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PROTEOMICS INVESTIGATIONS OF HDL. CHALLENGES AND PROMISE

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Abstract

High density lipoprotein (HDL) is recognized as the major negative risk factor of cardiovascular disease and number of anti-atherogenic functions has been ascribed to HDL. HDL is an assembly of a neutral lipid core and an outer shell consisting of polar lipids and proteins. It has been defined many different way based on various distinct properties including density flotation, protein composition, molecular size, and electrophoretic migration. Overall the studies characterizing HDL clearly demonstrate that it is a complex heterogeneous mixture of particles. Furthermore several studies convincingly demonstrated that certain populations of HDL particles have a distinct functionality suggesting that HDL may serve as a platform for assembly of protein complexes with very specific biological functions. Indeed recent proteomics studies described over 100 proteins associated with HDL. Here we review approaches to isolation and proteomic analysis of HDL and discuss potential problems associated with isolation methods which may confound our understanding of the relation of the HDL composition and its biological function.

INTRODUCTION

It is well established by many clinical, epidemiological and genetic studies that while increased levels of high density lipoprotein (HDL) cholesterol are associated with decreased the risk of cardiovascular disease (CVD) low levels of HDL are associated with increased risk.[1, 2] These data strongly suggest that HDL may have important anti-atherogenic properties. Probably the most studied HDL property is its ability to remove cholesterol from lipid loaded macrophages, a cellular hallmark of atherosclerosis, in a process termed reverse cholesterol transport.[3, 4] Many *in vitro* and *in vivo* studies in animal models show that this property is mediated by interaction of HDL with two receptors on the cell surface of macrophages, the adenosine triphosphate (ATP)-binding cassette transporters (ABCA1 and ABCG1), transmembrane proteins upregulated in macrophages by cholesterol uptake. While the ABCA1 is thought to mediate the transport of cholesterol and phospholipids from cells to lipid-poor apolipoproteins,[5] the ABCG1 facilitates efflux of cholesterol to mature HDL particles.[6–10] Several recent studies indicate that HDL has also anti-inflammatory properties *in vivo* [11, 12] while other studies show that HDL may have anti-oxidant properties perhaps due to presence of paraoxonase 1, PON1.[13, 14] It has been proposed that HDL's anti-atherogenic properties depend on the types of particles present *in vivo* rather than on the HDL-C level and that HDL in humans with established CVD may lose these protective properties.[12,13] Such proposals are supported by animal studies in mice deficient in scavenger receptor B-I (SRB1) and apoA-II, showing advanced atherosclerosis even though the plasma levels of HDL-C are elevated.[15–18] Moreover, in recent study Rader et al showed that HDL from people with the same HDL-C level can differ significantly in its sterol efflux capacity and that the sterol efflux capacity of the serum HDL negatively correlates with intima-media thickness independent of HDL-C level.[19] It has been also demonstrated that HDL from patients with CAD contains significantly increased concentration of oxidized tyrosine and *in vitro* studies showed that oxidative modifications

of apoA-I significantly impair sterol efflux.[11, 20–25] Several other lines of evidence also weaken the hypotheses that HDL-C levels *perse* relate to CVD status and that elevating HDL-C is necessarily therapeutic. For example, genetic variations that associate with altered HDL-C do not strongly associate with altered CVD risk,[26] certain drugs that elevate HDL levels, such as fibric acid derivatives, show no clear clinical benefit,[27] , a cholesteryl ester transfer protein (CETP) inhibitor, torcetrapib, that increased HDL-C by ~75% associated with an increased risk of CVD events, although this effect may be due to off-target activity, [28] and recently, the AIM-HIGH study was stopped prematurely due to lack of additional benefit of extended-release niacin in combination with aggressive statin treatment.

However, the precise molecular determinants of the HDL anti-atherogenic properties have not been firmly established. Unraveling of these determinants is also compounded by the heterogeneity of HDL particles and many ways the HDL is defined and isolated. Significantly, currently used methods of the HDL isolation disrupt integrity of the particles and our lack of understanding of the relation of the isolated HDL to the HDL as it exists in circulation limits our ability to address the HDL structure-function relationship.

