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Unbiased and targeted mass spectrometry for the HDL proteome

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Abstract

Purpose of review—Mass spectrometry (MS) is an ever evolving technology that is equipped with a variety of tools for protein research. Some lipoprotein studies, especially those pertaining to HDL biology, have been exploiting the versatility of MS to understand HDL function through its proteome. Despite the role of MS in advancing research as a whole however, the technology remains obscure to those without hands on experience, but still wishing to understand it. In this review, we walk the reader through the co-evolution of common MS workflows and HDL research, starting from the basic unbiased MS methods used to profile the HDL proteome to the most recent targeted methods that have enabled an unprecedented view of HDL metabolism.

Recent findings—Unbiased global proteomics have demonstrated that the HDL proteome is organized into sub-groups across the HDL size fractions providing further evidence that HDL functional heterogeneity is in part governed by its varying protein constituents. Parallel reaction monitoring, a novel targeted MS method, was used to monitor the metabolism of HDL apolipoproteins in humans and revealed that apolipoproteins contained within the same HDL size fraction exhibit diverse metabolic properties.

Summary—MS provides a variety of tools and strategies to facilitate understanding, through its proteins, the complex biology of HDL.

Keywords

Apolipoprotein; label-free; multiple reaction monitoring; parallel reaction monitoring; spectral counting; stable isotopes

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None.

INTRODUCTION

Despite the importance of lipoproteins, VLDL, LDL and HDL, to human physiology and the on-going pursuit to manage their levels in dyslipidemic patients, research is still needed to fully understand these complex particles. Lipoproteins vary in macromolecular content, protein, triglycerides, and cholesterol and lipids. The protein constituents of HDL in particular have been heavily dissected in recent years, primarily through liquid chromatography mass spectrometry (LC-MS), revealing several more proteins additional to the classical apolipoproteins, such as apoA-I, apoA-II, apoE, and apoC3, whose biological activities are diverse [1] but still encompass the role for HDL as anti-atherogenic particle. Moreover, LC-MS technology has extended well beyond a tool for identification. Changes in apolipoprotein abundance can now be readily assessed using one or more of a number of strategies including label-free and label-based strategies [2, 3]. Quantitative proteomics provides several innovative workflows that are poised to address many of the unknowns of apolipoprotein function and metabolism. In this review, we detail the most recent developments in quantitative proteomics and how we and others have applied them to investigate the complex nature of the HDL proteome.

UNBIASED PROTEOMICS APPLIED TO HDL BIOLOGY

In the last ten years, several proteomics studies have reported between 18 to >170 HDL associated proteins (Fig. 1a). The discrepancy in protein number is due to one or more factors: each HDL isolation and separation strategy may introduce unique protein contaminants or deplete bona fide HDL proteins; protein fractionation and proteolysis methods vary in peptide recovery and yield; recent studies that have capitalized on improved ionization technology, speed, sensitivity and accuracy of mass spectrometers would have observed lower abundant proteins; and the criteria for reporting HDL proteins vary from study to study. The health status and lipid profile of the study subjects also impact the composition of the HDL proteome, biological variables that are often evaluated in HDL proteomics studies. Nonetheless, despite all of the sources of variability listed above, approximately 90 proteins have persisted, and are thought to comprise the consensus HDL proteome [4]. We analyzed the consensus proteome using the Markov cluster algorithm (MCL) provided by the String data base (<http://string-db.org/>) that sorted HDL's proteins into two major functional networks, one pertaining to lipid metabolism, and the other grouping several biological processes including protein activation cascades, regulation of wound response and regulation of protease activities (Fig. 1b). The diversity in both HDL's protein constituents and their associated biological processes have repositioned HDL research to extend beyond its canonical role in lipid metabolism [5]. Fortunately, LC-MS technology and proteomics workflows have been evolving rapidly and are poised to accompany HDL research into the next phase of new findings.

Data-dependent acquisition MS

Unbiased proteome profiling is primarily done using “bottom-up” proteomics, a method that identifies proteins based on their proteolytic forms, peptides. In contrast, “top-down” proteomics is the analysis of intact proteins and is more suitable for studying protein

isoforms or post-translational modifications. ApoA-I isoforms, for example, have been studied using top-down approaches [7, 8]. For numerous reasons including a significantly simpler mass spectrometric signal to interpret, bottom-up is the standard approach for routine proteomics. The majority of proteomics studies are done using data-dependent acquisition (DDA) and will be the focus of this review. The other acquisition method, data-independent acquisition, which is not as common as DDA, is reviewed elsewhere [9, 10].

