

Thematic Review Series: High Density Lipoprotein Structure, Function, and Metabolism

Proteomic diversity of high density lipoproteins: our emerging understanding of its importance in lipid transport and beyond¹

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Abstract Recent applications of mass spectrometry technology have dramatically increased our understanding of the proteomic diversity of high density lipoproteins (HDL). Depending on the method of HDL isolation, upwards of 85 proteins have been identified, and the list continues to grow. In addition to proteins consistent with traditionally accepted roles in lipid transport, HDL carries surprising constituents, such as members of the complement pathway, protease inhibitors involved in hemostasis, acute-phase response proteins, immune function mediators, and even metal-binding proteins. This compositional diversity fits well with hundreds of studies demonstrating a wide functional pleiotropy, including roles in lipid transport, oxidation, inflammation, hemostasis, and immunity.¹ This review summarizes the progression of our understanding of HDL proteomic complexity and points out key experimental observations that reinforce the functional diversity of HDL. The possibility of specific HDL subspecies with distinct functions, the evidence supporting this concept, and some of the best examples of experimentally defined HDL subspecies are also discussed. Finally, key challenges facing the field are highlighted, particularly the need to identify and define the function of HDL subspecies to better inform attempts to pharmacologically manipulate HDL for the benefit of cardiovascular disease and possibly other maladies.—Shah, A. S., L. Tan, J. L. Long, and W. S. Davidson. **Proteomic diversity of high density lipoproteins: our emerging understanding of its importance in lipid transport and beyond.** *J. Lipid Res.* 2013. 54: 2575–2585.

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HDL PROTEIN HETEROGENEITY: A BRIEF HISTORY

Working at the Pasteur Institute in Paris in 1929, Michel Macheboeuf reported the first detailed isolation of a lipid-rich “ α -globulin” from horse serum, a fraction that would later become known as high density lipoprotein (HDL) (1). Although relatively rapid progress was made at characterizing the lipid composition of this fraction - phospholipids (PL, ~25%), cholesterol (~4%), triglycerides (TG, ~3%) and cholesteryl esters (CE, ~12%) - it took nearly 40 years to begin to sort out the complexities of the protein component. Part of the delay may have resulted from the widespread belief that “apo-HDL” was likely single protein entity, much like low-density lipoprotein (LDL), which was dominated by a single polypeptide later identified as apolipoprotein (apo)B. Using amino acid analyses, early electrophoresis methods, and a variety chromatographic techniques, the first HDL protein moiety was identified in the late 1960s. Various referred to as α -protein, “R-Thr” peptide (2), fraction III (3), and fraction II peptide (4), it later became known as apoA-I (5). Working in parallel, these laboratories also first recognized the possibility for heterogeneity within the HDL protein complement. This led to the identification of a second protein later known as apoA-II (5). The significance of this observation was not lost on these early pioneers. For example, the Shores

Abbreviations: APR, acute-phase response; CAD, coronary artery disease; CE, cholesteryl ester; CETP, CE transfer protein; CVD, cardiovascular disease; LBP, LPS-binding protein; LPS, lipopolysaccharide; PAF-AH, platelet-activating factor aryl hydrolase; PL, phospholipid; PLTP, PL transfer protein; PON1, paraoxonase 1; RCT, reverse cholesterol transport; SAA, serum amyloid A; SERPIN, serine protease inhibitor; TG, triglyceride.

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observed, "The existence of multiple forms of polypeptides may be of considerable significance in the physiological and biochemical functions of the lipoproteins...." (6).

Around the same time, the Alupovic laboratory documented still further complexity in the HDL proteome by identifying the first of the minor constituents, the apoC peptides (5). Soon after, Mahley and colleagues noted the presence of the arginine-rich apoE in light fractions of HDL in dogs (HDL-1) (7), while others found apoD in the more dense HDL fractions (8). ApoF was also identified (9). Improvements in SDS-PAGE technology in the early 1980s revealed serum amyloid A (SAA) (10) and apoA-IV (11) in human HDL. Immunological studies using antibodies raised against isolated HDL showed that paraoxonase 1 (PON1) coeluted with apoA-I upon gel filtration of plasma and that immunoabsorption of apoA-I removed 90% of PON1 from plasma (12). Similarly, immunoabsorption of clustrin (apoJ) pulled down apoA-I, indicating its presence on HDL. It also became clear that several plasma enzymatic activities were associated with HDL, at least transiently. Single-spin vertical ultracentrifugation studies showed that lecithin:cholesterol acyl transferase (LCAT) was present in the higher density subfractions of HDL and also in LDL (13). Other factors known to remodel HDL included cholesteryl ester transfer protein (CETP) (14), phospholipid transfer protein (PLTP) (15), and platelet-activating factor aryl hydrolase (PAF-AH) (16), also known as lipoprotein-associated phospholipase A2.

By the early 1990s, HDL was generally thought to contain somewhere around 15 proteins. Indeed, one of the first two-dimensional gel electrophoresis studies of human plasma HDL referred to a set of "established HDL proteins" that included apoA-I, A-II, A-IV, C-II, C-III, D, and E, with mention of about 8 others that "can associate" with HDL (17) (six other protein spots were observed but not identified). In general, these fell into four major functional groups: *i*) proteins associated with lipid transport or lipoprotein integrity, i.e., the "apos"; *ii*) lipolytic enzymes, such as LCAT and PON; *iii*) lipid transfer proteins, such as CETP and PLTP; and *iv*) acute-phase response proteins, such as SAA and apoJ/clustrin. The presence of these proteins fit well into the general dogma of a primary HDL function as a lipid transport vehicle.

