)		Phospholipids: UHPLC: column (2.1 × 100 mm, 1.7 uM particle), flow rate: 0.7 mL/min, ESI	Qtrap: MRM scanning, negative and positive mode	Multiquant software functions, JMP (SAS Institute)	LC-MS analysis of serum/lipoproteins	[45]
23 Healthy volunteers: serum	Gel filtration chromatography, sequential ultracentrifugation, immunoassay	UPLC: column (UPLCR BEH C8), floe rate: 450 uL/min., 60°C, autosampler: 4°C ESI	Triple-quadrupole: positive ion mode. Quantification of plasmalogens: Capillary voltage: 3500 V, source temperature 80°C, desolvation: 400°C, cone voltage: 35 V. CE: 20–32 eV		LCMS: distribution of each molecular species in plasmalogen and choline plasmalogen	[46]

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Source	**Methods**	**chromatographic technique = Instrumentation (method)**	**Mass analyzer = Instrumentation (method)**	**Database search**	**Number of lipoproteins and apoproteins**	**References**
Healthy volunteers: plasma (VLDL, LDL, HDL)	Ultracentrifugation, SDS-PAGE, size exclusion chromatography, circular dichroism, spectroscopy, spectropolarimeter		MALDI-TOF: 337 nm nitrogen laser, positive ion: 20 kV		Identification of apolipoproteins released from VLDL by mass spectrometry	[47]
20 Patients with lipoproteins (a) (18–70 years): plasma (LDL, HDL)	Ultracentrifugation, ELISA	LC: solid-phase extraction	Triple Quadrupole-linear ion tramp: SRM. (energy collision: 34 V, Q1: 786–788; Q2: 1069–1072 *m/z)*		LC-MS system: concentrations of lipid, lipoprotein and apolipoprotein	[48]
Healthy donors (nonlipidemic, 24–65 years, purchased samples): plasma (HDL, LDL)	Ultracentrifugation (330,000 g, 6 h), SDS-PAGE and Western blotting, Negative stain electron microscopy	UPLC: column (Kinetex EVOC18), ESI	Triple-quadrupole: lipid species were analyzed by selected reaction monitoring (from 141 to 369 *m/z,* from 0 to 50 eV)	Extraction and ionization efficacy by calculating analyte/ISTD ratios (AU) and expressed as AU/mg protein	LCS permit the separation of a mixture in HDL protein	[49]
12 Healthy male (36–67 years): plasma (HDL, LDL)	Ultracentrifugation (40,000 rpm, 44 h, 15°C), fractioned, apoA-I was detected by Western blotting, internal standars	HILIC-UHPLC-FLD: Glycan chromatography column, 150 × 2.1 mm i.d., 1.7 μm BEH particles, flow rate of 0.56 mL/min	MALDI-TOF-MS: 25 kV, acceleration voltage: 140 ns extraction delay, mass window: 1000–5000 *m/z.* For each spectrum: 10,000 laser shots, laser frequency of 2000 Hz		LDL: 18 HDL: 22 N-glycome of human plasma lipoproteins	[50]
16 Healthy adults: plasma (HDL, apoA-I, apoB)	Ultracentrifugation. PRM analysis (shotgun proteomics experiments).	UPLC: flow rate: 0.6 uL, column (Xbridge BEH C18) ESI	Orbitrap: PRM mode, isolation window: 2 Th, HCD: 27%, orbitrap analyzer: 15,000 resolution, AGC: 5×10^4, maximum ion time 30 ms	PeptideAtlas mass spectral database	Meal macronutrient content HDL composition in the postprandial state	[51]

Pre-analysis		Chromatography	Mass spectrometry	Analysis	Post-analysis	
Source	**Methods**	**chromatographic technique = Instrumentation (method)**	**Mass analyzer = Instrumentation (method)**	**Database search**	**Number of lipoproteins and apoproteins**	**References**
47 Volunteers: serum (HDL, non-HDL).	Anti-apoAI magnetic nanoparticles (10 mg) and serum (5 μl) were mixed FTIR, X-ray diffraction	ID/LC/MS system: LC: column (waters symmetry C18), flow rate: 0.3 mL/min. APCI: corona current: 5 uA, source temperature: 450°C	API 4000 tandem mass spectrometer (triple-quadrupole): Collision energy: 26 eV, collision exit potential: 6 V		ID/LC-MS permit the monitoring serum in clinical settings to dyslipidemia and atherosclerosis	[52]

