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## The Difference Between High Density Lipoprotein Subfractions and Subspecies: An Evolving Model in Cardiovascular Disease and Diabetes

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### Abstract

**Purpose of review:** The term high density lipoproteins (HDL) refers to an eclectic collection of subparticles that play diverse roles in physiology. Here, we define the term 'HDL subspecies' and review recent work on their molecular characterization and relation to disease, focusing on cardiovascular disease and diabetes.

**Recent findings:** The HDL family contains over 200 proteins and nearly 200 lipids that partition into different particles in plasma. Simple subfractionation of HDL based on a particular physicochemical property has not risen to the challenge of revealing the roles of specific particles in disease. However, by targeting minor protein or lipid components, a handful of compositionally defined HDL subspecies have been described and characterized.

**Summary:** By combining targeted particle isolation techniques with the power of large human studies, progress is being made in understanding HDL subspecies functions and implications for

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Author notes:

In our view, subspecies and subparticles essentially mean the same thing. We prefer to use the term subspecies as subparticle tends to connote a static molecularly defined particle which probably is not common given the exchangeability of many HDL components. For consistency, we use the term subspecies throughout this review.

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disease. However, much work remains before these advancements can be translated into disease mitigation strategies.

### Keywords

High density lipoproteins; subspecies; subfractions; cardiovascular disease; type 2 diabetes; pre-beta HDL; trypanosome lytic factor; lipoprotein; lipoprotein subspecies

## Introduction: The HDL-cholesterol fallacy and the need for a deeper dive

From the Framingham Heart Study in the 1970s [1] to large scale meta analyses performed four decades later [2], epidemiologic studies have consistently shown that low serum HDL-cholesterol is a risk factor for atherosclerotic cardiovascular disease (ACVD). The concept that HDL plays a direct role in mediating this protection was bolstered by many studies in animal models showing that raising HDL-cholesterol reduces atherosclerosis. There was also a massive body of *in vitro* work showing that isolated HDL has functional properties predicted, based on our best understanding of the disease pathology, to be anti-atherogenic. This ethos prompted the pharmaceutical industry to invest heavily in deriving novel small molecule therapies to raise HDL-cholesterol in humans. Unfortunately, the resulting interventional clinical trials have largely failed to alter key cardiovascular endpoints in human beings - despite significantly raising HDL-cholesterol concentrations in plasma (for a good review, see ●[3]). Since interventional clinical trials are considered the ultimate test for the validity of a scientific principle as a foundation for medical treatment, some scientists and clinicians have written off HDL as a therapeutic target.

However, many argue that these trials have not impugned HDL itself, but are rather an indictment of the concept that its cholesterol content is a suitable proxy for the functionality of all HDL particles. Indeed, HDL comprises a family of lipoproteins whose individual particles differ widely in density, size, charge, protein, and lipid composition. Some are cholesterol rich, while others contain almost no cholesterol at all. Some 40 proteomics studies have identified more than 200 individual proteins present on lipoproteins in this density range, as tracked by the HDL Proteome Watch (<http://homepages.uc.edu/~davidswm/HDLproteome.html>). The situation is even more complex when the nearly 200 different lipid species are considered [4]. It is now clear that these components differentially segregate into HDL subspecies with distinct and stable proteomic/lipidomic makeups. HDL has well documented roles in lipid transport, endothelial protection, anti-inflammation, anti-oxidation, anti-thrombosis, and the acute phase response [5]. There is also growing appreciation for its roles in complement pathway activation, anti-infection, and protease inhibition [6, 7]. Rationalizing HDL as such a jack-of-all-trades is difficult as a singular entity. But this is easier to understand in the context of a collection of distinct particles with different functions, all of which happen to have a similar density and therefore co-isolate in an ultracentrifuge. Unlike VLDL or LDL, which largely carry out lipid transport and delivery functions, HDL appears to be an extracellular lipid platform that serves as a nexus for the coordination of proteins that mediate a large amount of biology that goes beyond lipid transport.

This model of particle specialization predicts a role for HDL in different disease states which may go well beyond traditional links to ACVD. Some particles may participate in cholesterol transport while others may be tasked with supporting the innate immune system or hemostasis. Some may be anti-inflammatory, others may be pro-inflammatory. Relating specific HDL subspecies with disease progression may reveal better biomarkers to track disease and perhaps reveal new targets for therapies – therapies more discriminating, and hopefully more fruitful, than the generic HDL-cholesterol raising compounds that have thus far disappointed. In this brief review, we will focus on recent progress in identifying true HDL subspecies and their potential contributions to disease using ACVD and T2D as examples.

### **The difference between HDL ‘subfractions’ and HDL ‘subspecies’.**

The terms HDL subfraction and HDL subspecies have been used interchangeably for many decades. The concept of HDL heterogeneity was recognized soon after the lipoprotein class was discovered and most early sub characterizations relied on some physicochemical property of the particles, usually driven by a separation strategy that is easily performed in the laboratory. It was quickly established that human plasma HDL could be further divided into density subfractions such as HDL<sub>2</sub> and HDL<sub>3</sub> [8]. These have been refined into many nomenclatures with HDL<sub>2b</sub>, HDL<sub>2a</sub>, HDL<sub>3a</sub>, HDL<sub>3b</sub>, and HDL<sub>3c</sub> being one of the most widely recognized [9]. There have been many attempts to relate these subfractions to ACVD. However, an honest look at this extensive literature reveals much inconsistency with respect to their roles in disease. HDL has also been subfractionated based on its major scaffold protein content with LpA-I particles containing the major protein APOA1, but not the second most abundant protein APOA2, while LpA-I/A-II particles contain both [10]. Again, numerous studies have attempted to relate these to ACVD with conflicting results. HDL has also been subfractionated by charge (pre-beta, alpha-HDL) and size (large, medium, small, etc.). For an excellent review on HDL subfractionation and nomenclature, see [11]. These separations led to the rise of advanced lipoprotein testing (ALT) techniques [12], in which analytical approaches such as differential ion mobility, 2-dimensional (2D) gel electrophoresis, and nuclear magnetic resonance (NMR) spectroscopy have been used to track lipoprotein subfractions in clinical samples. Additionally, clever use of whole particle mass spectrometry has resulted in the identification of HDL size categories [13].