APPLICATIONS OF MODERN PROTEOMICS TO HDL

Classical biochemical and immunological approaches were typically hampered by sensitivity issues or the fact that one needed a preconceived notion of what one was looking for prior to the experiment. However, the development of soft ionization techniques, such as electrospray (18) and matrix-assisted laser desorption ionization (MALDI) (19), for introducing large molecules into a mass spectrometer (MS) allowed for the development of sensitive and unbiased tandem MS approaches capable of identifying components of complex protein mixtures.

In general, ionized proteolytic peptides in the gas phase are sorted by mass and then subjected to a controlled fragmentation, typically cleaving at the peptide bonds. The fragments are then evaluated by a second mass analyzer, and the resulting patterns are bioinformatically compared with theoretical patterns for all known proteins, effectively sequencing the peptides and producing a redundant list of proteins present in the initial mixture. Given that modern MS instrumentation can identify proteins across a 10,000-fold difference in concentration with sensitivities down to the nano- to picomole range, it is not uncommon to identify hundreds of proteins in a given biological sample. Although strategies can vary significantly between laboratories, MS-based proteomic approaches applied to HDL fall into two rough categories. In the first, HDL proteins are first separated by gel electrophoresis either by size only (1D) or by charge and then size (2D). The resulting gel spots are excised, digested with a protease, such as trypsin, and then identified by tandem MS. The second approach, sometimes referred to as the "shotgun" technique, starts by trypsinizing all proteins together in solution. The peptides are then separated by HPLC (typically by reverse phase) and subjected to electrospray ionization (ESI) as they elute from the column and then analyzed by tandem MS. A more detailed description of proteomic technologies can be found in Ref. 20.

One of the first applications of these technologies toward the HDL proteome was pursued by Karlsson et al. (21). Using a 2D electrophoresis/tandem MS approach, they identified 13 proteins in HDL₂ and HDL₃ separated by density ultracentrifugation from healthy donors. Of these, 11 were previously suspected HDL components from the biochemical studies summarized above. In addition, this study highlighted a key advantage of the charge dimension of the electrophoretic approach in that multiple isoforms of apoA-I, apoA-II, apoC-III, apoE, apoM, SAA, and SAA-4 were identified that varied with respect to sequence differences or posttranslational modifications. The next year, Rezaee et al. (22) used a multipronged approach of 1D and 2D electrophoresis, shotgun proteomics, and immunological assays to study centrifugally isolated total HDL from normal donors. This study first identified key mediators of the complement system - C3, C1 inhibitor, and complement factor H - implicating HDL in innate immunity. Likely, the most high-profile proteomic analysis of HDL was performed by Vaisar et al. (23). Using centrifugally isolated total HDL or HDL₃ from normal subjects, they identified 48 HDL proteins. These included additional members of the complement family, strengthening the argument for a role of HDL in innate immunity. Others, such as the serine protease inhibitor (SERPIN) family, showed a clear theme of protease inhibition, including those involved in hemostasis. Overall, the Rezaee and Vaisar studies clearly demonstrated that the proteins associated with HDL are not limited to those involved in lipid transport. These concepts drove tremendous interest in further exploring the HDL proteome.

By the end of 2012, several laboratories had published some 14 studies that applied various MS techniques to

understand the human HDL proteome. As sensitivity of the instrumentation has increased, so has the number of proteins that are proposed to be associated with HDL. These studies included HDL samples isolated by traditional density ultracentrifugation (21–31), immunoaffinity capture (32), size exclusion chromatography (33), and even anion-exchange and isoelectric focusing (S. M. Gordon and W. S. Davidson, unpublished observations). A summary of the key experimental details for each of these studies is given in **Table 1**. As a result of differences in instrumentation, sensitivity, HDL isolation technique, patient donors, and protein identification algorithms, the total list of putative HDL proteins can vary dramatically from study to study. Several estimates of the total

number of HDL proteins have reached into the hundreds and have included hits that may induce skepticism. For example, certain intracellular and cell-surface proteins and even human skin keratin have been detected in HDL preparations. In an effort to manage this large amount of information and attempt to filter out instrument- or laboratory-specific artifacts, we have initiated the HDL Proteome Watch (<http://homepages.uc.edu/~davidswm/HDLproteome.html> or <http://www.hdlforum.org/resources/links>). This project tracks published reports that have used modern MS methodologies to study human HDL samples that have been physically separated in some way from plasma or serum. Currently, 204 individual proteins have been detected in human HDL samples. Of