SRM: selected reaction monitoring. HPLC: high performance liquid chromatography. HILIC-UHPLC-FLD: hydrophilic-interaction ultra-high-performance liquid chromatography with fluorescence detection. LC: liquid Chromatography. UPLC: ultra-performance liquid chromatography. MALDI: matrix-assisted laser desorption/ionization. TOF: time of flight. Qtrap or LTQ linear trap: triple quadrupole-linear ion trap. CE: collision energy. MS/MS: tandem mass spectrometry. PRM: parallel reaction monitoring. AGC: automatic gain control. ID/LC/MS: isotope dilution liquid chromatography mass spectrometry. ESI: electrospray. nESI: nano-electrospray. FPLC: fast protein liquid chromatography. MRM: multiple reaction monitoring. HILIC: silica-based and solid-core reverse phase after hydrophilic interaction. ICP-MS: Inductively coupled plasma-mass spectrometer. SPE: solid phase extraction. SPE-HPLC-TIS-MS/MS: solid phase extraction coupled to high performance liquid chromatography-turbo ion spray ionization-tandem mass spectrometry. SEC-FPLC: size exclusion chromatography by fast protein liquid chromatography. APCI: atmospheric pressure chemical ionization. CID: collision-induced dissociation. LDI-MS: laser desorption/ionization mass spectrometry, EDX: dispersive X-Ray Spectroscopy. CSH: calcium silica hydrate. WT: wild-type. KO: knockout. FTIR: Fourier transformation infrared.

Table 1.
Chromatographic and spectrometric methodologies used recently in lipoproteomic analysis.

Pre-analysis |		Spectroscopy experiment NMR type	Post-analysis	References
Source	isolation method			
133 Caucasian participants (T2DM, >18 years): serum (VLDL, LDL, and HDL)		1H: FT, 400 MHz	Determinate the lipoprotein subtraction characteristics	[56]
98 people (T2DM/ nor T2DM): plasma (GlycA vs. Lp-PLA2)	Centrifugation (1400 g, 15 min)	^{1}H: 400 MHz, 47° C, CaEDTA resonance at 2.519 ppm was used as the internal chemical	GlycA is correlated with LP-PLA2 in plasma (person without T2DM/ MetS)	[57]
23 patients with primary aldosteronism: plasma (HDL, VLDL, LDL, ApoB, and ApoA-I)	Immunoturbidimetric assay	^{1}H: 400 MHz, 47° C. (LipoProfile-3 algorithm)	Circulating LDL may contribute to adrenal steroidogenesis in humans	[58]
115 nondiabetic women (35–55 years, mediterranean diet, physical exercise, 2 years): plasma (HDL. LDL)		^{1}H	Lipoprotein size, particle and subclass concentrations	[54]
Human serum (purchased) spiked into phlebotomy tubes (LDL, HDL)	Centrifugation (3000 g, 5 min, 4 h)	^{1}H: 600 MHz	Lipoprotein subclass analysis standardized by tube type and tube size to prevent risk of analytical interference.	[59]
Patients with HFrEF (782), HFpEF (1004), and no HF (4742): plasma (HDL)		NMR LipoProfile-3 algorithm	Quantify concentrations of HDL. Phenotyped cohorts of HFrEF, HFpEF, and patients without HF	[60]
4897 subjects: plasma (LDL)		^{1}H: 400 MHz	Differentiate in the size of particle in LDL profile	[61]
309 patients (MACE): plasma (HDL, LDL, and VLDL). Control:902		^{1}H	Neither baseline HDL nor the change in HDL on treatment with dalcetrapib or placebo was associated with risk of MACE after ACS	[62]
Normal volunteer: plasma (HDL, LDL, andVLDL)	Sequential ultracentrifuge	1H, ^{13}C, ^{15}N: 600.55 MHz, 47° C, different pressures	Show the spatial arrangement, phase behavior and molecular dynamics in the particle core	[55]
3446 participants (HDL, LDL, and VLDL)		^{1}H: LipoProfile-3 algorithm	Association between FGF21 and NAFLD	[63]

MHz: megahertz (10^{6}). MetS or MS: metabolic syndrome. T2DM: type 2 Diabetes mellitus. FT: Fourier transforms. CaEDTA: EDTA mono calcium. GlycA: glycoprotein acetylation. Lp-PLA2: lipoprotein-associated phospholipase A2. HFrEF: reduced ejection fractions. HFpEF: preserved ejection fraction. HF: heart failure. MACE: major adverse cardiovascular events. FGF21: fibroplast growth factor21. NAFLD: nonalcoholic fatty liver disease.