Unfortunately, these strategies have not yet led to a specific subfraction that has a universally accepted relationship to ACVD. Why? Proteomics studies have offered a clue. Most of the subfractions described above retain significant proteomic heterogeneity, even when the highest resolution separation techniques are employed. For example, preparations of HDL<sub>3c</sub>, the smallest and most dense HDL subfraction, contain upwards of 25 separate proteins [14]. Given the limited surface areas available, these cannot all reside on the same particle at the same time. Thus, the subfraction must be composed of a number of distinctly composed particles of similar density. This is not surprising given that combining most lipophilic proteins with nonvesicular phospholipid (in the absence of significant amounts of neutral lipid esters) will produce a particle with size and density characteristics that typically fall within the HDL range [15]. The same can be shown for LpA-I and LpA-I/A-II particles which have distinct, but still highly heterogeneous proteomes [16]. Therefore, separation

techniques that exploit a general physicochemical characteristic (like density or charge) or a common protein/lipid constituent (like APOA1, APOA2 or cholesterol) lack sufficient resolving power to identify the roles of individual HDL family members. For the purposes of this review, we define the term HDL subfraction as a partially purified collection of particles that share a specific physicochemical property or abundant protein/lipid constituent and are easily isolated together, but probably do not fully represent functional HDL entities. The term HDL subspecies (or subparticles\*), on the other hand, is defined as a particular particle or set of related particles that are distinguished based on a specific *minor* component of HDL. The use of a minor component is critical in this regard. For example, defining HDL by the presence of APOA1 is problematic as > 90% of HDL particles contain APOA1 with all their accompanying heterogeneity. A similar argument can be made against using the second most abundant protein, APOA2 [16]. Additionally, common lipid components such as phosphatidylcholine or cholesterol also lack the required distinguishing power as these lipids are present at some level on most HDL particles. It is tempting to try to define HDL subspecies as molecularly defined entities (i.e. a particular particle with X number of proteins and Y number of lipids) rather than limiting to the presence or absence of a particular component. However, relatively few of these have been isolated and characterized to date. Given the known exchange dynamics between HDL particles, it is likely that a given defining component may be present on different sized particles. Indeed, the best example of a defined HDL subspecies, trypanosome lytic factor (see below), appears as two separate size species in human plasma. HDL subspecies, as defined here, can be evaluated by methods that target a specific component such as immunoaffinity chromatography, or can result from traditional purification techniques that have narrowed the class down to a specific molecular entity (see pre-beta 1 HDL below).

Figure 1 illustrates the difference between some current subfraction nomenclatures vs. the ideal of component-defined subspecies. We suggest that the field needs to move beyond the relatively superficial strategy of HDL subfractionation and take on the more difficult task of developing ways of isolating and functionally characterizing minor component-defined HDL subspecies. Fortunately, laboratories are beginning to take this approach. Recently, Furtado et al. ●● [17] used a relatively high throughput immunoaffinity isolation protocol to track the compositions of 15 HDL isolates that targeted specific apolipoproteins ranging from apolipoproteins A-II, A-IV, E, C's, J, L-I to other minor HDL proteins like  $\alpha$ -1-antitrypsin,  $\alpha$ -2-macroglobulin, plasminogen, fibrinogen, ceruloplasmin, haptoglobin, paraoxonase-1, and complement C3. These subspecies were found to range widely in plasma concentration (from less than 1 to 18%) of total plasma APOA1. Importantly, the subspecies were stable over time and mass spectrometry-based proteomic analyses showed distinct proteomes among most. Some of these subspecies have been connected to major disease states using samples from large prospective human studies, as discussed in more detail below.

### Examples of component-specific HDL subspecies.

**Trypanosome lytic factor (TLF) 1 and 2:** The prototypical HDL subspecies, TLF was identified in the early 80's as a factor in primate plasma that lyses the cattle pathogen *Trypanosoma brucei brucei* [18]. The activity was localized to two HDL subspecies, TLF1 and TLF2 [19]. TLF1 is a 500 kDa particle containing APOA1, APOL1, and haptoglobin-

related protein (HrP). The trypanolytic activity of TLF1 results from a fascinating interplay between its resident proteins. Current understanding [20, 21] holds that HrP mediates TLF1 binding to a heme receptor in the flagellar pocket of a susceptible trypanosome. Once internalized in an endosome, APOL1 forms a channel in the vesicle membrane that is kept closed by the acidic environment of the vesicle. When cycled to the plasma membrane, the channel activates, causing a cascading osmotic pressure imbalance that lyses the cell [22]. APOA1 itself is not toxic to trypanosomes but plays a key role by packaging HrP and APOL1 into a lethal, mobile package that can access the parasite when in the circulation. TLF2 is much larger (about 1000 kDa) and contains less lipid than TLF1. In addition to APOA1, APOL1, and HrP, it also contains immunoglobulin M (IgM) [19]. This subspecies may kill trypanosomes by a similar mechanism to TLF1, but it is much less efficiently taken up by the heme receptors in the flagellar pocket. The IgM component may mediate uptake by other mechanisms [23]. Although of less interest to those studying HDL's role in ACVD, the presence of these well characterized HDL subspecies makes two points very clear: *i)* HDL contains subspecies that have specialized missions in disease and immunity that have nothing to do with lipid transport and *ii)* individual proteins sequestered on an HDL particle can synergize to execute biological functions.