HETEROGENEITY OF HDL

Lipoproteins represent a large class of protein-lipid complexes which are broadly classified by their flotation in density ultracentrifugation as high-density lipoproteins (HDL), low density lipoproteins (LDL), intermediate density lipoproteins (IDL) and very-low density lipoproteins (VLDL). Alternatively they can also be classified by their migration in agarose gel electrophoresis as pre-alpha, alpha-, pre-beta and beta-lipoproteins.[29, 30] When clinicians refer to the level of HDL they typically refer to plasma or serum HDL cholesterol concentration commonly determined by precipitation methods where plasma apoB containing lipoprotein particles are removed from plasma using dextrane sulfate, polyethylene glycol or heparin manganese precipitation or by homogenous methods where apoB particles are complexed with sulphated cyclodextrin and dextrane suphate, and HDL cholesterol concentration is then measured *in situ* without separation form plasma.[31, 32] However, these procedures do not isolate HDL particles and solely reflect plasma cholesterol concentration not associated with particles containing apoB.[30] To define and isolate HDL particles from plasma several distinct approaches were developed over years generally exploiting rather distinct (often orthogonal) properties of HDL. Classical definition of HDL developed in the 1950’s by Havel and coworkers[33]is based on flotation density. It defines HDL as particles in the flotation density between 1.21 and 1.063 g/ml, although sometimes denser particles floating in the density 1.21–1.25 g/ml are also considered HDL and are referred to as very high density lipoprotein (VHDL). Alternatively, HDL can also be also defined based on content of its main constitutive proteins, apoA-I and apoA-II. This approach was pioneered in 1970’s by Albers and Alaupovic who in a series of elegant studies defined several classes of HDL lipoproteins using sequential immunoaffinity chromatographic separations as Lp-A-I (all lipoprotein particles containing apoA-I), Lp-AI:A-II (particles containing apoAI and apoAII) and Lp-A-II (particles with apoA-II without apoA-I). It is notable that similar approach applied to larger (LDL and VLDL) apoB containing particles resulted in definition of five distinct subclasses of apoB containing particles as well (Lp-B, Lp-B:E, Lp-B:C, Lp-B:C:E, and Lp-A-II:B:C:D:E).[34–37] Furthermore, it has also been shown that apoA-I, a protein considered to be a defining feature of HDL is also present in VLDL.[38] As discussed above the lipoprotein particles can also be separated and defined based on electrophoretic migration properties as well as by size exclusion chromatography [39] and isoelectric focusing, [40] and isotachophoresis. [41, 42] Collectively, studies focused on isolation of HDL particles clearly show the complexity of defining lipoprotein classes, their polydispersion and the challenges associated with their proteomic analysis.[30]

ISOLATION OF HDL AND ITS CHARACTERIZATION BY MASS SPECTROMETRY

While clinically the HDL cholesterol has been the central focus of the studies focusing on the role of HDL in atherogenesis and cardiovascular disease, the HDL protein component has been investigated primarily from the perspective of its role in lipid metabolism. Only with the recent major advances of mass spectrometric techniques mainly the soft ionization techniques, matrix assisted laser desorption laser ionization (MALDI)[43] and especially electrospray ionization (ESI)[44] over last 20 years, which allowed direct analysis of proteins, has global characterization of the HDL proteome moved into the focus. Since the late nineties a number of studies have explored the protein composition of HDL using various isolation and separation techniques.[45] Both the isolation approach and the separation of the isolated proteins in HDL have major consequences for the eventual findings of such studies. As discussed above the method of HDL isolation affects the type of particles isolated as well as their integrity since each methods isolates different population of the particles. At the same time separation of HDL proteins and analytical approach to protein identification in the isolated HDL particles have also major influence on the results of the HDL proteomics studies. One of the major issues of global protein characterization of HDL proteome is establishing specificity of association with HDL as opposed to contamination with plasma proteins as well as with other lipoprotein classes, mainly LDL and lipoprotein (a) (Lp(a)). For instance, while ultracentrifugation is well established as a method of HDL isolation it does not provide complete separation from either plasma proteins or LDL, and Lp(a). In fact populations of small dense LDL particles as well as Lp(a) particles may appear in the density range of large buoyant HDL_2 particles (1.063–1.125 g/ml).[46, 47] This is reflected in many HDL proteomics studies where small amounts of apoB, and or apo(a) as well as albumin are detected with albumin predominantly detected in the HDL_3 fraction and apoB and apo(a) in the HDL_2 fraction. Furthermore, the classical ultracentrifugation exposes the HDL particles to high ionic strength and high sheer stress conditions which result in stripping off multiple proteins during isolation and contribute to particle scrambling.[48] Although a low ionic strength buffer approach has been developed using D_2O/sucrose density gradient it does not alleviate the stress imposed on the particles by high-speed centrifugation.[49] In contrast to ultracentrifugation, specificity of immunoaffinity isolation of HDL with antibody against apoA-I is easily controlled for with proper control columns coupled with non-specific or pre-immunization antibody.[50] While both ultracentrifugation and immunoaffinity isolate HDL based on lipoprotein specific property thus providing great degree of specificity, the isolation of HDL based on its size by size exclusion chromatography (SEC) relies only on the HDL size for separation. This approach is therefore highly non-specific since many proteins and protein complexes in plasma also have apparent molecular size in the range of 100,000–200,000 Da with many of them being more abundant in plasma than apoA-I, e.g. immunoglobulins, complement C3, and are co-eluting with HDL.[51, 52] Although it can be argued that elution of these proteins across a wide size range outside of their apparent molecular weight range may indicate association with HDL, these proteins form multimers as well as multiprotein complexes, which will result in elution across a wide range of MW at levels easily detected by mass spectrometer.[52] Thus without high resolution FPLC (e.g. multiple columns employed in series) and quantitative analysis of co-elution with constitutive apolipoproteins (e.g. apoA-I) conclusions about protein association with HDL cannot be made from SEC. Recently, Gordon et al proposed a novel way to characterize HDL as “lipoprotein particles in the HDL size range (~120–250 kDa) with high-phospholipid content”.[53] The approach is based on the combination of extensive SEC separation (3 Supradex200 columns in series) with selective capture of phospholipids on calcium silicate beads from fractions in the HDL size range. The captured HDL particles are then digested with trypsin directly on the silicate

beads and digested peptides are eluted for further identification by liquid chromatography-mass spectrometry (LCMS) analysis. The method thus provides an alternative way for identification of HDL associated proteins and allows insights into specific associations of proteins in HDL. Unfortunately the approach does not allow isolation of the intact HDL particles due to harsh conditions necessary to elute captured particles from the silicate beads and thus prevents their functional characterization.

