

## REVIEW ARTICLE

**A Closer Look at Cardioprotective Function of HDL:  
Revise the HDL – Cholesterol Hypothesis?**Anna Meiliana<sup>1,2,\*</sup>, Andi Wijaya<sup>1,2</sup><sup>1</sup>Postgraduate Program in Clinical Biochemistry, Hasanuddin University, Jl. Perintis Kemerdekaan Km.10, Makassar, Indonesia<sup>2</sup>Prodia Clinical Laboratory, Jl. Cisangkuy No.2, Bandung, Indonesia

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**Abstract**

**BACKGROUND:** The strong inverse association of plasma levels of high-density lipoprotein (HDL) cholesterol with coronary heart disease (CHD) found in human epidemiological studies led to the development of the ‘HDL cholesterol hypothesis’, which posits that intervention to raise HDL cholesterol will result in reduced risk of CHD. A number of recent developments have brought the potential protective role of HDL into question. Several clinical trials of agents that substantially raise HDL-C have been demonstrated to not reduce CHD event rates.

**CONTENT:** For decades, HDL and HDL-cholesterol (HDL-C) levels were viewed as synonymous, and modulation of HDL-C levels by drug therapy held great promise for the prevention and treatment of cardiovascular disease. Nevertheless, recent failures of drugs that raise HDL-C to reduce cardiovascular risk and the now greater understanding of the complexity of HDL composition and biology have prompted researchers in the field to redefine HDL. As such, the focus of HDL has now started to shift away from a cholesterol-centric view toward HDL particle number, subclasses, and other alternative metrics of HDL. Many of the recently discovered functions of HDL are, in fact, not strictly conferred by its ability to promote cholesterol flux but by the other molecules it transports, including a diverse set of proteins, small RNAs, hormones, carotenoids, vitamins, and bioactive lipids. Based on HDL’s ability to interact with almost all cells and deliver fat-soluble cargo, HDL has the remarkable capacity to affect a wide variety of endocrine-like systems.

**SUMMARY:** There is a significant need to redefine HDL and its benefit. HDL transports a diverse set of functional

**Abstrak**

**ATAR BELAKANG:** Penelitian epidemik pada manusia menunjukkan hubungan terbalik yang kuat antara kolesterol *high-density lipoprotein* (HDL) plasma dengan penyakit jantung coroner (PJK). Hal ini memicu perkembangan “hipotesis kolesterol HDL” yang menyatakan bahwa peningkatan kadar HDL dapat mengurangi risiko PJK. Banyak perkembangan saat ini yang mempertanyakan potensi proteksi HDL. Beberapa uji klinis menunjukkan bahwa peningkatan kolesterol HDL secara substansial ternyata tidak mengurangi laju kejadian PJK.

**ISI:** Selama beberapa dekade, kadar HDL dan kolesterol HDL dianggap sama, dan terapi obat yang dapat meningkatkan kadar kolesterol HDL diharapkan dapat digunakan untuk mencegah dan mengobati penyakit kardiovaskular. Namun, kegagalan obat peningkat kadar kolesterol HDL dalam mengurangi risiko kardiovaskular, ditambah dengan pemahaman yang lebih baik mengenai kompleksitas komposisi dan biologi HDL, menuntut para peneliti untuk mendefinisikan ulang HDL. Dengan demikian, fokus HDL saat ini bergeser dari kolesterol menuju ke jumlah partikel HDL, subklas dan metrik HDL lainnya. Beberapa penemuan akhir-akhir ini menunjukkan fakta bahwa fungsi HDL sesungguhnya bukan meningkatkan keluaran kolesterol melainkan melalui molekul lain yang dibawa, HDL membawa bermacam protein yang berlainan, *small* RNA, hormon, karotenoid, vitamin, dan lemak bioaktif. Berdasarkan kemampuan HDL untuk berinteraksi dengan hampir semua sel dan mengantarkan muatan larut lemak, HDL memiliki kapasitas luar biasa untuk mempengaruhi sebagian besar sistem dalam tubuh yang menyerupai endokrin.

**RINGKASAN:** Perlu definisi ulang mengenai HDL dan

proteins, including many binding proteins. HDL transports and deliver vitamins, carotenoids, and other small molecules. Moreover, HDL transports hormones, steroids and bile acids, and can modulate multiple endocrine pathways. HDLs also transport and deliver microRNAs to recipient cells and control gene expression. Likewise, HDLs carry bioactive lipids and can activate signaling cascades and receptors that control endothelial apoptosis, migration, survival and activation. Many of HDL's alternative non-cholesterol cargo likely confer many of HDL's alternative functions.