TABLE 1. Experimental details of recent MS-based proteomic studies of HDL

Reference	Patient Population	HDL Type and Separation Technique	MS Approach	MS Database Searched	Gel Separation?
(21)	Pooled plasma from healthy adults (n = 4)	HDL ₂ , HDL ₃ isolated by density gradient UC (KBr)	MALDI-TOF	NCBI and SwissProt	Yes 2D gel
(24)	Pool of plasma from >10,000 donors	Total HDL isolated by one-step density gradient UC (KBr)	MALDI-TOF	UniProt	Yes 2D gel
(25)	1 healthy donor	Total HDL isolated by density gradient UC (KBr)	HPLC to separate peptides MALDI-TOF	Details not given	No
(22)	Healthy donors (n not reported)	Total HDL isolated by one-step density gradient UC (KBr) and immunoisolation	MALDI-TOF, isotope coded affinity tag plus Western blot analysis	Details not given	Yes 1D and 2D gel
(23)	Studies of total HDL: 20 males; studies of HDL ₃ : 6 healthy males and 7 with CAD	Total HDL and HDL ₃ isolated by density gradient UC (KBr)	LC-ESI-MS/MS	International Protein Index	No
(26)	9 healthy normolipidemic males and 3 samples, each consisting of a pool from 20 healthy normolipidemic males	HDL _{2b} , 2a, 3a, 3b, and 3c isolated by density gradient UC (KBr)	LC-ESI-MS/MS	SwissProt	No
(33)	3 healthy normolipidemic males	“HDL” isolated by high resolution size exclusion chromatography followed by lipid removal agent	LC-ESI MS/MS	UniProt and SwissProt	No
(27)	10 healthy adults, 10 with stable coronary disease and 10 with acute coronary syndrome. Age-matched males	Total HDL separated by density gradient UC (KBr)	LC-ESI MS/MS	UniProt	Yes 1D gel
(32)	Aged-matched females with rheumatoid arthritis: 4 with anti-inflammatory HDL index, and 4 with proinflammatory HDL index	Total HDL isolated by immunoaffinity capture	LC-ESI MS/MS	UniProt	Yes IEF gel
(28)	19 healthy adults and 27 with end stage renal disease on hemodialysis. Males and females	Total HDL isolated by one-step density gradient UC (KBr)	LC-ESI-MS/MS	NCBI	No
(29)	15 healthy adults and 15 with psoriasis. Males and females	Total HDL isolated by two-step density gradient UC (KBr)	LC-ESI-MS/MS	SwissProt	No
(30)	10 healthy adults and 10 with end stage renal disease. Males and females	Total HDL isolated by density gradient UC (KBr)	LC-ESI-MS/MS	SwissProt	No
(31)	30 healthy adults and 30 with end stage kidney disease on hemodialysis. Males and females	Total HDL isolated by three-step density gradient UC (KBr)	MALDI-TOF and iTRAQ labeling prior to LC-ESI MS/MS	UniProt	Yes IEF gel

IEF, isoelectric focusing; iTRAQ, multiplexed isobaric-tagged reagents produced by Sciex; UC, ultracentrifugation.

these, 85 proteins have appeared in at least three different studies (from independent laboratories), representing the best current estimate of the HDL proteome. These proteins are listed in **Fig. 1**.

Figure 2 shows a gene ontology enrichment analysis showing the general functional classifications of the consensus proteins listed in Fig. 1. Although many HDL proteins fall within the general area of lipid metabolism, proteins with numerous other functions are also present, including proteins involved in hemostasis, such as fibrinogen, and several of the SERPINS involved in the clotting cascade. There are a striking number of HDL proteins involved in the inflammatory/immune response, including numerous members of the complement system and its associated proteolysis inhibitors, apoJ, and vitronectin. Also clearly represented are acute-phase response proteins, such as SAA and lipopolysaccharide (LPS)-binding protein (LPB). Surprisingly, there are also proteins involved in heme and iron metabolism, such as hemoglobin, transferrin, and hemopexin, as well as those with a host of additional and enigmatic functions, rang-

ing from platelet regulation to vitamin binding and transport.

HDL FUNCTIONAL DIVERSITY MATCHES ITS PROTEOMIC DIVERSITY

HDL is most widely recognized for its ability to shuttle cholesterol from the periphery to the liver for catabolism/excretion during the process of reverse cholesterol transport (RCT). Numerous studies have shown that HDL and most of its apolipoproteins can promote lipid efflux from cells via a number of mechanisms (34) and can deliver cholesteryl esters to the liver in the process of selective uptake (35). The importance of this process has been demonstrated by in vivo models of RCT showing that genetic lowering of plasma HDL decreases the appearance of macrophage-derived cholesterol in the feces (36). HDL also has well documented antioxidative properties and has been shown to prevent oxidative modification of LDL, thus reducing macrophage foam-cell generation in the