Second dimension of complexity in the HDL proteome characterization arises from the plethora of methods used to identify HDL associated proteins once the HDL particles are isolated. In general three approaches have been used so far to characterize HDL proteome. First approach uses two-dimensional gel electrophoresis with isoelectric focusing in the first dimension and SDS-PAGE size separation in the second dimension.[54, 55] Proteins separated are then visualized by protein staining, detected spots are excised and proteins identified after in-gel digestion with trypsin followed by mass spectrometric analysis on matrix assisted laser desorption ionization mass spectrometers (MALDI-MS) or by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with electrospray ionization (ESI). While MALDI-MS provides rapid analysis it tends to fail if more than few (3–4) proteins are present in the spot on the gel (surprisingly frequent situation), the LC-MS/MS is much more time consuming and generates complex data, but it can easily identify multiple proteins in the same sample. While the sensitivity and throughput of the two-dimensional electrophoresis (2DE) approach is limited, it has a unique ability to detect protein isoforms, protein fragments as well as allows for separation post-translational modified proteins. Relative quantitation for the 2DE is based on the protein staining with limited dynamic range and sensitivity. Furthermore, matching the same spots in multiple gels is complicated by variability of protein migration in both dimensions of the separation.

Second approach is based on direct LC-MS/MS analysis of enzymatic (largely tryptic) digests of the HDL proteins without prior protein separation. This approach is commonly referred to as “shotgun proteomics” or bottom-up proteomics.[56] The key to this approach is the ease with which small peptides (6–20 amino acids long) are analyzed and sequenced by liquid chromatography combined with tandem mass spectrometry. Peptides generated by tryptic digestion are separated by liquid chromatography and tandem mass spectra of the peptides are acquired using data-dependent acquisition. The sequence information of individual peptides is then obtained by matching of the experimental mass spectra to the mass spectra generated *in silico* from protein sequence databases using variety of search engines (e.g. Sequest, MASCOT, X!tandem, etc.). Peptide identifications are then evaluated for validity (e.g. probability of the true identifications, estimation of false discovery rate) and identity of proteins is inferred from combination of peptide identifications. While this approach provides unprecedented sensitivity and dynamic range for protein identification, it is important to note that for majority of proteins the identification is based on only a small number of peptides therefore reflecting only small fraction of protein sequence. Consequently, very often the information about protein sequence integrity (full length protein vs. protein fragment), protein isoforms and post-translational modifications is lost. Since the shotgun proteomics is the most popular proteomics method, number of approaches to relative quantitation have been developed over the years (see below).

Lastly, a group of approaches which directly analyze intact proteins in the sample has been applied to HDL. Such approach can be based on laser desorption ionization techniques MALDI and its close relative - surface enhanced laser desorption ionization (SELDI).[57] Both of these approaches are fast and easily amenable to high throughput analysis and could easily analyze hundreds of samples a day, which could make them useful as global screening tools. Although these approaches were used for HDL analysis they lack the ability to identify proteins and in general rapidly loose sensitivity with increasing molecular weight

with practical limit of 30–40 kDa. Furthermore, the resolution and precision of the mass measurement further limits their utility. Direct analysis of intact proteins can also be accomplished using LC-MS/MS with electrospray ionization in so call top-down approach.[58] Although this approach would appear the most intuitive since it does not loose information about the protein structure and modifications there are several significant drawbacks to its practical use. In contrast to the shotgun proteomics both chromatographic separation and tandem mass spectrometric analysis of intact proteins are much more complicated than analysis of peptides and while top-down proteomics has seen great advances over the past decade it is still limited to simple protein mixtures and employed by only a small group of laboratories.

PROTEIN COMPOSITION OF HDL

Traditionally, HDL has been viewed only as a component of the cholesterol homeostasis machinery, a concept reflected by naming the proteins identified in HDL (and other lipoprotein particles) as apolipoproteins. Perhaps first indication that HDL may carry proteins with other functions than those related to lipid metabolism came with identification of apoJ (Clusterin), a component of the membrane attack complex of the complement system. In recent years a number of studies focused on identification of HDL associated proteins. Up today over 110 proteins were identified in HDL by various approaches (Table 1). Strikingly however, the overlap in protein identifications between more than 2 different studies is less than 30 proteins. This significant discrepancy between different studies reflects the multiplicity of HDL definitions, isolation approaches as well as analytical approaches discussed in the previous sections. While majority of the studies used ultracentrifugation as means of isolation of the HDL particles prior to proteomics analysis, individual procedures varied significantly as did steps of the sample preparation following HDL isolation (e.g. direct analysis or delipidation) and the analytical methods used to identify proteins. In one of the first proteomics studies Heller et all used single-step ultracentrifugation followed by combination of SDS-PAGE, native PAGE, 2DE and LC-MS/MS identification of selected spots in the gels to identify 24 proteins in HDL from a single pool of plasma.[59] Rezaee et al. used single step ultracentrifugation and immunoaffinity chromatography against apoA-I and apoA-II to isolate HDL from plasma and characterized it by SDS-PAGE or 2DE combined with MALDI-MS analysis, or by LC-MS/MS combined with isotope coded affinity tags (ICAT) methodology.[55] Although overall they identified 57 proteins in HDL by various techniques, majority (33) were identified by the ICAT approach rising concerns about validity of many of these protein identifications. Because ICAT approach selects only cysteine containing peptides for the LC-MS/MS analysis, the identifications are often based on a single peptide MS/MS resulting in large number of false positive identifications. Protein identifications based on a single peptide have been deemed by proteomics community as unacceptable for unequivocal protein identification.[60, 61] In addition, several other studies used ultracentrifugation isolated HDL for focused studies aimed at identification of specific proteins in HDL (Table 1).[62–66] To characterize HDL proteome globally we employed two-stage ultracentrifugation to isolate HDL_3, the dense sub-fraction of HDL, from plasma of seven apparently healthy subjects and used shotgun proteomics and two-dimensional LC-MS/MS with minimal sample manipulation to characterize HDL proteome.[56] After applying stringent protein identification criteria which minimized false discovery rate to less than 2%, we reproducibly identified 48 proteins associated with HDL_3. Significantly we were able to detect 22 of 23 proteins known to reside in HDL. We classified the identified proteins using Gene Ontology (GO) analysis, an unbiased approach testing enrichment of a set of proteins for specific functional categories compared to the whole proteome. Strikingly, only 22 proteins were associated with lipid metabolism, while the majority of the proteins were associated with other biological functions including inflammation, complement activation,