Table 2.
NMR methodology applied recently in lipoprotein-based analyses.

Lipoprotein-associated proteins are not easy to study by proteomic methods, mainly the embedded in the lipoprotein membranes. These latter have rigid transmembrane domains that contain α-helices or β-barrels, which stabilize the protein by strong secondary structural characteristics and these regions can resist proteolytic digestion [6, 36]. Thus, the protein identification for lipoproteomes has been mainly performed by two methods: mass spectrometry and resonance magnetic nuclear.

Modern mass spectrometry (MS) techniques have allowed for thorough characterizations of the lipoproteins [37]. However, different experimental conditions have been reported to avoid contamination of the biological samples as well as selective and optimized methods to detect lipoproteins, usually liquid-phase separation techniques, prior to spectrometric techniques [36, 37]. Thus, different spectrometric experimentation conditions have been reported for the study of lipoproteins and apoproteins, respectively (**Table 1**).

There are several challenges in the lipoproteomic analyses performed with the most advanced mass spectrometry methods. Some of them are the abundance of proteins from the biological source and the lipoprotein(s) purification steps, which conditioned the protein content and constitution. However, if this obstacle can be overcome, the mass spectrometry analysis has been demonstrated to be a useful tool to identify a diverse array of proteins related to lipoproteins, avoiding aberrant integration of unexpected proteins by reducing the suppression of ionization at high peptide resolution [53].

On the other hand, the nuclear magnetic resonance (NMR) technique permit, besides protein identification, can provide information of the lipoproteins at both molecule and atomic levels under physiological or 'near-physiological' conditions. The signal most used to quantify lipoproteins is the methyl signal because give a specific response in the lipids that travel inside the lipoproteins [54, 55]. In this way, proton spectroscopy has been the most used nuclear magnetic resonance technique to quantify lipoproteins, but it is not the only one as will be seen later (**Table 2**).

Furthermore, not only ^{1}H NMR has been the technique used for the study of lipoproteomics but also the two-dimensional NMR techniques have been used. The two-dimensional heteronuclear $^{13}C—^{1}H$ chemical-shift made it possible to analyze macromolecular complexes like HDL, but with a limited resolution in reduced peaks above 50 ppm and the limited resolution in the 29–33 ppm region, inclusive with artifacts that would later be discarded by the spectra of one dimension (^{1}H and ^{13}C) [64].

In addition, the material to be used for the spectroscopic analysis should be the optimal one to avoid contamination, as in the case of a tube used for the collection of biological material and used in clinical research, which should be specifically for the analysis of lipoproteins [59].

Also, the experimental conditions do not always favor the use of NMR for the analysis of lipoproteins. Thus, mass spectrometry permits the particle identification via LC-MS system in contrast to NMR spectroscopy, which failed. Due to that, the NMR spectroscopy makes HDL particle quantification only in a physiological setting: full serum or plasma but not in HDL-containing suspensions [49]. Therefore, the technician must consider the biological and technical variables for an assertive lipoproteomic analysis.

5. Clinical significance of lipoproteomic analyses

The lipoproteomic analyses have been focused on understanding the functional mechanisms underlying apolipoproteins and lipoprotein-associated proteins that

can be used to develop new diagnostic and/or prognostic biomarkers for many lipoproteins' metabolism-related illness.

The HDL lipoprotein fraction has been the most studied according to lipoproteomic relationships with different diseases. Among the HDL-associated proteins, ApoC-III levels have been seen increased in the patients with either a lupus nephritis (lupus erythematosus) or with a cerebral lacunar infarction, which could be related with a reduced anti-inflammatory activity of HDL particles [65, 66].

However, HDL-carried ApoC-III has been more implicated in cardiovascular disease (CVD) risk. One of the first evaluations was performed with coronary artery disease patients, which exhibit increased levels of ApoC-III [67]. Recently, in a cohort study, it was demonstrated that HDL-carried ApoC-III is related to a higher risk for coronary heart disease [68].