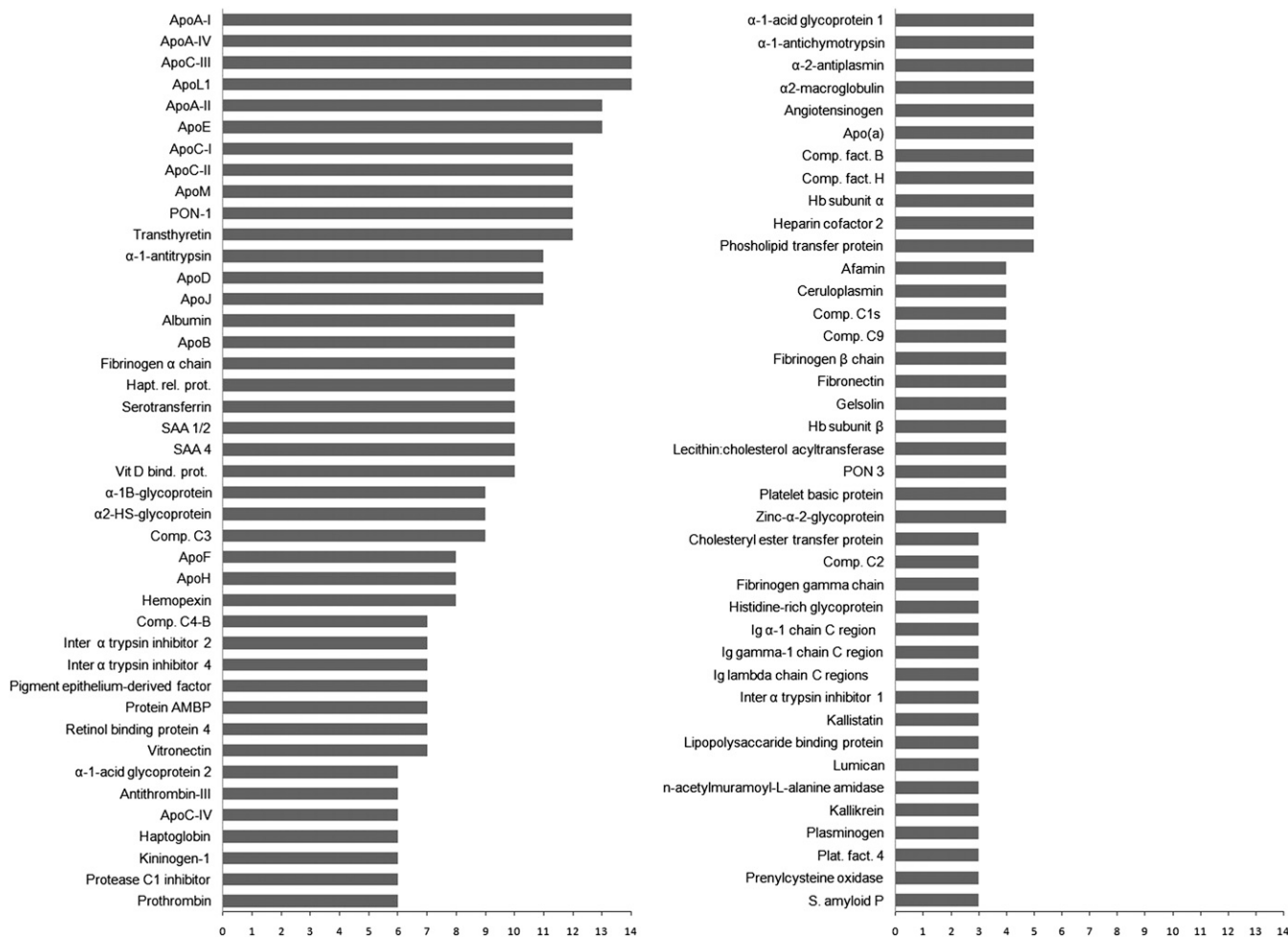


Fig. 1. Frequency of detection of HDL-associated proteins in MS-based proteomic studies. By our count, there are currently 14 published studies that have used soft-ionization MS techniques on human plasma HDL in which an effort has been made to separate them from nonlipid-containing proteins. Proteins that have appeared in at least three of the studies (from different laboratories) are shown. The bar represents the number of studies in which the protein was observed. Although not quantitative by any means, proteins that are more frequently observed likely reflect the most abundant species in HDL.

Lipid Metabolism and Transport	Hemostasis	Immune Response	Metal Binding	Vitamin Transport
ApoA-I, ApoA-II ApoA-V, ApoD ApoE, ApoF ApoC-I, ApoC-II ApoC-III, ApoC-IV ApoO, Apo(a) LCAT, PLTP, CETP PAFAH, SAA4 Pon1, Pon3 Albumin, Transthyretin ApoM, ApoB ApoL-I	Fibrinogen α Fibrinogen β Fibrinogen γ ApoH α -2 macroglobulin	Platelet basic prot. Vitronectin Haptoglobin rel. prot. Ac-muramoyl amidase Ig α -1 chain C Ig γ -1 chain C Ig κ -1 chain C Ig λ -1 chain C AMB, AZGP1 Serpins G1 Platelet factor 4 ApoL-I	HB α , HB β Ceruloplasmin Hemopexin Serotransferrin His-rich glycoprot. AMB	Afamin Vit. D bind. prot. Retinol bind. prot.
ApoA-IV α -1-acid glycoprot. 1 α -1-acid glycoprot. 2 SAA1/2	Prothrombin Kininogen Kallistatin	ApoA-IV LPS-binding protein α -2 antiplasmin	α -1 anti-trypsin Serum amyloid P ITIH4 α -1 anti-chymo. Fibronectin α -2 HS glycoprot.	Acute Phase Response/Inflammation
	Anti-thrombin III Plasminogen Serpins G1 Serpins D1 Kallikrein	α -2 antiplasmin	Pre-nyl-Cys-oxid. α -1 anti-trypsin Hep. cofactor 2 ITIH4, ITIH1, ITIH2 α -1 antichymo. Serpins F1, AMBP	Proteolysis/Inhibition
ApoJ	Comp. C9	Comp. 1S, Comp. C2 Comp. 4B, Comp. B Comp. H, Comp. C3 ApoJ		Complement