**KEYWORDS:** HDL, ApoA1, RCT, ABCA1, ABCG1, miRNA, HDL Lipidome, HDL Proteome

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## Introduction

In population studies, high-density lipoprotein cholesterol (HDL-C) is inversely related to the risk of myocardial infarction and death.(1-4) Of note, in patients fully treated according to current guidelines with intense statin therapy and low-density lipoprotein cholesterol (LDL-C) at target levels, HDL-C remains predictive of outcome for major adverse cardiovascular events.(5) HDL and HDL-C levels are often considered to be one and the same, referred to as “good” cholesterol, mainly due to the epidemiologically observed inverse association of HDL-C to cardiovascular disease (CVD) risk. As a consequence, billions of dollars and herculean effort were invested into HDL biology and pharmacological strategies to raise HDL-C levels for the prevention and treatment of CVD.(6) However, interventions that increase the concentration of HDL-C in humans have not yet been shown to translate into a reduction in clinical cardiovascular events. Indeed, recent human clinical trials investigating the effects of raising the level of HDL-C by treatment with cholesteryl ester transfer protein (CETP) inhibitors or with niacin failed to demonstrate any clinical cardiovascular benefit.(7-9)

A reasonable assumption that has been made from these studies is that the cholesterol content of HDLs is not the factor that protects. Thus, while the concentration of HDL-C is generally an excellent marker of the HDL functions that do protect, it does not invariably reflect their cardioprotective functions.(10) Thus, the pathogenic role and, hence, suitability of HDL as a therapeutic target has

funksinya. HDL mentransportasikan berbagai macam protein fungsional, meliputi beberapa protein pengikat. HDL membawa dan mengantar vitamin, karotenoid, dan molekul kecil lainnya. Lebih dari itu, HDL juga mengantar hormon, steroid dan asam empedu, dan dapat mengatur berbagai jalur endokrin. HDL juga mengantar *microRNA* ke sel penerima dan mengendalikan ekspresi gen. HDL mengangkut lemak bioaktif dan dapat mengaktifkan kaskade sinyal dan reseptor yang mengendalikan apoptosis, migrasi, pertahanan hidup dan aktivasi endotel. Beberapa muatan non kolesterol yang dibawa HDL tampaknya juga menambah fungsi alternatif HDL.

**KATA KUNCI:** HDL, ApoA1, RCT, ABCA1, ABCG1, miRNA, HDL Lipidome, HDL Proteome

increasingly been questioned. In fact, it has been argued that low HDL-C may only represent a marker for proatherogenic risk factors, rather than HDL being a mediator protecting against atherogenesis.(11) Such a conclusion neglects the structural and functional heterogeneity of HDL, which is neither reflected by the nonfunctional clinical biomarker HDL-C nor has yet been targeted by randomized controlled trials (7,9,12,13) or Mendelian randomization studies (14,15).

Several well-documented functions of HDL and apolipoprotein AI (ApoA-I) report the potential to protect against CVD. The most extensively studied was the ability of HDLs to promote efflux of cholesterol from macrophages in the artery wall.(16) HDL inhibits vascular inflammation (17,18), and has antioxidant (17) and antithrombotic (19) properties. HDL also enhances endothelial function (20), promotes endothelial repair (21,22), increases angiogenesis (23), suppresses the production and mobilization of monocytes and neutrophils from bone marrow (24), and have recently been reported to have antidiabetic property (25,26). Which of these HDL functions are clinically important is not known. Nor is it known which HDL component(s) or subpopulations are responsible for these potentially cardioprotective properties. Until we know which HDL components and subpopulations relate to specific potentially cardioprotective functions and until we have much more information about the effects of HDL-raising therapies on HDL composition, HDL subpopulation distribution, and HDL function, it will be difficult to predict how any specific HDL-targeted therapy will impact on human cardiovascular risk.(10)