In a standard DDA run, the mass spectrometer surveys the incoming peptide precursor ion signals (MS1) to select a user-defined number of peptides to be fragmented in the subsequent scans (MS2). The number of peptides fragmented per cycle varies from study to study, however up to the 20 most abundant peptides are generally selected per cycle (~2 seconds). The isolation window around the precursor's m/z (mass-to-charge) of the peptide is as narrow as possible (for example, ± 1 m/z) in order to increase specificity of the target peptide by decreasing the interference by other peptide signals (Fig. 1c), but not too narrow as signal loss will occur. When the isolation window is optimal, the fragment ions are more likely to belong to the isolated precursor and thus the quality of peptide sequencing is improved (Fig. 1c). We will return to the relevance of the isolation window size when we overview recent advancements in targeted platforms below.

Label-free proteomics provides insight into HDL heterogeneity

Quantitative proteomics is generally categorized as label-free or label-dependent. Label-free employs either spectral counting (the total number peptide-spectrum matches per protein) or the area under the curve of the peptides' chromatographic peaks [2], under the observation that the more abundant a protein is in the sample, the more likely its peptides are detected and sequenced (Fig. 1d). A majority of the quantitative HDL proteomics studies have relied on spectral counting (Fig. 1a). Some of these studies have specifically addressed whether proteins are differentially localized across HDL size or density fractions [2, 4, 11, 12].

For instance, spectral counting was used to evaluate the co-migration of HDL proteins across various analytical separation methods under the assumption that proteins residing on the same particle are more likely to be identified together within sub-fractions of each method. Human plasma HDL was fractionated using one of three methods, size exclusion chromatography, anion exchange chromatography or isoelectric focusing, followed by a calcium silicate hydrate-dependent enrichment of the phospholipid compartment from each fraction per method [11]. The proteins were found to be differentially localized across the fractions for all three methods. For example, 106 proteins were reported from the size exclusion experiment whose fractions 13 to 29 represented the largest to smallest HDL sizes, respectively. ApoA-I signal was detected in all 17 fractions but predominated in fractions 20 to 27. ApoA-II, the second most abundant HDL protein, was only detected in fractions 22 to 25. Some proteins, such as fibrinogen alpha and beta chains (FGA and FGB), predominated in the larger sizes, whereas albumin and apoA-IV predominated in the smallest sizes [11].

We profiled the proteome of human plasma HDL that was isolated by immunopurification of apoA-I followed by fractionation by non-denaturing polyacrylamide gel electrophoresis [2]. Five size fractions, prebeta, alpha3, alpha2, alpha1 and alpha0 (Fig. 2a), were in-gel proteolyzed for parallel quantitative proteomics approaches including spectral counting. We

limited the proteins to those supported by two or more peptides per donor (between 76 to 105 proteins across three donors), then filtered the list down to those in common to the three donors (58 proteins). Fig. 2b features a heat map from one donor's normalized spectral counts from 23 of the 58 HDL proteins, sorted from alpha0 to prebeta. ApoA-I signal was detected in all five fractions, but predominated in alpha2 and alpha3; apoA-II's distribution was similar to that of apoA-I; FGA and FGB predominated the larger sizes, alpha0 and alpha1; and apoA-IV and albumin the smaller size fractions, alpha3 and prebeta (Fig. 2b). Similar to the gel filtration data above, we too observed that the HDL proteome was organized in sub-groups of proteins whose peak abundances were for the most part distributed within one or two adjacent size fractions [2]. These and other global proteomics studies have thus provided the groundwork for further exploration on the extent of HDL particle heterogeneity [1, 13]. The differences in distribution of the HDL proteins across, for instance, the size fractions, indicate that subsets of proteins are more likely to co-occupy any given HDL particle.

Stable isotope labeling of apolipoproteins for their accurate quantification

Label-free proteomics is practical to gain an overview of a protein's relative abundance across samples, but not for the relative abundances among different proteins. This restriction is a consequence of differences in ionization efficiency - the number of (peptide) ions generated to the number of molecules consumed at the ionizing source of the instrument [14]. A peptide's intensity is therefore also dependent on its ionization efficiency. To account for differing ionization efficiencies for accurate quantification, label-based approaches are needed. Stable isotope-labeled peptide or protein standards can be spiked into to samples at known quantities [2, 3, 15]. The ionization efficiency of the endogenous "light" peptide and its labeled "heavy" standard is the same, thus the endogenous peptide's abundance is known by its relative abundance to its standard using the area under the curve method (Fig. 3a).