Fig. 2. General functional relationships of the HDL proteins in Fig. 1. We performed a gene ontology (GO) search for every protein shown in Fig. 1 as listed in UniProt (<http://www.uniprot.org>). We determined the eight most frequently reoccurring biological process or molecular function annotations and distributed the proteins among these as appropriate in the figure. Note that most proteins had a large number of GO annotations, and this figure is not meant to be a comprehensive representation of all potential functions for a given protein. Six proteins (α -1B glycoprotein, haptoglobin, angiotensinogen, gelsolin, platelet basic protein, and lumican) are not listed because their entries did not include these top eight annotations, but they are associated with additional entries that further attest to the functional diversity of HDL.

vessel wall (37). Aside from lipid transport functions, HDL has clear anti-inflammatory traits (reviewed in Ref. 38). It can inhibit the expression of cell-adhesion molecules on endothelial cells that sequester circulating monocytes during injury (39). HDL can also reduce the activity of macrophage chemotactic factor 1, which signals the infiltration of surface-adhered monocytes into the vessel wall (40). In rabbits fitted with carotid periarterial collars, HDL administration resulted in 40% reductions in VCAM-1 expression and monocyte infiltration within one week (41). As an in depth discussion of the well-studied lipid transport, antioxidative, and anti-inflammatory functions of HDL is beyond the scope of this review, readers interested in more detail are directed to the excellent recent treatise on HDL by Kontush and Chapman (42).

Under conditions of infection, inflammation, or tissue injury, the acute-phase response (APR) is triggered, causing huge alterations in liver protein synthesis patterns that translate to remarkable changes in HDL protein composition. Negative APR proteins (i.e., those decreasing in APR) include apoA-I, transthyretin, and retinol-binding protein. Their rapid reduction may quickly release “acute-booster reactant” or free ligands, such as metal ions and vitamin A, needed for repair processes (43). These proteins tend to be replaced in HDL by positive APR proteins, which include SAA, apoJ/clustrin, and lipopolysaccharide-binding protein (LBP). SAA can increase in expression up to 1000-fold within 24 h of onset of APR in certain animal models (44). SAA appears to work toward preserving the cholesterol content of peripheral tissues by reducing HDL’s ability to promote cholesterol efflux (45) and whole-body transfer of macrophage cholesterol to the feces (46). This may enhance local repair processes. However, SAA increases both holoparticle uptake of HDL (46) and selective uptake of cholesteryl ester by the liver and adrenals via SR-BI (47). Because the adrenals rely heavily on HDL-derived cholesterol for steroid hormone production,

enhanced cholesterol delivery from HDL may allow the adrenals to better produce anti-inflammatory glucocorticoids in response to stress. As it is known to bind vascular proteoglycans (48), SAA may also tether HDL in the vascular matrix, thereby preventing RCT and inhibiting oxidation (49). ApoJ/clustrin is predominantly found in HDL₃ (26). It may prevent rampant tissue injury by inhibiting complement activation (50). LBP, as its name suggests, is an avid LPS-binding factor. It is known to increase host-cell sensitivity to LPS by presenting it to its cell-surface receptor, CD14 (51). Thus, positive and negative APR proteins residing in HDL appear to coordinate short-term inflammatory and repair processes. (For a more complete review of HDL protein changes in the APR, see Ref. 52).

HDL is also known to play important roles in hemostasis (for a recent review, see Ref. 53). Several studies have shown that HDL-C levels correlate inversely with different pathological modes of thrombosis (54, 55). HDL can oppose LDL induction of platelet aggregation, serotonin release, and thromboxane B2 production (56), and it can block ox-LDL inhibition of nitric oxide synthase (57). Griffin et al. (58) showed that HDL, but not LDL, could inhibit the activation of coagulation factor Va by activated protein C, an important early step of the clotting cascade, although this activity did not appear to coelute with the majority of apoA-I upon gel filtration separation (59). Recent evidence has also revealed that the cholesterol efflux functions of HDL and apoA-I may significantly impact platelet function. Mice lacking the SR-BI receptor, known for mediating lipid efflux, among other functions, produce platelets that aggregate poorly due to high levels of membrane cholesterol (60). In humans, when type II diabetes patients were infused with reconstituted HDL preparations, they experienced a 50% decrease in platelet aggregation versus controls (61). Overall, these effects on hemostasis are consistent with the discovery of HDL protein groups involved in clotting, such as thrombin, PAF-AH,

and clot propagating/inhibiting proteases, although much more work is needed to understand the precise roles they play.