and proteolysis. These findings further support the hypothesis that HDL proteins may contribute to other HDL functions than cholesterol homeostasis. Discovery of number of protease inhibitors (serpins) in HDL suggests that HDL may be involved in control of proteolysis, a critical process involved in the atherosclerotic plaque rupture. Association of several complement and coagulation cascade proteins suggest potential for HDL role in limiting activation of coagulation cascade and mitigating tissue damage by dampening of complement activation. Recent advances in separation and mass spectrometric technology facilitated further improvements in the detection limits and ability to probe the HDL proteome in more detail and we are now able to routinely and reproducibly detect over 60 in HDL (unpublished results). Identification of such a large number of proteins raises important question about the stoichiometry of the HDL particles. Indeed it is hard to imagine that the 60+ proteins can coexist on a single HDL particle. As discussed above number of studies show that HDL is a heterogeneous mixture of particles. Recent studies of Asztalos and Schaefer clearly demonstrated that apoA-I in plasma forms number of distinct particle populations and that other apolipoproteins specifically associate with subsets of these apoA-I particle populations.[67] Furthermore, particles isolated by immunoaffinity with antibodies against apoA-I and apoA-II can be separated by size to several broad peaks suggesting multiple populations of particles within each Lp-A-I and Lp-A-I:A-II.[68] Collectively, these studies support the novel concept of the HDL as a platform for assembly of protein complexes with specific function. This hypothesis is further supported by recent discovery that HDL carries three proteins– apoA-I, apoL1 and haptoglobin-related protein, which form the trypanosome lytic factor (TLF), a complex capable of killing protozoan parasites responsible for African Sleeping Sickness.[69, 70] Furthermore, we recently used immunoaffinity chromatography to isolate HDL particles containing phospholipid transfer protein (PLTP), a protein involved in remodeling of HDL.[50] Strikingly, these complexes consisted of three main protein components – PLTP, apoJ and apoA-I with an unexpected stoichiometry 1:5:1 and contained only low amount of lipid, consistent with earlier findings which showed majority of PLTP activity in the density range of 1.25–1.21 g/ml.[71] The immunoaffinity isolation of specific proteins can only address a predetermined hypothesis about the protein associations. To investigate the assembly of specific protein complexes in HDL globally, Davidson et al used two unbiased approaches. Using combination of HDL fractionation by isopycnic ultracentrifugation, LC-MS/MS and bioinformatics analysis they identified 5 specific groups of HDL proteins which correlated in their distribution over the 5 density fractions. Moreover, they demonstrated that distribution of PON1 and PON3 strongly correlated with ability of the HDL fractions to attenuate LDL oxidation suggesting that such specific protein associations may be important for various HDL functions.[72] To extend their studies they used combination of SEC and phospholipid capture (see above).[53] While apoA-I and to a large part apoA-II were observed across the whole range of HDL molecular sizes, other proteins eluted in very specific groups suggesting possible associations in specific complexes. Indeed, haptoglobin-related protein (HPR) and apoL1, components of trypanosome lytic factor, tightly correlated, suggesting that this approach is valid and has great promise for unraveling of the protein assembly in HDL.[53] Yet unexplored by global proteomics approaches remain the HDL particles isolated by immunoaffinity. Several studies of such particles isolated by immunosorption using antibodies against apoA-I have shown that they are more polydisperse and contain more total protein compared to the particles isolated by ultracentrifugation.[73] Moreover, several proteins including apoJ and haptoglobin are found at much higher relative abundance in LpA-I particles compared to ultracentrifuge isolated HDL providing further evidence for alteration of the HDL during ultracentrifugation isolation.[74] The gentle elution conditions and the ability of immunoaffinity to isolate intact particles from plasma for further functional investigations make this approach a viable alternative to ultracentrifugation. Collectively, the data from the proteomics investigations of HDL provide strong evidence that HDL is much more complex lipoprotein than previously thought and many lines of

evidence suggest that it may serve as a platform for assembly of protein complexes which may have specific functions related to atherogenesis as well as immunity.