**Pre-Beta 1 HDL:** Immunosorption studies by Kunitake et al. [24] identified a lipid-poor particle that contains predominantly APOA1 that migrates to the pre-beta position (slightly more positively charged than the bulk of HDL, which migrates to the alpha position) by agarose gel electrophoresis. Technically, this subspecies does not qualify as HDL because it is more dense than the accepted HDL density range (1.063–1.210 g/ml) and is usually not observed in ultracentrifugal HDL preparations. This species has a MW of about 65 kDa and currently thought to be composed of two molecules of APOA1 with small amounts of phospholipid, free cholesterol and cholesteryl esters, but little triglyceride. This appears to be a precursor of more lipidated HDL subspecies as it preferentially interacts with the cell surface transporter ATP binding cassette A1 (ABCA1) [25, 26], a primary driver of cholesterol efflux out of cells and HDL lipidation. As such, pre-beta 1 HDL was postulated to be anti-atherogenic. This drove the development of a variety of tools for measuring its levels in plasma including ultrafiltration-isotope dilution [27], 2-D gel electrophoresis [28] as well as a clinical assay based on an antibody that recognizes a unique APOA1 conformation in these particles [29]. Work by Lamon-Fava et al. using a high throughput 2-D electrophoresis technique showed that pre-beta 1 HDL levels were higher in subjects with ACVD vs controls [30]. Others have shown that pre-beta 1 HDL levels are also increased in multiple dyslipidemic states [31] and reduced upon treatment with statins [32]. These results were surprising in light of the expected protective nature of pre-beta 1 HDL, giving rise to the so called 'Pre-β 1 Paradox'. Asztalos et al. showed that pre-beta 1 particles derived from individuals with documented coronary artery disease were functionally defective in terms of cholesterol efflux, the initial step in reverse cholesterol transport [33]. It was proposed that a compositional difference was responsible for the defect. Alternatively, or in conjunction, high levels of pre-beta 1 may reflect dysfunction in cholesterol efflux pathways at the cellular level in ACVD. Others have speculated that high levels of this subspecies may indicate a defect in the normal process of HDL maturation and recycling that may contribute to ACVD development [31, 34].

**Myeloperoxidase (MPO), APOA1 and PON1 ternary complex:** Huang and colleagues reported a ternary complex of these three proteins and phospholipid in mice and humans by immunoprecipitation [35]. They went further and identified the sites on APOA1 where MPO and PON1 bind. MPO is a source of reactive oxygen species and impairs anti-inflammatory functions of HDL by oxidizing APOA1. Conversely, PON1 blocks the buildup of lipid peroxides in lipoproteins. Interestingly, MPO and PON1 were shown to inhibit each other's activity, likely as a result of their close proximity on the HDL particle. Given the expectation that PON1 is involved in several anti-inflammatory and anti-atherogenic processes, the presence of MPO may render this complex a dysfunctional HDL subspecies [35].

**HDL-S1P:** Minor lipid components of HDL can also be a useful marker to distinguish particular subspecies. The best current example is HDL particles that contain shingosine-1-phosphate (S1P), a bioactive sphingolipid that is a ligand for a family of receptors that mediate a variety of cellular processes. In endothelial cells, one S1P signaling pathway controls cellular nitric oxide production to control vasodilation. Persegol et al. [36] assayed the ability of various HDL density subfractions to regulate vasodilation in rabbit aortic rings. They found that the densest subfraction, HDL<sub>3c</sub>, displayed outsized effects compared to the other subfractions. This subfraction turned out to contain the highest levels of S1P. Further work showed that this lipid is sequestered in HDL subspecies that contain apolipoprotein M, a minor apolipoprotein known to bind S1P [37]. APOM-containing HDL particles were shown to produce an anti-inflammatory response in human primary endothelial cells including limiting the expression of certain cell adhesion molecules. Importantly, S1P delivered in other ways, such as complexed to albumin, was much less efficient in modulation cell adhesion molecule expression [36]. There is also an extensive literature indicating that S1P/APOM subspecies play a key role in liver inflammation/regeneration (reviewed here [38]).

#### **HDL subspecies in disease states - ACVD.**

As summarized above, there have been many studies that have attempted to relate semi-purified HDL subfractions to risk for ACVD with varying success. However, the work of relating protein-defined HDL subspecies (as defined here) to ACVD has lagged behind. In 2018, Jensen et al. used immunoaffinity chromatography to measure the concentrations of APOC3-containing HDL subspecies and relate them to ACVD development in four large prospective human studies ●●[39]. APOC3 was targeted because of reports showing that humans with reduced levels of this are protected from ACVD [40, 41]. They found that APOC3 HDL accounted for about 6–8% of total plasma APOA1. Interestingly, while levels of non-APOC3 HDL were associated with a lower disease risk, agreeing with years of data with total HDL-cholesterol, levels of APOC3-containing HDL subspecies were surprisingly positively associated with ACVD. A follow up study showed that APOC3 HDL levels were also positively correlated with higher carotid intima-media thickness in otherwise generally healthy adults [42]. Mechanistically, the APOC3 in HDL has been proposed to affect APOE-mediated clearance mechanisms in both APOB-containing lipoproteins and in HDL itself. APOE-rich HDL, another component-defined HDL subspecies, may be an important player in reverse cholesterol transport with APOC3 potentially working to inhibit its uptake.



Morton et al. ●●[43] tracked the metabolic fate of endogenously labeled proteins on HDL isolated by immunoaffinity chromatography. They found that APOE-HDL could expand in size in circulation and be quickly cleared, consistent with a role in reverse cholesterol transport. Non-APOE HDL did not expand and was cleared more slowly. However, when APOC3 was present on APOE-HDL, their expansion was curtailed and particle clearance was inhibited. The authors speculated that APOE-HDL plays important roles in the transport of cholesterol out of the vessel wall and is thus atheroprotective, but the presence of APOC3 on these particles negates that protection. When this was investigated in a large prospective population-based study, they found that APOE-HDL was inversely related to ACVD risk, but only the fraction that lacked APOC3. More recently, this same group compared the levels of 15 component-defined HDL subspecies to ACVD risk in four prospective studies. Increased levels of HDL subspecies defined by alpha-2-macroglobulin, complement C3, haptoglobin and plasminogen were associated with higher disease risk vs HDL particles lacking these components. Consistent with the earlier studies, HDL containing APOE were associated with lower risk. Particles containing APOC1 were also found to be protective ●●[44]. Overall, studies like these are beginning to reveal that component-defined HDL subspecies play complicated roles that may be protective or harmful in multifactorial diseases like ACVD.