Intriguingly and somewhat underappreciated in the lipoprotein field, HDL also plays important roles in host defense. It has been shown to be a major bacteriocidal factor in several species of fish (62) as well as humans (63, 64). HDL can also deactivate particular oxidized phospholipids accumulating in macrophages that have been infected with *Mycobacterium leprae*, the organism that causes leprosy (65). Similarly, HDL components can neutralize toxins released during infection, including enterohemolysin (66), LPS, and lipoteichoic acid (67–71). This sequestration prevents the activation of toll-like receptors (TLR) on macrophages and their subsequent secretion of proinflammatory cytokines (for a more in-depth review on the role of HDL in innate immunity, see Ref. 72). Recent work has also demonstrated that specific components of HDL can play a highly intriguing role in neutralizing the protozoan *Trypanosoma brucei* (*T. brucei*) (more on this below). In fact, the importance of HDL in host defense is highlighted by the fact that some invading pathogens have evolved offensive capabilities that specifically target HDL. For example, *Streptococcus pyogenes*, a group A streptococcal bacterium responsible for tonsillitis, pharyngitis, and toxic shock syndrome, secretes a protein called serum opacity factor (SOF). SOF specifically targets sHDL particles by binding to apoA-I and apoA-II, causing a dramatic redistribution of the HDL neutral lipid cargo into large protein-poor micro-emulsions (73). Thus, SOF might have evolved as a virulence factor designed to subvert the antibacterial properties of intact HDL particles. Given the tight linkage between infection and inflammation, the ability of HDL to regulate the amplitude of the inflammatory response may work in conjunction with these host-defense effects to resolve infections. Indeed, since cardiovascular disease (CVD) and other chronic derangements of lipid metabolism typically affect mortality at postreproductive ages, one can argue that selective pressures related to host defense and inflammation were likely the dominant evolutionary factors that have shaped HDL composition and function.

Overall, the huge amount of work on HDL has produced a diverse functional portfolio that closely mirrors the known functions of its recently identified protein constituents. It follows that the tremendous functional heterogeneity inherent to HDL is driven in large part by its compositional heterogeneity. This raises an important question: Does the HDL proteome undergo changes in the face of disease?

ALTERATIONS IN THE HDL PROTEOME IN DISEASE STATES

With the complexity of the HDL proteome largely established, investigations have begun to focus on monitoring its changes in various disease states. Vaisar et al. was the first to compare HDL proteomic profiles between normolipidemic subjects and patients with documented coronary

artery disease (CAD) (23, 74, 75). They identified several proteins that were enriched in the CAD patients, including apoE, apoC-IV, PON1, complement C3, and apoA-IV, all involved in vascular inflammation. Moreover, pattern recognition analyses showed a promising ability to differentiate mass signatures from normal and CAD subjects, particularly from mass markers found in apoA-I, apoC-III, and apoC-I (74). Alwaili et al. (27) performed a proteomic comparison in control, stable CAD, and acute coronary syndrome (ACS) subjects. Here, significant differences in SAA, apoA-IV, and complement C3 levels were noted in the ACS patients, indicating a shift to an inflammatory profile. These studies illustrate the promise of HDL proteomics for deriving new biomarkers for CAD diagnosis and, perhaps more importantly, for ways to measure the effectiveness of current and future treatment regimens. Indeed, Vaisar et al. compared the HDL₃ proteome of subjects with stenotic lesions verified by angiography before and one year after combination treatment with a statin and the HDL-raising drug niacin (75). The treatment was found to reduce apoE levels while increasing apoF and PLTP, partially remodeling the stenotic proteomic profile toward that of control subjects. Alterations in the HDL proteome were also noted after treatment with the CETP inhibitor anacetrapib, though the impact of these changes on CVD is unclear (76).

The state of the HDL proteome has been evaluated in other conditions as well. Asking similar questions in the context of type II diabetes, Hoofnagle et al. looked at the levels of five proteins previously implicated in cardioprotective effects of HDL (77). They found that clusterin concentration in HDL was negatively associated with insulin resistance, potentially implicating it in HDL cardioprotection. Because renal disease is associated with low HDL-C, two laboratories have monitored the HDL proteome in response to chronic dialysis. Holzer et al. (29) found that dialysis patients have increased levels of the acute-phase inflammatory proteins SAA, PAF-AH, and apoC-III in HDL along with decreases in phospholipid and increases in triglyceride content. These changes corresponded with impaired cholesterol efflux function. Weichart et al. (30) showed that HDL from advanced renal disease patients lacked normal anti-inflammatory properties and correlated this with HDL enrichment of several proteins, including SAA. These studies suggest a link between HDL dysfunction and increased risk of CAD in renal disease.

Turning to other chronic inflammatory diseases, Holzer et al. investigated the HDL proteome from a cohort of patients suffering from psoriasis, an inflammatory skin disease (28). These patients exhibited a reduction in apoA-I levels relative to controls, but they had increased levels of apoA-II and proteins involved in APR. Interestingly, the ability of HDL to promote cholesterol efflux from macrophages was negatively correlated with psoriasis severity.

Sex steroid withdrawal in men was shown to increase HDL-associated levels of clusterin (apoJ) while increasing the capacity of HDL to promote cholesterol efflux from macrophages (78). A follow-up study showed that testosterone replacement in hypogonadal men promoted significant

increases in PON1 and fibrinogen α -chain while lowering apoA-IV, but it had no effect on HDL-C levels or cholesterol efflux functionality (79).

Overall, these studies indicate that the HDL proteome can change in a variety of disease states and that these changes are often related to at least in vitro measures of HDL function. However, it remains to be seen whether these changes are secondary to other processes occurring during disease progression or whether the HDL particles themselves contribute to the disease etiology. This will be an important question to pursue going forward.