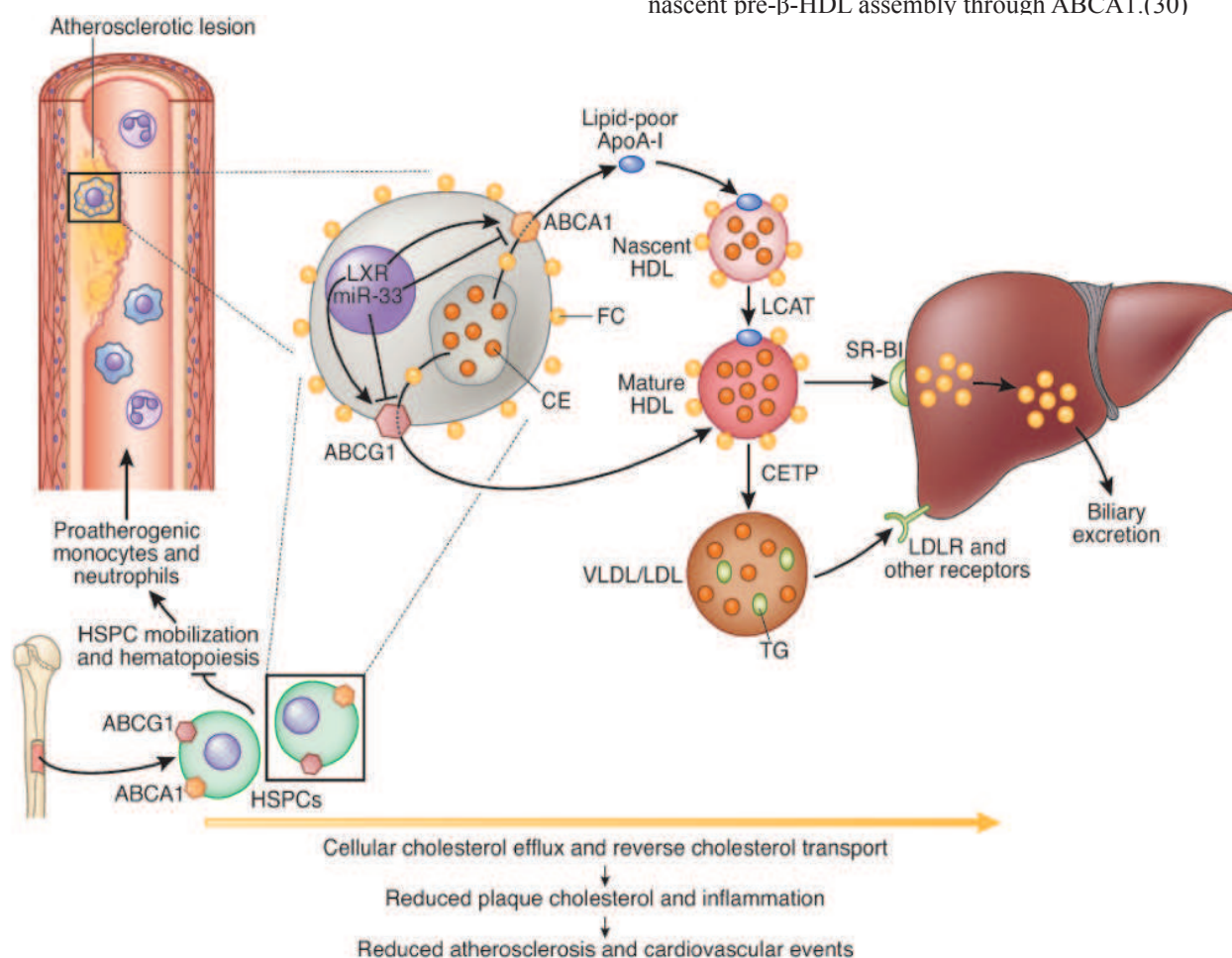
What has become apparent in the course of these sobering messages regarding HDL-C is that cholesterol-based measures of HDL may only tell us part of the story. HDL is known to circulate in plasma as a heterogeneous cohort of particles, varying markedly in terms of size, shape, and composition of proteins and lipids. To what degree this influences the relative functional properties of these particles and their ability to protect the artery wall remains unknown. (27) Given that interventions that increase the concentration of HDL-C may not necessarily be accompanied by an enhancement of HDL function, it is clear that we need to understand much more about how HDL function relates to HDL subpopulation distribution and cardiovascular risk in order to develop rational HDL-based therapeutic strategies for preventing atherosclerosis. (10)

### HDL Metabolism and Heterogeneity

HDLs are typically defined as lipoprotein particles with buoyant densities from 1.063 to 1.21 g/mL and having

ApoA-I as the major apolipoprotein species. It has become increasingly appreciated that the HDL population consists of a collection of particles with diverse sizes, structures, and composition and functional properties that are thought to influence atheroprotectiveness. The compositional complexity reflects not only multiple species of proteins and lipids but also other macromolecules (*e.g.*, microRNA (miRNA)).(28)