### HDL subspecies in disease states - Type 2 Diabetes

APOC3 HDL also appears to play important roles in Type 2 Diabetes (T2D), a glucose metabolic disorder associated with a high degree of dyslipidemia. Aroner et al. noted that APOC3 levels in plasma were strongly associated with risk of T2D in the Danish Diet, Cancer and Health Study [45]. They followed up by using the immunoisolation techniques described above to probe the relationship between APOC3 HDL levels and diabetes risk in the Multi-Ethnic Study of Atherosclerosis ●[46]. The subspecies was found to be strongly associated with T2D risk and lower insulin sensitivity whereas HDL lacking APOC3 was inversely associated. The authors speculated that APOC3's effects on TG metabolism may explain some of the association, though accounting for TG concentrations did not fully eliminate the association potentially suggesting alternative functions of APOC3.

Recently, Kurano and colleagues [47] showed that S1P-HDL (or APOM-HDL) levels were negatively correlated with BMI and insulin resistance in humans. Mechanistic work showed that APOM/S1P can activate the AKT and AMPK insulin signaling pathways through S1P receptors. In conjunction, APOM was shown to improve mitochondrial function in liver and adipose cells. This dovetails nicely with their previous work which showed that APOM overexpression in mice stimulated insulin secretion *in vivo* and *in vitro* [48].

Considerable work has been done to understand the changes in HDL subfractions (i.e. not subspecies) that occur as a result of diabetes. Early studies using ultracentrifugation found lower levels of cholesterol-rich, larger HDL<sub>2</sub> in participants with ACVD and T2D but higher levels of cholesterol-poorer HDL<sub>3</sub> compared to patients without T2D and ACVD [49]. Two-dimensional gel separation studies have shown that women with T2D exhibit lower levels of large  $\alpha$ -1,  $\alpha$ -2, and pre- $\alpha$ -1 particles and higher levels of smaller particles such as lipid-poor  $\alpha$ -3, consistent with the ultracentrifugation studies [50]. Similarly, nuclear magnetic

resonance spectroscopy studies that quantitate HDL particle size and concentrations show that adults with T2D exhibit reduced levels of medium (9.0–11.5 nm) and large (11.5–18.9 nm) HDL particles but have an enrichment of small (7.8–9.0 nm) HDL compared to adults without diabetes [51–53]. Thus, in diabetes there appears to be a shift in favor of smaller CE-poor HDL subfractions.

In an attempt to go beyond HDL subfractions to explore HDL subspecies in more detail in T2D and obesity, our laboratory used a size exclusion chromatography technique to isolate lipoproteins by size across 15 fractions [54], a technique that corresponds well to NMR analysis [55]. In age- and sex-matched adolescents who were either lean, obese, or obese with T2D, we found that youths with T2D exhibited dramatically lower phospholipid content in large HDL compared to lean youths. The loss of these species correlated to increases in vascular stiffness measured by pulse wave velocity. With the ability to recover the particles for compositional analysis, we found that these were enriched in APOE, APOC1 and paraoxonase (PON)1. These same fractions from T2D and obese individuals exhibited diminished ability to promote cholesterol efflux and protect LDL from oxidation [56, 57] vs leans. Further work showed that surgical weight loss with vertical sleeve gastrectomy (VSG) could restore these APOE-rich HDL subspecies nearly to levels observed in lean subjects [56]. Current work is focusing on using immunoisolation techniques to further define these APOE-rich particles.

## Conclusions and ways forward

If one were to point to a single flaw in logic that has hampered our understanding of HDL biology, a strong case can be made for the tendency to over generalize what HDL actually is. Consider the plasma membrane (PM) of a cell. Most agree that the PM plays an important barrier function that maintains cellular integrity. But another critical role is to provide a diffusion medium that anchors lipid bound proteins and allows them diffuse in only two dimensions, dramatically increasing the odds that they will interact vs. Brownian diffusion in 3 dimensions. Receptor subunits, for example, can assemble within certain membrane domains producing a fully active receptor to mediate a biological function. This occurs for thousands of membrane-associated proteins in a given cell. Thus, one cannot reasonably argue that the PM has only one or two functions; it may have thousands of functions based on its mediation of protein:protein interactions (and protein:lipid interactions). The evidence summarized above indicates that HDL acts as an anchor point for extracellular proteins much like the PM does for cellular proteins. APOA1 and its solubilized phospholipids allows lipophilic proteins to segregate for the purpose of mediating different functions across a range of biochemical processes.

Accepting this paradigm, it is not surprising that a relatively ubiquitous component like cholesterol was a found to be a poor proxy for the entirety of HDL functionality. Subfractionating HDL by physicochemical properties initially seemed like a better solution to this problem. However, these too are unacceptably heterogeneous. Referring back to the PM analogy, if one uses ultracentrifugation to isolate PM vesicles from cells, it comes as no surprise that those vesicles, despite having similar density and size characteristics, contain a multitude of different PM proteins and receptors that are targeted toward very different



biological processes. Indeed, the Cell Surface Protein Atlas project has documented nearly 1500 cell surface glycoproteins in over 40 human cell lines [58]. The same seems to be true for HDL, if on a smaller scale. By extension, alternative lipid testing techniques such as NMR particle sizing and 2-D electrophoresis suffer from the same limitation that plagues HDL-cholesterol as a measurement. Fundamentally, they retain an unacceptable degree of compositional heterogeneity within each arbitrary designation (large vs. small HDL, pre-beta vs. alpha HDL, etc.).