EVIDENCE FOR HDL PROTEIN SUBSPECIATION AND COOPERATIVE PROTEIN FUNCTION

When it was becoming clear that HDL contained more than a single protein constituent, Scanu and colleagues asked as early as 1969 “whether HDL represents a single lipoprotein species having distinct polypeptide chains or whether HDL, as we prepare it in the ultracentrifuge, is a mixture of HDL species each with distinct peptide moieties....” (3). Is HDL essentially a single entity with numerous interchanging protein components, or is it a collection of individualized species with distinct functionalities that happen to have similar physicochemical properties that are easily isolated together? This is a critical question with respect to pharmacological approaches to altering HDL function. Under the single entity scenario, it may be possible to raise HDL levels generically and thereby achieve improvements on a majority of its functions. Alternatively, if individual species perform distinct functions, it may be most advantageous to pharmacologically raise only certain ones to achieve benefits, particularly if altering other sub-species might have deleterious effects on other important functions.

Careful in vitro studies have documented the movement of apolipoproteins between VLDL and HDL (80). In fact, many of the apolipoproteins associated with HDL have been termed “exchangeable” due to their ability to exist in a soluble lipid-poor state that allows them to ping-pong between different lipoprotein species. Therefore, one school of thought views HDL as a transient ensemble of randomly exchanging proteins. This view is reflected in the clinic where the HDL-C measurement is commonly taken as a surrogate for the totality of HDL species and function. However, there is significant evidence that many apolipoproteins do in fact segregate into compositionally stable particles. Asztalos and colleagues used antibodies to visualize individual protein migration patterns in a native 2D gel electrophoresis system. They found apoA-I in 11 distinct spots representing variously charged and sized species (81). However, apoA-II associated with apoA-I only in the α_2 and α_3 species but not in the others. ApoE was found on larger particles that did not completely overlap with apoA-I, and apoA-IV was also found in distinct locations (82). Similarly, our group applied a shotgun ESI-MS/MS approach to learn about the distribution of proteins across five density subfractions (HDL_{2b,a} and HDL_{3a,b,c})

isolated from humans by density-gradient ultracentrifugation (26). Using an abundance pattern analysis, we categorized different HDL proteins into five groups based on their distribution. Some proteins preferred small, dense particles whereas others preferred large, light ones. In a follow-up study, we separated human lipoproteins into 17 individual fractions by size exclusion chromatography and again saw highly distinct elution patterns for individual proteins (33). In more recent work, we separated human plasma using three different separation techniques (gel filtration, ion exchange chromatography, and isoelectric focusing) and tracked protein elution patterns across them. The results show that numerous pairs of proteins tend to comigrate across the different separation techniques (83), suggesting the existence of discrete and stable subparticles. These reports indicate that, although some apolipoproteins and HDL-associated lipid remodeling factors undoubtedly exchange between HDL particles, others clearly do not. The mechanisms driving such segregation are as yet unknown but likely involve some combination of the following: *a*) the affinity of a given protein to a particular lipid composition; *b*) the affinity for a certain degree of surface curvature on different-sized HDL particles; or *c*) specific protein-protein interactions on the particle surface that promote and maintain protein segregation.

The idea of protein-protein interactions or at least protein colocalization within specific HDL particles opens the possibility that some proteins have cooperative functions. In fact, there are several examples of cofactor interactions within the HDL proteome. On its own, LCAT is inefficient in mediating cholesterol esterification in lipoproteins, but in the presence of apoA-I, LCAT activity is stimulated by several orders of magnitude (84). ApoC-II is an important cofactor for efficient lipolysis by lipoprotein lipase (85). One role of apoA-II may be to modulate endothelial lipase (EL), an important enzyme for the physiological regulation of HDL-C levels, perhaps via effects on apoA-I conformation (86). In terms of reciprocal regulation, ApoF (also known as lipid transport inhibitor protein) can inhibit the CETP-mediated exchange of CE between HDL and TG-rich LPs, possibly by modulating CETP's affinity for the HDL particle surface (87). There is also emerging evidence that HDL may rely on cooperative interactions to carry out antioxidative functions. HDL can prevent LDL oxidation (88–90) via associated PON1 (91, 92), a calcium-dependent esterase that closely associates with apoA-I and is thought to prevent LDL oxidation by hydrolyzing oxidized phospholipids (90) and cholesteryl linoleate hydroperoxides (93). Recent work by Hine et al. (94) demonstrates PON1 may interact with apoA-I and LCAT to inhibit LDL oxidation, with the combination preventing LCAT inactivation.

However, the most striking and best documented example of HDL protein-protein cooperation relates to HDL's role in innate immunity. Dense fractions of HDL are well known to mediate the lysis of *T. brucei*, a trypanosome related to the one that causes African sleeping sickness in humans. This activity was termed trypanosome lytic factor (TLF) (95, 96). Immunoprecipitation studies

demonstrated that TLF is a distinct and minor HDL particle that contains apoA-I, apoL-I, and haptoglobin-related protein (HRP) (97). The current model for TLF lysis of *T. brucei* holds that the HDL particle is taken up by the trypanosome in a receptor-mediated pathway, possibly via the HRP moiety. The complex is then targeted to the lysosome where apoL-I, via a colicin-like pore-forming domain, permeates the organelle to kill the organism (98). TLF's unique composition is the strongest evidence yet that distinct particles within classically defined HDL exist and perform highly specialized functions that are quite distinct from traditional lipid transport roles (99).