HDL PROTEOME IN DISEASE: FUNCTIONAL DETERMINANT OR BIOMARKER?

The identification of over 100 proteins in HDL opens up an important question of the relation of these proteins to the HDL's anti-atherogenic properties. While the traditional concept links levels of HDL cholesterol with protection against atherosclerosis and cardiovascular disease, recent papers from Rader's laboratory [19, 75] convincingly separate HDL cholesterol level from sterol efflux capacity suggesting that proteins in HDL may be related to this major protective function of HDL. Several lines of evidence relate cardiovascular disease to HDL remodeling. First, analysis of plasma distribution of apoA-I by the two dimensional native gels showed that larger alpha- and pre-alpha migrating particles are decreased in the subjects with cardiovascular disease while the concentration of smaller alpha particles increases significantly.[76] Second, in normolipidemic as well as hyperlipidemic subjects with CAD the distribution of immunoaffinity purified lipoprotein particles Lp-A-I A-II as well as Lp-A-I without apoA-II was significantly shifted towards smaller particles in the size range of 7.0–8.2 nm.[77] Lastly, we have recently showed that proteome of the HDL_3, the smaller, denser HDL particles, is significantly altered in the subjects with stable coronary artery disease (CAD). In the HDL_3 of 7 subjects with stable CAD several proteins including apoE, complement C3, PON1, apoA-IV and apoC-IV were significantly enriched compared to control subjects.[56] We then confirmed the elevated levels of apoE in an independent cohort of 32 subjects with CAD and 32 matched controls. Significantly, elevation of apoE in HDL with CAD was also independently observed in the CARE study.[78] In a separate study we have investigated HDL_2 proteome of people with CAD using MALDI- MS of HDL_2 tryptic digests and pattern recognition analysis in a population of 19 CAD subjects and 20 apparently healthy controls. Model built using partial least square discriminate analysis based on MALDI-MS signature of HDL clearly differentiated control from CAD subjects. Furthermore, targeted LC-MS/MS analysis of specific spectral features distinguishing the two classes showed elevated levels of apo(a), apoC-III as well as increased levels of methionine oxidation in apoA-I.[79]

Therapeutic interventions have been used to increase HDL-C levels, however little evidence has shown how these therapies affect HDL composition and its function. Combined statin and niacin therapy provide modest increases in HDL-C. We therefore investigated whether the therapy also alters protein composition of HDL. To address this question we used shotgun proteomics to analyze HDL isolated from 7 subjects with stable CAD before and after 1 year of combined simvastatin and niacin therapy.[80] Strikingly, we found that the therapy partially reversed the changes in HDL associated with cardiovascular disease. Significantly, we observed decrease of apoE content in HDL3 while other proteins previously implicated in cholesterol transport, including phospholipid transfer protein (PLTP), apoF and apoJ, were significantly enriched after therapy. While the elevation of HDL-C after the combined therapy is likely due to the known effects of niacin, given the widespread use of statins it would be extremely important to investigate the effects of each drug alone.

Cholesteryl ester transfer protein (CETP), an HDL associated protein, plays critical role in remodeling of HDL by mediating trafficking of cholesterol ester from HDL to LDL. CETP deficiency results in significant increase of HDL-C and CETP inhibitors have been aggressively pursued as a way of HDL-C elevation.[81] Study of HDL in people with CETP deficiency demonstrated that this HDL has improved ability to efflux cholesterol from lipid loaded macrophages.[82] These findings were also extended to people treated with CETP

inhibitor torcetrapib.[83] Although torcetrapib failed in clinical trials,[28] likely due to off-target effects,[84] CETP inhibitors are actively pursued and latest results for dalcetrapib and anacetrapib showed significant increases of HDL-C accompanied by improvements of reverse cholesterol transport.[85] However, the understanding the mechanism of the effects of CETP inhibition on HDL function and its protein composition are currently limited.

Collectively, the observations of changes in the HDL proteome associated with cardiovascular disease and the positive effects of lipid lowering therapy raise the possibility that the analysis of HDL proteome may serve both as a surrogate measure of HDL function as well as a diagnostic tool and a measurement of treatment efficacy.

FUTURE DIRECTIONS IN HDL PROTEOMICS

Although immense body of data exists on HDL isolated by ultracentrifugation conclusive evaluation of the relevance of the isolated HDL to the HDL as it exists in the circulation remains one of the important areas of HDL research. While many HDL functional assays can be easily adapted to high throughput format, the HDL isolation represents a significant bottleneck for performing large scale clinical studies which would be required to establish HDL protein composition–function relationship in humans. Immunoaffinity isolation offers an attractive alternative to ultracentrifugation which can be adapted to 96-well format for parallel processing in high-throughput format. However, its relevance to plasma HDL remains to be addressed.