In addition to ApoC-III and ApoC-II, other HDL-associated apolipoproteins, were proposed as biomarkers for CVD risk in patients with chronic hemodialysis [69]. The ApoC-III/ApoC-II/ApoE levels in VLDL lipoproteins, independent of HDL, were associated with incident CVD, which supports the concept of targeting triacylglycerol-rich lipoproteins to reduce the CVD risk [70].

Other HDL-associated apolipoproteins have been associated with cardiac pathologies. For example, ApoA-I, ApoA-IV, ApoE, and ApoL1 levels have been seen enriched in HDL3 fraction from patients with acute coronary syndrome (ACV), with a concomitant reduction of these apolipoproteins in the HDL2 fraction [71]. Furthermore, ApoC-I was significantly decreased in the HDL particles of coronary heart disease (CAD) patients in comparison to normal individuals [72]. The existence of CAD has recently been correlated with an HDL apolipoproteomic score, independent of circulating ApoA-I and ApoB rates and other typical cardiovascular risk factors [73].

Regarding HDL-associated proteins, many of them have been related to heart illness. For example, serotransferrin, haptoglobin, hemopexin, complement factor B, ras-related protein Rab-7b, and paraoxonase-3 (PON3) levels have been seen reduced in HDL from patients with some cardiac pathology. Meanwhile, PON1, alpha-1B-glycoprotein, vitamin D-binding protein, alpha-1-antitrypsin (A1AT), acid ceramidase, serum amyloid A and P proteins, sphingosine-1-phosphate, filamin A, and pulmonary surfactant-associated protein B are increased in HDL fractions from patients with cardiometabolic disorders [69, 71, 72, 74, 75].

Diabetes is, perhaps, the major controllable risk factor for CVD. In particular, related to the HDL fraction, ApoA-I, ApoA-II, ApoA-IV, ApoE, ApoJ, as well as PON1, transthyretin, complement C3, and vitamin D-binding protein have shown changes in patients with type 2 diabetes (T2D) [76, 77]. In contrast, individuals with type 1 diabetes (T1D) had proteomic alterations of their HDL particles. For example, the complement factor H-related protein 2 was elevated, independent of glucose control, in T1D patients in comparison to healthy controls. Also, the optimal glucose control corrected the elevated levels of the alpha-1-beta glycoprotein and inter alpha trypsin inhibitor 4. Furthermore, the HDL particles in T1DM individuals, independent of glucose control, exhibit a higher abundance of irreversible post-translational modifications of HDL-associated apolipoproteins [78, 79].

Also, in psoriatic patients, the levels of HDL-associated ApoA-I exhibit a significant reduction, whereas levels of apoA-II, serum amyloid A, C3, and A1AT, among other proteins were increased [80]. Besides C3 and C9, complement factor B, as well as ApoJ, fibrinogen, haptoglobin, and serum amyloid A have been also increased in HDL fraction from patients with rheumatoid arthritis [81]. Taken together, these results suggest that HDL-associated proteins could be involved in anti-inflammatory properties in chronic illness.

Concerning other diseases, the proteome of HDL particles has been used to identify protein markers. In nonalcoholic fatty liver disease, including individuals with nonalcoholic steatohepatitis, changes in the abundance of HDL-associated proteins such as antithrombin III and plasminogen have been identified [82]. Although not directly to HDL particles, proteomic analysis of some HDL-related apolipoproteins has been associated with viral diseases. For example, a change in the expression level of ApoA-I was suggested as a specific and appropriate alternative to conventional HIV diagnosis and progression measurements in clinical research settings [83]. Increased concentration of ApoM in sera patients with HBV infection have been detected [84]. Recently, a downregulation of ApoA-I and ApoM levels have been associated with the severity of COVID-19 infection [85].

In addition to HDL, only other few lipoproteomic studies have been developed to discover changes in lipoproteins-associated proteins in diverse pathologies. Proteomic studies of LDL have reported that carry apolipoproteins AI, A-II, CI, C-II, C-III, C-IV, D, E, and F, in addition to apoB, as well as clusterin, C3, C4a, and C4b, and PON1 that are also associated to this particles [86]. Serum amyloid A levels were found to increase in all lipoprotein fractions, especially in LDL from atherosclerotic patients [24]. An enriched content of all the apoC-III isoforms and a lower content of apoA-I, apoC-I, and apoE were detected in the sdLDL from subjects with metabolic syndrome and subclinical peripheral atherosclerosis [87]. VLDL, IDL, and LDL fractions from Alzheimer patients exhibit low levels of complement C3 [88].