There are also examples of HDL-like particles that have been isolated without a specifically known function. Chueng et al. used immunoaffinity techniques to isolate particles that contain PLTP (100). MS analysis showed that they contained numerous proteins, including clusterin, coagulation factors, complement factors, and apoA-I. Interestingly, these particles were extremely lipid-poor, with only 3% of the mass attributed to phospholipid. Given the composition, the authors proposed that these particles may play roles in host defense and inflammation. This illustrates that the composition of some HDL particles may differ significantly from commonly envisioned lipid-dominated particles and that protein-protein interactions may play important roles in HDL subspeciation.

CONCLUSIONS AND CHALLENGES

The tremendous leaps that have been made in characterizing the HDL proteome have left little doubt as to the role of HDL in a range of processes, ranging from lipid metabolism to inflammation to host defense. Further work will undoubtedly confirm and expand the list of proteins known to associate with extracellular phospholipid in the plasma. Already new technologies are being developed, such as selected reaction monitoring (SRM), that allow not only the identification of HDL protein constituents but also their quantitation with precision approaching traditional immunological methods (30, 101). These technologies will be critical for determining alterations in the HDL proteome in the face of disease states such as CAD and diabetes. As stated above, these studies will be useful for identifying new biomarkers for early diagnosis of disease, pinpointing new pathways for therapeutic intervention, and assessing the effectiveness of current and new therapeutics.

Along with the promises of this field, there are also several challenges that need to be met. First, in terms of characterizing the HDL proteome, it will be important to distinguish artifacts from truly functioning HDL proteins. As MS technology continues to improve, it is certain that more and more proteins will be identified in HDL preparations. However, the sensitivity of MS has likely already outstripped the fidelity of the various HDL isolation strategies used to generate the samples. Thus, it is increasingly likely that minor non-HDL-associated contaminants, often resulting from highly abundant soluble proteins in plasma,

will be reproducibly detected as HDL constituents. It will be important to recognize these possibilities of contamination and develop better HDL isolation techniques as well as to cross-check results with different methodologies. It should also be kept in mind that HDL may be a repository for fragments of proteins that have been degraded in plasma (25). Therefore, the detection of peptides from some proteins may not reflect an intact and functioning protein in HDL. MS protein identifications should be backed up with immunodetection methods, such as Western blotting, that are capable of assessing whether the intact protein is present. Second, it will be important to determine the plasma distribution of many of these HDL factors. For example, complement C3 is consistently detected as an HDL constituent; however, it is also a highly abundant soluble entity in plasma. How much of plasma C3 is associated with HDL? Does HDL association make it functionally different from the soluble form? These are important questions that will need to be asked for many of the newly identified HDL proteins. Third, given the large number of proteins associated with HDL and the relatively few protein structural and functional interactions that we currently understand, it is easy to imagine that many more HDL subparticles await discovery and characterization. These particles may underlie a huge amount of unknown biology relating to a range of processes and diseases. Thus, it would seem prudent to devote resources to identifying these complexes and correlating them with specific functions. We may find that the levels of a given protein may not differ in a given disease state but that its distribution across HDL subspecies or its association with other proteins may change significantly. Put another way, particular HDL complexes could turn out to be better biomarkers than individual proteins. This principle was elegantly illustrated by the recent work of Jensen et al. (102). Using immunoaffinity separations, they separated HDL from two large, prospective CVD studies and quantified the cholesterol content of the HDL particles that contained apoC-III and those that lacked it. Strikingly, they found that apoC-III-containing HDL was associated with increased CVD risk, whereas the apoC-III-lacking fraction was associated with cardioprotection.

The recent failure of niacin (103) and two different CETP inhibitors (104, 105), all capable of significantly raising HDL-C, in cardioprotection has cast some doubt on whether HDL plays a truly protective role in CVD or represents a readout of other more directly operational processes. Indeed, the "HDL-cholesterol hypothesis" has begun to be replaced by the "HDL function/flux hypothesis" (106), reflecting the growing consensus that measurement of one minor HDL component (i.e., cholesterol) is not sufficient to capture the cardioprotective potential of HDL. Recent studies have clearly shown that specific functional readouts, such as capacity for cholesterol efflux (107) or anti-inflammatory index (108), may be better indicators than the HDL-C number for predicting CAD risk in an individual. Given the huge functional and compositional pleiotropy in HDL, individuals likely have a "portfolio" of HDL subspecies that are individually tasked

to different functions across lipid metabolism, inflammation, antioxidation, and host defense. The HDL-C number may represent these particles as a group, but it probably does not represent those subfractions that play the most important roles in cardioprotection. If this is correct, then it becomes extremely important to identify those subspecies that are most relevant to CVD protection and focus on therapeutics that specifically target those species. Raising HDL-C in the generic sense without such targeting may not elevate the truly cardioprotective species. Importantly, such treatments might even have deleterious effects on subspecies that are important for other pathways, such as host defense. Indeed, an underappreciated fact from the failed ILLUMINATE trial (104) is that a major cause of death in the torcetrapib treatment group, aside from the drug's off-target effect that contributed to increased CAD (109), was sepsis.¹¹⁶

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