Peptide standards were used to quantify 17 apolipoproteins (one standard per protein) in Lp(a), VLDL, LDL and HDL, isolated from healthy individuals by density centrifugation followed by size exclusion chromatography [16]. The relative distributions of the apolipoproteins within and among the lipoproteins varied; for example, after apoA-I, apoA-II and apoC-II were the second and third most abundant proteins on HDL and after apoB, apoC-II and apoC-III on VLDL and interestingly, apoA-I and apoC-II on LDL [16].

In parallel to the spectral counting analysis highlighted in Fig. 2b, we also used peptide standards in combination with an apoA-I ELISA (enzyme-linked immunosorbent assay) to determine the pool sizes for seven apolipoproteins, apoA-I, apoA-II, apoA-IV, apoC-III, apoD, apoE and apoM, in each HDL size fraction [2]. In alpha2 for instance, the apoA-I pool size average for the three donors was ~1800 mg, whereas apoA-II and apoE were ~400 and 200 mg, respectively, and apoD was the lowest at 10 mg. Other studies have used labeled standards to estimate the apolipoprotein molecule number [16] or to monitor the relative abundances of HDL proteins from clinical samples, such as those from subjects with or without lupus; a study that identified a loss in PON3 lupus subjects with a carotid artery plaque phenotype [17].

Despite their value for accurate quantification, peptide standards are costly, approximately \$500 per peptide. Stable isotope protein standards can also be synthesized at a lower cost, provided the time and means to clone the gene of interest into the expression system is available [15]. As a consequence of the increased cost of label-based proteomics, standards are often used for only a subset of proteins using as few as one or as many as all observable peptides (if a protein standard is used) for labeling. Stable isotopes have also been used to create peptide tags in order to pool peptides derived from separate samples for a single mass spectrometric acquisition. These are generally referred to as, tandem mass tagging approaches, since quantification information is contained in the tandem MS scan (MS2). More information on tandem mass tagging and its application to HDL proteome research is provided elsewhere and in more detail [2, 18, 19].

TARGETED PROTEOMICS APPLIED TO HDL BIOLOGY

Targeted proteomics is dependent on the ability for the mass spectrometer to enrich peptides of interests and filter out unwanted background signals. The approach is very sensitive, accurate and convenient if antibodies are not available; and is often used in validation experiments following global proteomics studies [2]. Multiple reaction monitoring (MRM), or sometimes referred to as selected reaction monitoring (SRM), is the most used targeted MS method [20]. MRM is best performed on a quadrupole instrument that is specialized for isolating, one at a time, a predefined list of peptides for subsequent fragmentation, followed by isolation of a single fragment ion (transition) for detection (Fig. 3b, top panel). MRM uses narrow isolation windows (~1 Da) in order to reduce interference by background peptides, and is repeated for as many transitions the experimenter wishes to monitor. The more transitions monitored, the more confident the peptide identification. At least three transitions are usually monitored per experiment with 50 to 100 transitions per second.

In order to perform MRM, the target peptide's features, collectively referred to as a peptide library, must be known: the precursor and fragment ion m/z values, and the elution time from the chromatographic gradient. These features would have been established in previous DDA studies. Stable isotope labeled peptide standards are often implemented in targeted methods (Fig. 3b, bottom panel) since 1) as standards, their transitions should be detected at the same elution time as the endogenous counterpart and 2) they can be used for absolute quantification. However, monitoring the light and heavy peptides requires two independent scans. An alternative to MRM that has emerged in recent years is PRM, parallel reaction monitoring. Unlike MRM that is performed on a triple quadrupole instrument that scans at unit resolution (~1Da), PRM is performed on the high resolution/accurate mass (HR/AM) quadrupole Orbitrap instrument (HR-MRM for the quadrupole time-of-flight instrument) that scans up to a resolution of 240 K (at 200 m/z) [20–22]. A major advantage of PRM over MRM is that it is permissible to larger isolation windows since its HR/AM scans can differentiate target ions from background ions (Fig. 3b). As a consequence, all transitions are scanned simultaneously, permitting confident peptide identification in a single scan. In addition, the endogenous and standard peptides can be co-isolated and relative quantification done at the same time (Fig. 3b). In addition, HR/AM scans are about 10-times slower than those of MRM, thus fewer peptides are monitored per second. Nonetheless, as we will

demonstrate below, the trade-off of HR/AM for speed, makes PRM an increasingly attractive alternative to MRM for future targeted-based research.