Reverse cholesterol transport is generally used to describe the transport of cholesterol by HDL from the vascular wall to the liver for excretion into bile as neutral sterol or bile acid.(29) *de novo* synthesis of HDL involves the secretion of ApoA-I by liver and small intestine into circulation, followed by a largely extracellular acquisition of phospholipids (PL) and cholesterol leading to the formation of nascent HDL. The early ApoA-I lipidation with free cholesterol (FC) and phosphatidylcholine (PC) occurs on its critical interaction with ATP-binding cassette sub-family A member 1 (ABCA1) and results in the formation of discoidal pre- $\beta$ -HDL particles. ApoM is an HDL-associated apolipoprotein that affects HDL biogenesis by affecting nascent pre- $\beta$ -HDL assembly through ABCA1.(30)



**Figure 1. Pathways influencing HDL cholesterol metabolism and flux and potential relationship to atherosclerosis.**(31) (Adapted with permission from Nature Publishing Group).

In the circulation, nascent disc-shaped HDL is, under normal conditions, thought to mature into larger spherical HDL. This process entails acquisition of cholesteryl ester (CE) in its hydrophilic lipid core, a step made possible through lecithin cholesterol acyltransferase (LCAT). In the circulation, several proteins and enzymes modulate HDL. In humans, these include CETP, PL transfer protein (PLTP), hepatic lipase (LIPC), endothelial lipase (LIPG), and secreted phospholipase A2 (sPLA2).(32) CETP accommodates the transfer of CE from HDL to ApoB-containing lipoproteins in exchange for triglycerides (TG). PLTP is crucial to HDL particle remodeling. PLTP facilitates the transfer of PL from TG-rich lipoprotein (TRL) to HDL with the formation of both larger and smaller particles, whereas it can also induce fusion of smaller HDL.(33,34) LIPC is involved in breaking-down HDL-TG and PL, thereby reducing HDL size and enhancing the dissociation of lipid-free/lipid-poor ApoA-I from larger HDL.(35) LIPG, a second lipolytic enzyme, is expressed in the liver, lung, kidney, and placenta. The enzyme has shown to exhibit more phospholipase activity than TG lipase activity with a major preference for HDL instead of TRL. sPLA2 is highly expressed in the liver, particularly during acute and chronic inflammatory states. This enzyme hydrolyzes the sn-2 ester bond of PL to release a lyso PL and a nonesterified free fatty

acid.(36)

Proteins and enzymes that affect HDL metabolism through their impact on plasma TG lipolysis. These mostly affect the activity of lipoprotein lipase (LPL), the sole enzyme capable of hydrolyzing plasma TG in plasma TRL. LPL is synthesized and secreted by parenchymal cells in metabolically active muscle and adipose tissue. At these sites, surface lipid (FC and PL) and apolipoproteins resulting from TRL hydrolysis are conveyed from TRL to HDL.(35,37) For its catalytic activity, LPL needs apoC-II as cofactor, a small protein of 79 amino acids present on TRLs and HDL. ApoA-V can be considered as a modulator of LPL activity. ApoA-V (11q23) is expressed in the liver and the protein is secreted into plasma, where it associates with very low-density lipoprotein (VLDL), chylomicrons, and HDL. It seems to be a key modulator of plasma TG homeostasis but the molecular mechanisms are not fully understood. (38,39) The LPL reaction is regulated in a spatiotemporal fashion by several inhibitory factors encoded by ApoC-III, angiopoietin-like 3 (ANGPTL3), and ANGPTL4, which all affect HDL metabolism. It has been suggested that ApoC-III increases the catabolism of HDL and is involved in other relevant lipid metabolic functions.(40) Both encoded proteins can act as inhibitors of LPL activity by promoting, in different ways, the dissociation of the active LPL