Fortunately, these limitations are being recognized and particle isolation strategies are being employed that target specific subspecies components. These targeted components may seem minor in the context of total HDL, but they are key distinguishers of potentially functional sub particles. Immunoaffinity has been the most popular approach employed thus far, but other approaches that target the HDL “interactome” have potential. For example, one can envision chemical cross-linking techniques that capture specific protein:protein interactions within HDL subspecies being exploited. Alternatively, it may be possible to derive antibodies that identify discontinuous epitopes across interacting proteins or can bind specific conformational features characteristic of specific subspecies, as described above for pre-beta 1 HDL. There are also promising applications of high-resolution sizing techniques such as asymmetric flow field-flow fractionation combined with mass spectrometry to help narrow down these individual HDL species [59]. Once these analytical methods are optimized, the hope is that we can continue to relate specific HDL subparticles to specific functions that impinge on disease. Primary interest may focus on high incidence metabolic diseases such as ACVD, obesity and diabetes, but it is easy to see applications for a host of other chronic inflammatory and immune dysregulation disease states. Once identified, it may be possible to design therapies that skew HDL subpopulations toward those that are beneficial or it may be possible to design therapies that replace or enhance beneficial roles played by certain subspecies. In any case, it is an exciting time to be studying HDL.

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### Abbreviations:

|              |  |
|--------------|--|
| <b>ABCA1</b> | ATP binding cassette transporter A1    |
| <b>ALT</b>   | advanced lipid testing                 |
| <b>apo</b>   | apolipoprotein                         |
| <b>CETP</b>  | cholesteryl ester transfer protein     |
| <b>ACVD</b>  | atherosclerotic cardiovascular disease |
| <b>HDL</b>   | high density lipoprotein               |
| <b>HrP</b>   | haptoglobin-related protein            |
| <b>LDL</b>   | low density lipoprotein                |

|             |                               |
|-------------|-------------------------------|
| <b>NMR</b>  | nuclear magnetic resonance    |
| <b>RCT</b>  | reverse cholesterol transport |
| <b>TG</b>   | triglyceride                  |
| <b>T2D</b>  | Type 2 Diabetes               |
| <b>TLF</b>  | trypanosome lytic factor      |
| <b>VLDL</b> | very low density lipoprotein  |

## References

Papers of particular interest, published recently, have been highlighted as:

• Of importance

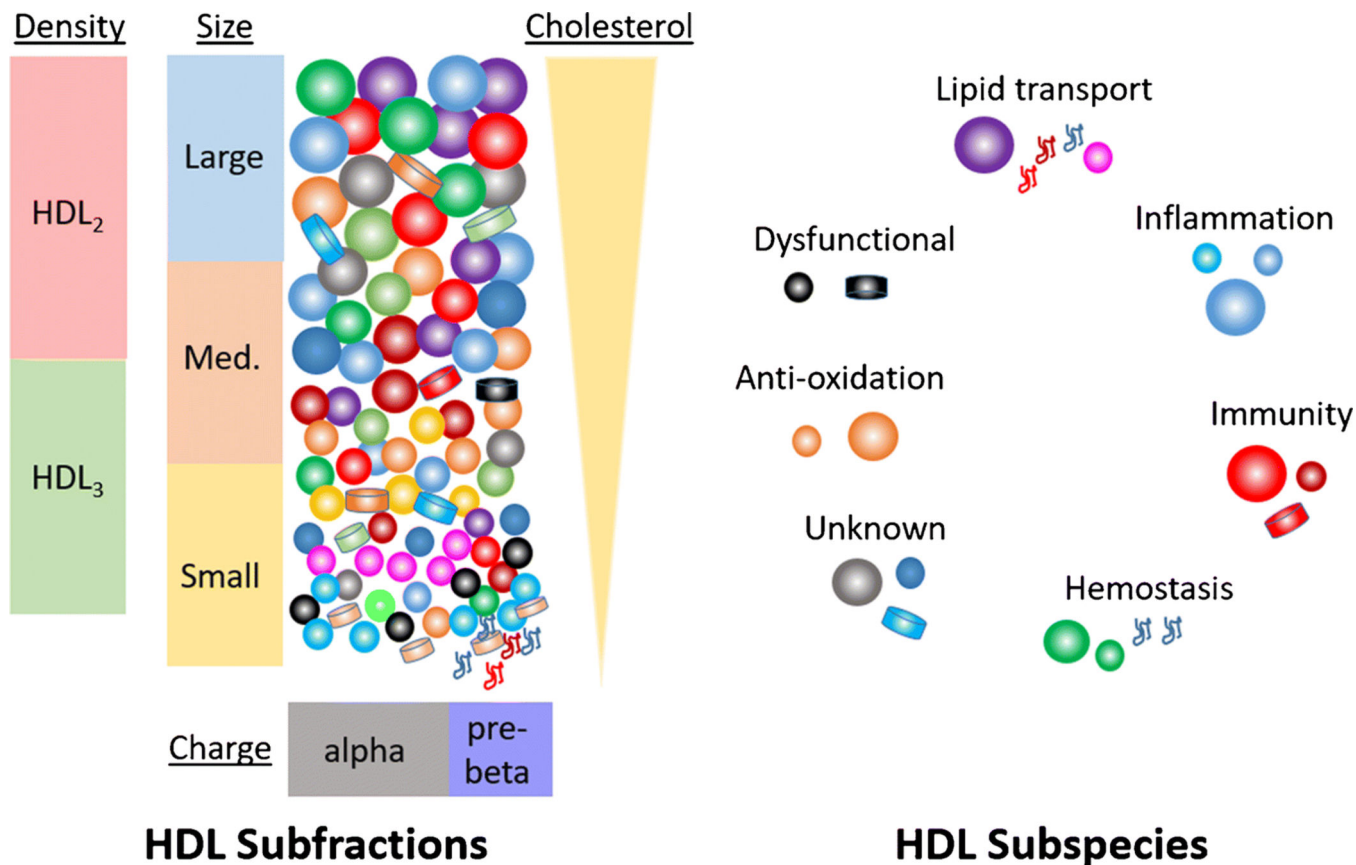
•• Of major importance

- Gordon T, Castelli WP, Hjortland MC, Kannel WB, Dawber TR. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. *AmJMed* 1977;62(5):707–14.
- Emerging Risk Factors C, Di Angelantonio E, Sarwar N, Perry P, Kaptoge S, Ray KK et al. Major lipids, apolipoproteins, and risk of vascular disease. *JAMA*. 2009;302(18):1993–2000. [PubMed: 19903920]
- Tall AR, Rader DJ. Trials and Tribulations of CETP Inhibitors. *Circ Res*. 2018;122(1):106–12. [PubMed: 29018035] ● A good review of the several existing clinical trials that have evaluated small molecule inhibitors of cholesteryl ester transfer protein. While these compounds clearly raise HDL-C, they have largely failed to alter major cardiovascular endpoints in humans
- Kontush A, Lhomme M, Chapman MJ. Unraveling the complexities of the HDL lipidome. *J Lipid Res*. 2013;54(11):2950–63. [PubMed: 23543772]
- Rye KA. High density lipoprotein structure, function, and metabolism: a new Thematic Series. *J Lipid Res*. 2013;54(8):2031–3. [PubMed: 23785125]
- Gordon SM, Remaley AT. High density lipoproteins are modulators of protease activity: Implications in inflammation, complement activation, and atherothrombosis. *Atherosclerosis*. 2017;259:104–13. [PubMed: 28242049]
- Creasy KT, Kane JP, Malloy MJ. Emerging roles of HDL in immune function. *Curr Opin Lipidol*. 2018;29(6):486–7. [PubMed: 30382953]
- De Lalla OF, Gofman JW. Ultracentrifugal analysis of serum lipoproteins. *Methods Biochem Anal*. 1954;1:459–78. [PubMed: 13193538]
- Kontush A, Chantepie S, Chapman MJ. Small, dense HDL particles exert potent protection of atherogenic LDL against oxidative stress. *ArteriosclerThrombVascBiol*. 2003;23(10):1881–8.
- Cheung MC, Albers JJ. Characterization of lipoprotein particles isolated by immunoaffinity chromatography. Particles containing A-I and A-II and particles containing A-I but no A-II. *J Biol Chem*. 1984;259(19):12201–9. [PubMed: 6434538]
- Rosenson RS, Brewer HB Jr., Chapman MJ, Fazio S, Hussain MM, Kontush A et al. HDL measures, particle heterogeneity, proposed nomenclature, and relation to atherosclerotic cardiovascular events. *Clin Chem*. 2011;57(3):392–410. [PubMed: 21266551]
- Chandra A, Rohatgi A. The role of advanced lipid testing in the prediction of cardiovascular disease. *Current Atherosclerosis Reports*. 2014;16(3):394. [PubMed: 24445969]
- Lutowski CA, Gordon SM, Remaley AT, Jarrold MF. Resolution of Lipoprotein Subclasses by Charge Detection Mass Spectrometry. *Anal Chem*. 2018;90(11):6353–6. [PubMed: 29756771]

14. Davidson WS, Silva RA, Chantepie S, Lagor WR, Chapman MJ, Kontush A. Proteomic analysis of defined HDL subpopulations reveals particle-specific protein clusters: relevance to antioxidative function. *Arterioscler Thromb Vasc Biol.* 2009;29(6):870–6. [PubMed: 19325143]
15. Remaley AT, Stonik JA, Demosky SJ, Neufeld EB, Bocharov AV, Vishnyakova TG et al. Apolipoprotein specificity for lipid efflux by the human ABCA1 transporter. *Biochem Biophys Res Commun.* 2001;280(3):818–23. [PubMed: 11162594]
16. Melchior JT, Street SE, Andraski AB, Furtado JD, Sacks FM, Shute RL et al. Apolipoprotein A-II alters the proteome of human lipoproteins and enhances cholesterol efflux from ABCA1. *J Lipid Res.* 2017;58(7):1374–85. [PubMed: 28476857]
17. Furtado JD, Yamamoto R, Melchior JT, Andraski AB, Gamez-Guerrero M, Mulcahy P et al. Distinct Proteomic Signatures in 16 HDL (High-Density Lipoprotein) Subspecies. *Arterioscler Thromb Vasc Biol.* 2018;38(12):2827–42. [PubMed: 30571168] ●● This paper takes what we believe is a much needed approach toward measuring specific specific component-defined HDL subspecies. This study clearly shows that multiple subspecies exist, are stable, and represent quite small fractions of the overall HDL particles in an individual.
18. Hawking F, Ramsden DB, Whytock S. The trypanocidal action of human serum and of baboon plasma. *Trans R Soc Trop Med Hyg.* 1973;67(4):501–16. [PubMed: 4206525]
19. Raper J, Fung R, Ghiso J, Nussenzweig V, Tomlinson S. Characterization of a novel trypanosome lytic factor from human serum. *Infect Immun.* 1999;67(4):1910–6.
20. Wheeler RJ. The trypanolytic factor-mechanism, impacts and applications. *Trends Parasitol.* 2010.
21. Vanhollebeke B, Pays E. The trypanolytic factor of human serum: many ways to enter the parasite, a single way to kill. *MolMicrobiol.* 2010;76(4):806–14.
22. Greene AS, Hajduk SL. Trypanosome Lytic Factor-1 Initiates Oxidation-stimulated Osmotic Lysis of *Trypanosoma brucei brucei*. *J Biol Chem.* 2016;291(6):3063–75. [PubMed: 26645690]
23. Currier RB, Cooper A, Burrell-Saward H, MacLeod A, Alsford S. Decoding the network of *Trypanosoma brucei* proteins that determines sensitivity to apolipoprotein-L1. *PLoS Pathog.* 2018;14(1):e1006855. ● Recent update on the protein species in TLF and how they may cooperate in the killing of trypanosomes.
24. Kunitake ST, La Sala KJ, Kane JP. Apolipoprotein A-I-containing lipoproteins with pre-beta electrophoretic mobility. *Journal of Lipid Research.* 1985;26(5):549–55. [PubMed: 3926924]
25. Favari E, Lee M, Calabresi L, Franceschini G, Zimetti F, Bernini F et al. Depletion of pre-beta-high density lipoprotein by human chymase impairs ATP-binding cassette transporter A1-but not scavenger receptor class B type I-mediated lipid efflux to high density lipoprotein. *J Biol Chem.* 2004;279(11):9930–6. [PubMed: 14701812]
26. Castro GR, Fielding CJ. Early incorporation of cell-derived cholesterol into pre-beta-migrating high-density lipoprotein. *Biochemistry.* 1988;27(1):25–9. [PubMed: 3126809]
27. O'Connor PM, Naya-Vigne JM, Duchateau PN, Ishida BY, Mazur M, Schoenhaus SA et al. Measurement of prebeta-1 HDL in human plasma by an ultrafiltration-isotope dilution technique. *Analytical Biochemistry.* 1997;251(2):234–40. [PubMed: 9299021]
28. Asztalos BF, Sloop CH, Wong L, Roheim PS. Two-dimensional electrophoresis of plasma lipoproteins: recognition of new apo A-I-containing subpopulations. *Biochimica et Biophysica Acta.* 1993;1169(3):291–300. [PubMed: 7548123]
29. Miyazaki O, Kobayashi J, Fukamachi I, Miida T, Bujo H, Saito Y. A new sandwich enzyme immunoassay for measurement of plasma pre-beta1- HDL levels. *J Lipid Res.* 2000;41(12):2083–8. [PubMed: 11108742]
30. Lamon-Fava S, Herrington DM, Reboussin DM, Sherman M, Horvath KV, Cupples LA et al. Plasma levels of HDL subpopulations and remnant lipoproteins predict the extent of angiographically-defined coronary artery disease in postmenopausal women. *Arterioscler Thromb Vasc Biol.* 2008;28(3):575–9. [PubMed: 18174456]
31. Stock EO, Ferrara CT, O'Connor PM, Naya-Vigne JM, Frost PH, Malloy MJ et al. Levels of prebeta-1 high-density lipoprotein are elevated in 3 phenotypes of dyslipidemia. *J Clin Lipidol.* 2018;12(1):99–109. [PubMed: 29198898] ● Shows that pre-beta 1 HDL levels are surprisingly elevated in a host of dyslipidemic states.