Large scale structure-function studies will also require major advances in the protein analysis. So far majority of HDL proteomics studies focused on qualitative description of HDL proteome and only limited number of studies focused on protein quantitation. Heller et all used protein identification index based on peptide identification scores to quantify several major HDL proteins is normolipidemic and hyperlipidemic subjects and showed that in hyperlipidemic subjects relative abundance in several of these proteins was altered.[86] We used spectral counting, a method where protein abundance is reflected by the number of instances (MS/MS spectra) a protein presence was detected, to quantify changes of protein abundance in subjects with CAD and in subjects treated with lipid lowering therapy. While both of these techniques are simple and readily available for typical shotgun proteomics experiments, they provide only semi-quantitative information. Moreover, these methods do not work very well for low abundance proteins with only small number of peptides identified and consequently low spectral counts. Thus these methods can only detect large changes with relatively large variability in the data (typical CV more than 25–30%). Recently, proteomics field adapted highly quantitative approach of multiple-reaction monitoring (MRM) used in the past extensively in pharmaceutical industry for quantification of small molecules. Unlike shotgun proteomics MRM-based quantitative methods are targeted and require prior knowledge of the molecules to be quantified, i.e. term targeted proteomics quantification. In targeted MRM proteomics, peptides produced by protease digestion, typically with trypsin, serve as surrogate markers of protein abundance. Targeted proteomic assays offer many advantages over traditional immunoassays used for protein quantification in biological samples, namely MRM-based methods can be rapidly developed and validated.[87] Furthermore, such assays are readily multiplexed for quantification of many proteins (>50) in a single analysis over wide range of relative concentrations without mutual interference often found in multiplexed immunoassays.[88] Furthermore, when combined with isotope dilution using stable isotope-labeled peptides as internal standards, MRM has been established as the most promising approach to precise relative protein quantification.[89–92] Thus targeted MRM protein quantification could provide accurate determination of essentially all proteins identified in HDL so far in a single analysis with a throughput compatible with hundreds of samples needed for analysis of samples from

clinical trials. When coupled with a high-throughput HDL isolation and HDL functional assays this approach could be a powerful tool for understanding the role of HDL as mediator and/or a biomarker of cardiovascular disease and a measure of efficacy of therapeutic interventions.

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Table 1

HDL associated proteins.

Name	Gene name	Gene I.D.	Accession #	IPI	Swiss Prot Entry name	Study count	Reference
alpha-1B-glycoprotein	A1BG	1	P04217	IPI00022895	A1BG_HUMAN	3	56,63,55
alpha-2-macroglobulin	A2M	2	P01023	IPI00478003	A2MG_HUMAN	2	59,53
actin, aortic smooth muscle	ACTA2	59	P62736	IPI00008603	ACTA_HUMAN	1	59
angiotensinogen	AGT	183	P01019	IPI00032220	ANGT_HUMAN	1	56
alpha-2HS-glycoprotein	AHSG	197	P02765	IPI00953689	FETUA_HUMAN	3	59,56,53
albumin	ALB	253	P02768	IPI00745872	ALBU_HUMAN	3	59,56,72
Protein AMBP	AMBP	259	P02760	IPI00022426	AMBP_HUMAN	1	56
alpha-amylase 1	AMY1A, AMY1B, AMY1C	276,277,278	P04745	IPI00300786	AMY1_HUMAN	1	54
serum amyloid P-component	APCS	325	P02743	IPI00022391	SAMP_HUMAN	1	55
apolipoprotein A-I	APOA1	335	P02647	IPI00021841	APOA1_HUMAN	7	59,56,63,55,54,65,72
apolipoprotein A-V	APOA5	116519	Q6Q788	IPI00465378	APOA5_HUMAN	1	66
apolipoprotein A-II	APOA2	336	P02652	IPI00021854	APOA2_HUMAN	7	59,56,55,54,74,72,53
apolipoprotein A-IV	APOA4	337	P06727	IPI00304273	APOA4_HUMAN	6	59,56,55,54,74,72
apolipoprotein B-100	APOB	338	P04166	IPI00022229	APOB_HUMAN	3	56,72,53
apolipoprotein C-I	APOC1	341	P02654	IPI00021855	APOC1_HUMAN	5	59,56,55,54,72
apolipoprotein C-II	APOC2	344	P02655	IPI00021856	APOC2_HUMAN	5	59,56,55,54,72
apolipoprotein C-III	APOC3	345	P02656	IPI00021857	APOC3_HUMAN	7	59,56,55,54,74,72,53
apolipoprotein C-IV	APOC4	346	P55056	IPI00022731	APOC4_HUMAN	2	56,72
apolipoprotein D	APOD	347	P05090	IPI00006662	APOD_HUMAN	5	59,56,55,74,72
apolipoprotein E	APOE	348	P02649	IPI00021842	APOE_HUMAN	7	59,56,55,54,74,72,53
apolipoprotein F	APOF	319	Q53790	IPI00299435	APOF_HUMAN	3	59,56,72
beta-2-glycoprotein 1	APOH	350	P02749	IPI00298828	APOH_HUMAN	2	56,53
apolipoprotein L1	APOL1	8542	O66791	IPI00186903	APOL1_HUMAN	7	59,56,63,55,54,72,53
apolipoprotein M	APOM	55937	O95445	IPI00030739	APOM_HUMAN	5	56,55,54,72,53
complement C1q (Chain A, Chain C)	C1QA	712	P02745	IPI00022392	C1QA_HUMAN	1	56
complement C1r	C1R	715	P00736	IPI00296165	C1R_HUMAN	1	56
complement C1s subcomponent	C1S	716	P09871	IPI00017696	C1S_HUMAN	1	53