6. HDL lipoproteomics for drug discovery

Target discovery, which is an important step in the drug development process, includes discovering and validating targets associated with the diseases. It is increasingly recognized that, in many instances, metabolomics is used to identify novel biomarkers, which can help in the discovery of therapeutic strategies for many diseases [89].

Until now, lipoproteomics has proved to be an effective method for identifying candidate cardiovascular disease markers. Studying the profiles of protein expression in drug-treated patients contributes to the discovery of multiple drug-specific targets. Traditionally, statin therapy has proven efficacy in reducing cardiovascular events, but as aforementioned, the identification of HDL-associated proteins is variable. The statin therapy has a notorious effect on the increment of A1AT associated to the large fraction of HDL particles enhancing its anti-inflammatory functionality, which may interfere with the statins outcome on reducing cholesterol levels [37]. In addition, a relationship between the CAD treatment and the HDL proteome was demonstrated using statin and niacin therapy, observing that ApoE and ApoC-II levels are enriched in the HDL3 fraction, which contains less ApoJ levels [90].

The increment in the HDL levels by CETP inhibitors is another biomarker that has continued to be disappointing in clinical research. In fact, in patients with deficiency of CETP (CETP-D), the HDL particles are enriched with ApoE, angiopoietin-like3 protein, and complement regulatory proteins such as C3, C4a, C4b, and C9 that could be associated to the increased atherogenic profile in CETP-D patients [91].

Thus, it is important to consider that among the diverse HDL populations not all possess a cardioprotective effect [92]. Therefore, we need a better knowledge of the protein cargo of the HDL populations with anti-atherogenic actions. A comprehensive understanding of the HDL proteomics can lead to the design of more effective

anti-atherogenic drugs based on the activity of HDL-associated proteins that will provide new therapeutic strategies at the molecular level.

In the dynamic drug discovery process, the different proteomic methods, which include MS-proteomics, expand beyond the general aim of target drug discovery. It must consider the drug-protein interactions as well as elucidate the mode of action of candidate drug molecules [93]. Thus, novel HDL-based therapeutic agents, besides the traditional statins, will need consider to the functional HDL lipoproteomics to characterize the interaction of the drug with the HDL-associated proteins. This is necessary as an attempt either to elucidate the mechanism of action by direct drug-protein interaction or as a biomarker for drug validation by monitoring the pharmacological effect through an increase of HDL populations with an anti-atherogenic protein cargo.

7. Conclusion

The lipoproteins can be involved in different pathologies related to lipid metabolism such as atherosclerosis, cardiovascular risk, obesity, metabolic síndrome, and diabetes, among others. However, the protein cargo of these particles has been associated with several functions, which differ from the amply recognized as structural composition and receptors recognition during their function as lipid transporters. For this reason, the identification of variants of apolipoproteins and lipoprotein-associated proteins has been an important referent to detect new biomarkers. In fact, as we described here, several methodologies have been developed to improve the lipoproteomic profile.

Despite these studies, the majority concord that both biological source and lipoprotein purification are important steps to avoid protein contamination, principally from samples that are used directly as serum or plasma. Also, the most used method to identify the lipoprotein-associated proteins is the mass spectrometry, but some limits are presented that depend on the platforms to apply this methodology. Also, it is important to highlight that not everyone has the facility to use this methodology and it is necessary to develop new methodologies to apply in clinical fields to ensure discoveries about these proteins both in new lipoproteins' fractions and new diseases.

Also, the lipoproteomic analyses could be a new clinical area to evaluate the therapy of the pathologies described here as prognostic analytes. In this sense, the HDL lipoproteomics is, perhaps, the more advanced field considering the evaluations of populations with statins' treatment. However, novel HDL therapeutic agents must consider the functional lipoproteomics of these particles. Finally, these lipoproteomes can help us to describe the molecular mechanism to understand the interaction of apolipoproteins as well as the lipoprotein-carried proteins to support the other omics such as lipidomics and metabolomics.

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Conflict of interest

The authors declare no conflict of interest.

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