32. Quinn AG, Schwemberger R, Stock EO, Movsesyan I, Axtell A, Chang S et al. Moderate statin treatment reduces prebeta-1 high-density lipoprotein levels in dyslipidemic patients. *J Clin Lipidol*. 2017;11(4):908–14. [PubMed: 28558949]
33. Asztalos BF, Horvath KV, Schaefer EJ. High-Density Lipoprotein Particles, Cell-Cholesterol Efflux, and Coronary Heart Disease Risk. *Arterioscler Thromb Vasc Biol*. 2018;38(9):2007–15. [PubMed: 30002062] ●● A study that directly addresses the “Pre-beta 1 Paradox”. They measured HDL functions in a case-control study of CHD cases. Among other interesting results, they found that pre-beta 1 particle levels were higher in CHD cases, but the particles were impaired with respect to promoting cholesterol efflux in vitro.
34. Guey LT, Pullinger CR, Ishida BY, O'Connor PM, Zellner C, Francone OL et al. Relation of increased prebeta-1 high-density lipoprotein levels to risk of coronary heart disease. *Am J Cardiol*. 2011;108(3):360–6. [PubMed: 21757044]
35. Huang Y, Wu Z, Riwanto M, Gao S, Levison BS, Gu X et al. Myeloperoxidase, paraoxonase-1, and HDL form a functional ternary complex. *J Clin Invest*. 2013;123(9):3815–28. [PubMed: 23908111]
36. Persegh L, Verges B, Foissac M, Gambert P, Duvillard L. Inability of HDL from type 2 diabetic patients to counteract the inhibitory effect of oxidised LDL on endothelium-dependent vasorelaxation. *Diabetologia*. 2006;49(6):1380–6. [PubMed: 16596357]
37. Christoffersen C, Obinata H, Kumaraswamy SB, Galvani S, Ahnstrom J, Sevvana M et al. Endothelium-protective sphingosine-1-phosphate provided by HDL-associated apolipoprotein M. *Proc Natl Acad Sci USA*. 2011;108(23):9613–8. [PubMed: 21606363]
38. Chen Z, Hu M. The apoM-S1P axis in hepatic diseases. *Clin Chim Acta*. 2020;511:235–42. [PubMed: 33096030]
39. Jensen MK, Aroner SA, Mukamal KJ, Furtado JD, Post WS, Tsai MY et al. High-Density Lipoprotein Subspecies Defined by Presence of Apolipoprotein C-III and Incident Coronary Heart Disease in Four Cohorts. *Circulation*. 2018;137(13):1364–73. [PubMed: 29162611] ●● Landmark study that showed that HDL is not all the same when it comes to predicting cardiovascular disease in large human studies. APOC3 containing HDL subspecies were shown to be deleterious
40. Jorgensen AB, Frikke-Schmidt R, Nordestgaard BG, Tybjaerg-Hansen A. Loss-of-function mutations in APOC3 and risk of ischemic vascular disease. *N Engl J Med*. 2014;371(1):32–41. [PubMed: 24941082]
41. Tg, Hdl Working Group of the Exome Sequencing Project NHL, Blood I, Crosby J, Peloso GM, Auer PL et al. Loss-of-function mutations in APOC3, triglycerides, and coronary disease. *N Engl J Med*. 2014;371(1):22–31. [PubMed: 24941081]
42. Yamamoto R, Sacks FM, Hu FB, Rosner B, Furtado JD, Aroner SA et al. High density lipoprotein with apolipoprotein C-III is associated with carotid intima-media thickness among generally healthy individuals. *Atherosclerosis*. 2018;269:92–9. [PubMed: 29351856]
43. Morton AM, Furtado JD, Mendivil CO, Sacks FM. Dietary unsaturated fat increases HDL metabolic pathways involving apoE favorable to reverse cholesterol transport. *JCI Insight*. 2019;4(7).●●Used stable isotopes to track the fate of various compositionally defined HDL subspecies in humans. Showed that apoE-containing HDL acts like a particle that is important in reverse cholesterol transport, but the presence of apoC-III on these particles negates any benefit.
44. Sacks FM, Liang L, Furtado JD, Cai T, Davidson WS, He Z et al. Protein-Defined Subspecies of HDLs (High-Density Lipoproteins) and Differential Risk of Coronary Heart Disease in 4 Prospective Studies. *Arterioscler Thromb Vasc Biol*. 2020;40(11):2714–27. [PubMed: 32907368] ●●Example of a study that combined component-specific isolation of HDL subspecies and related them to ACVD in large human studies. Both beneficial and potentially deleterious subspecies are identified.
45. Aroner SA, Yang M, Li J, Furtado JD, Sacks FM, Tjonneland A et al. Apolipoprotein C-III and High-Density Lipoprotein Subspecies Defined by Apolipoprotein C-III in Relation to Diabetes Risk. *Am J Epidemiol*. 2017;186(6):736–44. [PubMed: 28520887]
46. Aroner SA, Furtado JD, Sacks FM, Tsai MY, Mukamal KJ, McClelland RL et al. Apolipoprotein C-III and its defined lipoprotein subspecies in relation to incident diabetes: the Multi-Ethnic Study