Monitoring HDL proteins using MRM and PRM

In the last four years, there has been a steady growth in the use of MRM to study HDL proteins (Fig. 3c), however PRM made its debut in lipoprotein-related research in 2015 when it was demonstrated as an alternative to MRM for targeted HDL protein studies [46], and to monitor circulating PCSK9 in rabbits fed a dual CETP-PCSK9 small molecule inhibitor [44]. The studies in Fig. 3c have been used to monitor plasma levels of HDL proteins either related to their HDL biology directly, (ie. [2, 55] or to studies that identified HDL proteins in biomarker or target-discovery studies (ie. [58, 60]). Fig. 3d is a tree map that scales the 35 monitored HDL proteins according to the number of times they were featured in the studies in Fig. 3c. ApoA-I and apoE were the most frequently monitored using MRM or PRM (Fig. 3d).

Very recently, both MRM and PRM were used to determine the differences in the baseline proteome (before treatment) of HDL isolated from diabetic patients that either developed or were resistant to fenofibrate/rosiglitazone-induced hypoalphalipoproteinemia [55]. A [15N]-labeled apoA-I protein standard was spiked into HDL samples for subsequent proteolysis and targeted MS analysis. Its peptides served as a collective global standard for 37 other HDL protein peptides, a strategy that accounts for sample-to-sample variability without the cost of synthesizing standards for all proteins of interest. ApoA-I light and heavy peptides were measured by PRM in a manner similar to MRM, alternating the between light and heavy apoA-I target peptides in independent scans (Fig. 3b). Two peptides for the 37 other HDL proteins were also monitored, and their signals normalized to the apoA-I standard peptides. As a consequence, PON1, apoC-I, apoC-II and apoH were found to be increased in the hypoalphalipoproteinemia subjects [55].

HR/AM PRM provides a new outlook for in vivo metabolism studies

Targeted proteomics has also advanced lipoprotein metabolism studies that sometimes accompany clinical trials aimed to manage dyslipidemia [54, 64]. Typically, subjects are administered a stable isotope labeled amino acid (tracer), usually tri-deuterated-leucine (D3-Leu), that is taken up by organs such as the liver, and then incorporated into newly synthesized proteins such as apoA-I. The rate of appearance or disappearance of the tracer in circulating proteins provides information about their metabolism, and HDL itself by monitoring apoA-I metabolism [65, 66]. Gas chromatography (GC)-MS is the most common analytical method to monitor tracer enrichment by detecting the D0-Leu and D3-Leu pools; however, the MRM peptide-based detection method has been increasing in clinical use since it was introduced in 2006 [62] (Fig. 4a). In the last few years the metabolism of proteins such as apo(a), apoB, PCSK9, and CETP isolated from plasma or VLDL/LDL [27, 34, 54, 63, 64], or a subset of the classical apolipoproteins from HDL [52] have been studied in humans using MRM on the triple quadrupole platform (Fig. 4a).

However, at unit resolution, MRM cannot reliably measure tracer enrichment in slowly turning-over proteins such as most of those in HDL. Thus, the scope of in vivo metabolism

studies is limited to proteins that are rapidly metabolized (apoB, apoE) or highly abundant (apoA-I, apoB). On the other hand, as we recently demonstrated, PRM's ability to co-isolate and fragment multiple peptides (ie. D0- and D3-Leu peptides) (Fig. 3b), and perform HR/AM scans, permits the detection of tracer enrichment as low as 0.2 % [2]. Fig. 4b shows an example apoA-I fragment ion tracee peak (D0-Leu) and the m/z range that contains tracer peak (D3-Leu). The inset zooms into the tracer range where the absence (0 hour) and presence (2 and 6 hours) of the tracer, and its differentiation from numerous background peaks, is evident. The difference in mass between the tracer and the closest background peak is only 11 mDa. Using MRM, on the other hand, the tracer and background peaks would have emerged as a single peak, resulting in inaccurate enrichment calculation. Fig. 4c displays three prototypical enrichment profiles for apoA-I, apoA-II and apoE in alpha3 HDL. The panel highlights the remarkable contrast between the slowly turning over apoA-I and apoA-II, and the rapidly turning over apoE. Keeping in mind that all three proteins were isolated from the same HDL size fraction these findings underscore that research aiming to understand the extent of HDL particle heterogeneity will also have to consider the significance of the varying metabolism profiles.