Name	Gene name	Gene I.D.	Accession #	IPI	Swiss Prot Entry name	Study count	Reference
Cip1-interacting zinc finger protein	C1Z1	25792	Q9ULV3	IPI00220051	CIZ1_HUMAN	1	55
complement C2	C2	717	P06681	IPI00303963	CO2_HUMAN	1	53
complement C3	C3	718	P01024	IPI00783987	CO3_HUMAN	3	56,55,53
complement C4a	C4A	720	P0C0L4	IPI00032258	CO4A_HUMAN	1	56
complement C4b	C4B	721	P0C0L5	IPI00418163	CO4B_HUMAN	2	56,53
C4b-binding protein alpha chain	C4BPA	722	P04003	IPI00021727	C4BPA_HUMAN	1	74
complement C5	C5	727	P01031	IPI00032291	CO5_HUMAN	1	53
complement C9	C9	735	P02748	IPI00022395	CO9_HUMAN	1	56
T-cell surface glycoprotein CD5	CD5	921	P06127	IPI00025383	CD5_HUMAN	1	56
Cholesteryl ester transfer protein	CETP	1071	P11597	IPI00006173	CETP_HUMAN	3	56,55,74
complement factor B	CFB	629	P00751	IPI00218508	CFAB_HUMAN	1	53
complement factor H	CFH	3075	P08603	IPI00029739	CFAH_HUMAN	1	55
tetranectin	CLEC3B	7123	P05452	IPI00009028	TETN_HUMAN	1	53
clusterin	CLU	1191	P10909	IPI00291262	CLUS_HUMAN	5	56,55,74,72,53
ceruloplasmin	CP	5356	P00450	IPI00017601	CERU_HUMAN	1	55
C-reactive protein	CRP	6601	P02741	IPI00022389	CRP_HUMAN	1	55
macrophage colony-stimulating factor 1	CSF1	6635	P09603	IPI00015881	CSF1_HUMAN	1	55
prothrombin	F2	2667	P00734	IPI00019568	THRB_HUMAN	2	72,53
coagulation factor V	F5	2153	P12259	IPI00478809	FA5_HUMAN	1	56
coagulation factor VIII-associated protein	F8B	2157	Q66286	IPI00925597	Q66286_HUMAN	1	55
fibrinogen alpha chain	FGA	2243	P02671	IPI00021885	FIBA_HUMAN	5	56,74,72,53
fibrinogen beta chain	FGB	2244	P02675	IPI00298497	FIBB_HUMAN	1	63
fibrinogen gamma chain	FGG	2266	P02679	IPI00021891	FUBG_HUMAN	2	56
growth arrest-specific gene-6	GAS6	2621	Q66393	IPI00032532	GAS6_HUMAN	1	55
glutathione peroxidase 1	GPX1	2876	P07203	IPI00927606	GPX1_HUMAN	1	62
hemoglobin subunit alpha	HBA1, HBA2	3039, 3040	P69905	IPI00410766	HBA_HUMAN	1	65
hemoglobin subunit beta	HBB	3043	P68871	IPI00654755	HBB_HUMAN	1	65
histone H2A	HIST1H2AG	8329	P0C0S8	IPI00291764	H2A1_HUMAN	1	56
HLA class I histocompatibility	HLA-A	3105	P01892	IPI00876963	1A02_HUMAN	1	55

Name	Gene name	Gene I.D.	Accession #	IPI	Swiss Prot Entry name	Study count	Reference
antigen, A-2 alpha chain							
haptoglobin	HP	3240	P00738	IPI00641737	HPT_HUMAN	2	55,74
haptoglobin-related protein	HPR	3250	P00739	IPI00477597	HPTR_HUMAN	4	59,56,72,53
hemopexin	HPX	3263	P02790	IPI00022488	HEMO_HUMAN	2	56,53
histidine rich glycoprotein	HRG	3273	P04196	IPI00022371	HRG_HUMAN	1	56
isocitrate dehydrogenase [NAD] subunit alpha mitochondrial	IDH3A	3419	P50253	IPI00030702	IDH3A_HUMAN	1	59
insulin-like growth factor-binding protein	IGFBP1	3484	P08833	IPI00031086	IBP1_HUMAN	1	53
Ig lambda chain C regions	IGLC1		P0CG04	IPI00719373	LAC1_HUMAN	1	53
inter-a-trypsin inhibitor chain H1	ITIH1	3697	P19827	IPI00292530.	ITIH1_HUMAN	1	53
inter-a-trypsin inhibitor chain H2	ITIH2	3698	P19823	IPI00305461	ITIH2_HUMAN	1	53
inter-a-trypsin inhibitor chain H3	ITIH3	3699	Q06033	IPI00028453	ITIH3_HUMAN	1	53
inter-a-trypsin inhibitor chain H4	ITIH4	3700	Q66624	IPI00218192	ITIH4_HUMAN	3	56,63,53
plasma kallikrein	KLKB1	3818	P03952	IPI00654888	KLKB1_HUMAN	1	55
kininogen-1	KNG1	3827	P01042	IPI00032328	KNG1_HUMAN	2	56,53
lipopolysaccharide-binding protein	LBP	3929	P18428	IPI00032311	LBP_HUMAN	1	63
Phosphatidylcholine-sterol acyltransferase	LCAT	3931	P04180	IPI00022331	LCAT_HUMAN	2	56,74
galectin-7	LGALS7	3963	P47929	IPI00219221	LEG7_HUMAN	1	59
apolipoprotein(a)	LPA	4018	P08519	IPI00029628	APOA_HUMAN	1	72
lipoprotein lipase	LPL	4023	P06858	IPI00027847	LIPL_HUMAN	1	55
latent-transforming growth factor beta-binding protein 2	LTBP2	4053	Q66767	IPI00292150	LTBP2_HUMAN	1	55
lumican	LUM	4060	P51884	IPI00020986	LUM_HUMAN	1	53
bifunctional protein NCOAT	MGEA5	10724	O60502	IPI00465449.	NCOAT_HUMAN	1	55
neurogenic locus notch homolog protein 1	NOTCH1	4851	P46531	IPI00412982	NOTC1_HUMAN	1	55
alpha-1-acid glycoprotein 1	ORM1	5004	P02763	IPI00022429	A1AG1_HUMAN	1	59
alpha-1-acid glycoprotein 2	ORM2	5005	P19652	IPI00020091	A1AG2_HUMAN	1	56
platelet activating factor-acetyl hydrolase subunit 1B	PAFAH1B1	5048	P43034	IPI00218728	LIS1_HUMAN	1	72
prenylcysteine oxidase1	PCYOX1	56649	Q9UHG3	IPI00384280	PCYOX_HUMAN	1	56