- of Atherosclerosis. *Diabetologia*. 2019;62(6):981–92. [PubMed: 30949716] ● Showed that HDL subspecies may play an important role in type 2 diabetes
47. Kurano M, Tsukamoto K, Shimizu T, Kassai H, Nakao K, Aiba A et al. Protection Against Insulin Resistance by Apolipoprotein M/Sphingosine-1-Phosphate. *Diabetes*. 2020;69(5):867–81.
  48. Kurano M, Hara M, Tsuneyama K, Sakoda H, Shimizu T, Tsukamoto K et al. Induction of insulin secretion by apolipoprotein M, a carrier for sphingosine 1-phosphate. *Biochim Biophys Acta*. 2014;1841(9):1217–26. [PubMed: 24814049]
  49. Bakogianni MC, Kalofoutis CA, Skenderi KI, Kalofoutis AT. Clinical evaluation of plasma high-density lipoprotein subfractions (HDL2, HDL3) in non-insulin-dependent diabetics with coronary artery disease. *J Diabetes Complications*. 2001;15(5):265–9. [PubMed: 11522502]
  50. Russo GT, Giandalia A, Romeo EL, Alibrandi A, Horvath KV, Asztalos BF et al. Markers of Systemic Inflammation and Apo-AI Containing HDL Subpopulations in Women with and without Diabetes. *Int J Endocrinol*. 2014;2014:607924.
  51. Amor AJ, Catalan M, Perez A, Herreras Z, Pinyol M, Sala-Vila A et al. Nuclear magnetic resonance lipoprotein abnormalities in newly-diagnosed type 2 diabetes and their association with preclinical carotid atherosclerosis. *Atherosclerosis*. 2016;247:161–9. [PubMed: 26921744]
  52. Garvey WT, Kwon S, Zheng D, Shaughnessy S, Wallace P, Hutto A et al. Effects of insulin resistance and type 2 diabetes on lipoprotein subclass particle size and concentration determined by nuclear magnetic resonance. *Diabetes*. 2003;52(2):453–62. [PubMed: 12540621]
  53. Mora S, Otvos JD, Rosenson RS, Pradhan A, Buring JE, Ridker PM. Lipoprotein particle size and concentration by nuclear magnetic resonance and incident type 2 diabetes in women. *Diabetes*. 2010;59(5):1153–60. [PubMed: 20185808]
  54. Gordon SM, Deng J, Lu LJ, Davidson WS. Proteomic characterization of human plasma high density lipoprotein fractionated by gel filtration chromatography. *J Proteome Res*. 2010;9:523–949.
  55. Davidson WS, Heink A, Sexmith H, Melchior JT, Gordon SM, Kuklenyik Z et al. The effects of apolipoprotein B depletion on HDL subspecies composition and function. *J Lipid Res*. 2016;57(4):674–86. [PubMed: 26908829]
  56. Davidson WS, Heink A, Sexmith H, Dolan LM, Gordon SM, Otvos JD et al. Obesity is associated with an altered HDL subspecies profile among adolescents with metabolic disease. *Journal of lipid research*. 2017;58(9):1916–23. [PubMed: 28743729]
  57. Gordon SM, Davidson WS, Urbina EM, Dolan LM, Heink A, Zang H et al. The effects of type 2 diabetes on lipoprotein composition and arterial stiffness in male youth. *Diabetes*. 2013;62(8):2958–67. [PubMed: 23835332]
  58. Bausch-Fluck D, Hofmann A, Bock T, Frei AP, Cerciello F, Jacobs A et al. A mass spectrometric-derived cell surface protein atlas. *PLoS One*. 2015;10(3):e0121314.
  59. Bria CRM, Afshinnia F, Skelly PW, Rajendiran TM, Kayampilly P, Thomas TP et al. Asymmetrical flow field-flow fractionation for improved characterization of human plasma lipoproteins. *Anal Bioanal Chem*. 2019;411(3):777–86. [PubMed: 30470915]



**Figure 1. An illustration of the difference between HDL subfractions and functionally distinct HDL subspecies.**

The **left panel** shows a schematic representation of the numerous HDL particles from large to small spheres, discs, and poorly lipidated particles that one might encounter in a drop of peripheral blood. The problem with current subfractionation techniques is highlighted by the depicted ranges for methods that separate particles by size, density or charge. For example, isolated HDL<sub>3</sub> particles are homogeneous with respect to density, but clearly contain particles of highly varying composition. Sizing methods and charge based methods suffer from the same issue. The *yellow triangle* shows how the generic marker of cholesterol not only encompasses all of the heterogeneity, but also overemphasizes lipid-rich, light, and large HDL particles while underemphasizing lipid-poor, heavy and small HDL particles including minimally lipidated apolipoproteins. The **right panel** shows the concept of compositionally defined subspecies containing active protein or lipid components that perform specific functions in different physiological processes. These species can be targeted by applying isolation or analytical techniques that target a specific component of the subspecies. We argue that it is these component-defined subspecies that need to be isolated and characterized if we are to make progress in understanding how HDL may be leveraged for disease therapies.