In summary, PRM is a very powerful method to detect low abundant peptide signals with confidence. Although we highlight its ability to detect low tracer incorporation, other applications include monitoring the oxidized form(s) of dysfunctional apoA-I in plasma and atheroma, that are currently dependent on antibody-based technologies [67].

CONCLUSIONS

The overall finding from a number of global proteomics studies indicates that the constituents of the HDL proteome (~90 proteins) have likely all been identified. A subset of these studies have also revealed that the proteins are differentially localized across the HDL sizes supporting the notion that HDL comprises a heterogeneous population of particles, in part defined by protein content. In order to better understand the molecular interactions among all classes of lipoprotein particles, targeted MS methods such as MRM and PRM, in combination with stable isotope protein and peptide standards, are being used to quantify the relative proportions of their shared apolipoproteins. Moreover, due to the increased accuracy and sensitivity of PRM over MRM for tracer detection, PRM or other HR/AM-based technologies are likely to be more prominent in future in vivo metabolism studies in a clinical environment. Future or existing lipid management trials can now monitor the metabolism of HDL apoA-I subfractions and additional apolipoproteins in response to these therapies; potentially revealing unique and overlapping mechanisms of each therapy on HDL (and other lipoproteins') metabolism. Moreover, a similar approach can be taken using animal models for drugs currently in development, with the additional benefit of access to all compartments (lipoproteins and organs) providing a more comprehensive metabolism picture.

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KEY POINTS

- Global proteomics studies have demonstrated that the HDL proteome comprises between 90 to 100 proteins.
- The HDL proteome is differentially distributed across the HDL size fractions giving rise to a heterogeneous population of HDL particles
- There is an increase in targeted MS-based research to understand the regulation and metabolism of subsets of HDL proteins
- While MRM technology is the more commonly employed targeted MS method, PRM is likely to increase in its use for HDL protein studies
- PRM was instrumental for revealing the unique metabolic profiles of seven classical apolipoproteins across five HDL size fractions, underscoring the capability for this technology to address the many unknowns of HDL function and metabolism in basic and clinical research.

Figure 1.

An overview of data-dependent acquisition mass spectrometry for HDL proteome profiling.

a) The number of HDL proteins reported from global proteomics studies. Updated from the Davidson <http://homepages.uc.edu/~davidswm/HDLproteome.html>. b) Output from the String Database - Markov clustering of 92 HDL proteins with an inflation factor set to 2 - groups the HDL proteome into two major networks (blue, lipid metabolism; red, non-lipid metabolism related). The top four Gene Ontology biological processes are listed for each network. c) DDA mass spectrometry attempts to reduce interference of background signals by using a narrow isolation window around the selected peptide. d) An illustration of spectral counting and its dependence on the intensity of the peptide signal.

Figure 2.

Global proteomics profiling of five HDL size fractions. a) A gel lane depicting the borders for the five HDL size fractions that were proteolyzed for subsequent proteomic analysis [2]. The molecular mass marker and the corresponding size ranges are also indicated. b) Modified from [2], the proteome architecture across the HDL size fractions. 23 of the 58 reported proteins from one donor are presented.

Figure 3.

The use of stable isotope labeling to study HDL proteins. a) Stable isotope amino acids (upper panel) are introduced into peptide or protein standards, incurring a predictable mass shift (heavy form). The relative abundance of the endogenous light peptide to its heavy counterpart, which is spiked into the sample at a known quantity, provides the absolute quantity of the endogenous peptide. b) Comparison between selected reaction monitoring (SRM) and parallel reaction monitoring (PRM). c) The targeted MS strategy is growing in popularity for HDL proteome research. Publications were extracted from PUBMED when search terms “HDL protein”, “apolipoprotein”, “targeted mass spectrometry”, “SRM”, “MRM” and “PRM” were used in varying combinations. Taken from the studies in panel (c), APOA1 and APOE are the most frequent HDL proteins appearing in targeted MS studies.

Figure 4.

Targeted MS for in vivo apolipoprotein metabolism studies. a) Timeline for the use of LC-MS-based detection of tracer enrichment in apolipoproteins. b) A zoom into the mass range of an apoA-I peptide fragment scanned by PRM. The theoretical m/z values for the peptide precursors (tracee and tracer) and their respective fragments are provided. The tracee peak is very abundant, however, further zoom into the expected m/z range for the tracer is needed (inset). Using PRM, the tracer peak is easily detected at the sampled time points after injection of the D3-Leu. c) Modified from [2], example PRM-enabled enrichment curve data from alpha3 HDL.