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platelet-activating factor acetylhydrolase	PLA2G7	7941	Q53093	IPI00011588.	PAFA_HUMAN	1	64
plasminogen	PLG	5340	P00747	IPI00019580	PLMN_HUMAN	1	56
phospholipid transfer protein	PLTP	5360	P55058	IPI00217778	PLTP_HUMAN	2	56,72
serum paraoxonase/arylesterase 1	PON1	5444	P27629	IPI00218732	PON1_HUMAN	5	59,56,55,72,53
serum paraoxonase/lactonase 3	PON3	5446	Q64626	IPI00299778	PON3_HUMAN	2	56,72
platelet basic protein	PPBP	5473	P02775	IPI00022445	CXCL7_HUMAN	1	72
pregnancy-specific beta-1-lycoprotein 1	PSG1	5669	P62664	IPI00623720	PSG1_HUMAN	2	63, 55
retinol-binding protein 4	RBP4	5950	P02753	IPI00022420	RET4_HUMAN	1	56
SAA	SAA1,SAA2	62855, 6289	P02735	IPI00552578	SAA_HUMAN	3	56,54,72
SAA4	SAA4	6291	P35542	IPI00019399	SAA4_HUMAN	5	59,56,55,54,72
alpha-1-antitrypsin inhibitor	SERPINA1	5265	P01009	IPI00553177	A1AT_HUMAN	5	56,63,54,72,53
alpha-1-antichymotrypsin	SERPINA3	12	P01011	IPI00550991	AACT_HUMAN	1	53
kallistatin	SERPINA4	5267	P29622	IPI00328609	KAIN_HUMAN	1	53
antithrombin-III	SERPINC1	462	P01008	IPI00032179	ANT3_HUMAN	1	53
heparin cofactor 2	SERPIND1	3053	P05546	IPI00879573	HEP2_HUMAN	1	53
pigment epithelium-derived factor	SERPINF1	5176	P36955	IPI00006626	PEDF_HUMAN	2	56,53
alpha-2-antiplasmin	SERPINF2	5345	P08697	IPI00879231	A2AP_HUMAN	2	56,53
plasma protease C1 inhibitor	SERPING1	710	P05645	IPI00291866	IC1_HUMAN	2	55,53
SH2 domain-containing protein 1A	SH2D1A	4068	O60880	IPI00032401	SH21A_HUMAN	1	55
sialic acid binding Ig-like lectin 5	SIGLEC5	626532	Q96RL6	IPI00385641	SIG11_HUMAN	1	55
serotransferrin	TF	7018	P02787	IPI00022463	TRFE_HUMAN	3	59,56,55
tissue factor pathway inhibitor	TFPI	7035	P10646	IPI00021834	TFPI1_HUMAN	1	55
transthyretin	TTR	7276	P02766	IPI00022432	TTHY_HUMAN	5	56,63,55,72,53
vitamin D binding protein	VTDB	2638	P02774	IPI00555812	VTDB_HUMAN	3	59,56,53
vitronectin	VTN	7448	P04004	IPI00298972	VTNC_HUMAN	2	56,53
C-type lectin super family member1			gi 5036237			1	55
desmocollin			gi53435361			1	55
dj675G8.1(novel zinc finger protein)			gi 62437835			1	55

Curr Vasc Pharmacol. Author manuscript; available in PMC 2013 July 01.

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dj733D64.1			gi 3702537			1	55
dj758N20.1 (protein kinase)			gi 62693357			1	55
HSA			gi 178345			1	55
hypothetical protein dj1057B20.2			gi 11034845			1	55
IgG			gi10334541, P99007			1	55
insulinoma-associated protein I A-6			gi 66211925			1	55
KIAA 0675 gene product			gi 53643803			1	55
KIAA 1095			gi 5689527			1	55
KIAA 1730 protein			gi 12698005			1	55
latent transforming growth factor beta			gi 3327808			1	55
lymphocyte antigen			gi 553540			1	55
POU 5 domain protein			gi 12382246			1	55
protein tyrosine phosphatase			gi53645209			1	55
ryanodine receptor 2			gi 53638463			1	55
TAT-interactive protein, 72-kDa			gi 6627566			1	55
unknown protein			gi 12802992			1	55
unknown protein			gi 12653035			1	55
unnamed protein product			gi 10435007			1	55