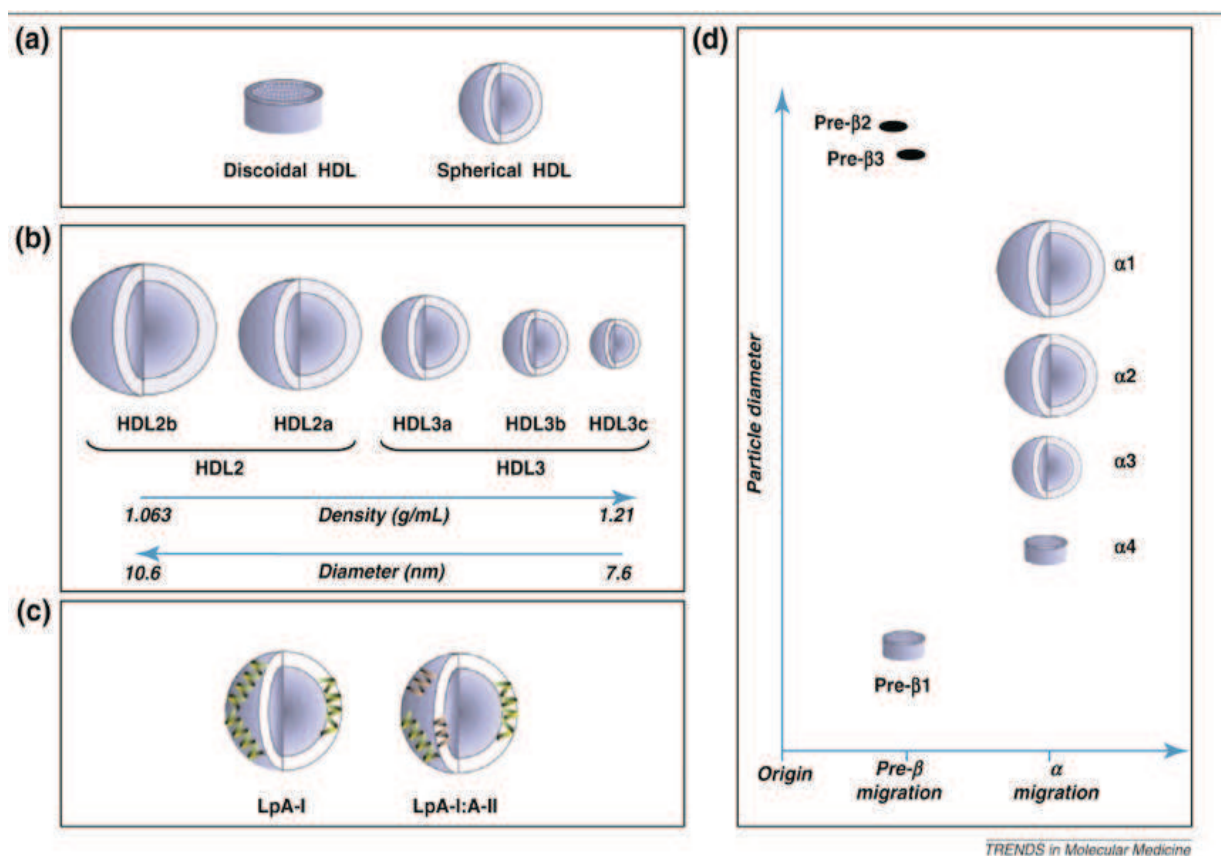


Figure 2. Heterogeneity of HDL particles. (42) (Adapted with permission from Elsevier Ltd.).



homodimer into inactive monomers. Scavenger receptor class B type I (SR-BI), as the main high-affinity receptor for HDL, enables the selective uptake of CE from circulating HDL via ApoA-I recognition. This occurs, however, without mediating the degradation of HDL, as is the case for LDL.(41) Plasma HDL particles are, however, highly heterogeneous in structure, composition and biological function.

Such heterogeneity results from differences in the relative content of apolipoproteins and lipids in HDL.(42) A topic of considerable clinical and biological interest is whether specific subfractions of HDL are more informative in assessing the benefits of a therapeutic intervention targeted to HDL.

### HDL Metabolome, Lipidome and Proteome

HDL's ability to participate in the reverse cholesterol transport (RCT) pathway is, likely an important part of its anti-atherogenic role. We are now beginning to realize the full breadth of HDL's function and cargo; thus, there is a significant need to begin to rethink and possibly focus on these other aspects of HDL besides its role in transporting cholesterol. Although it is not clear what the new focus on HDL should be, we review here the latest findings related to the metabolome, transcriptome, lipidome, and proteome of HDL.

Besides lipids, we know relatively little about the repertoire of small molecules circulating on HDL; however, this will likely be an emerging new field for HDL research. As HDL is possibly rebranded as a general transporter of cargo between cells, a comprehensive characterization of HDL's metabolome is warranted. Some of the best studied small metabolites carried by HDL are the fat-soluble vitamins.(43-46) A majority of  $\alpha$ -tocopherol (vitamin E), for example, is transported in plasma by lipoproteins, including HDL, and delivered to recipient cells by way of lipoproteins. Most interestingly, HDL delivery of vitamin E to epithelial cells was found to be independent of SR-BI, as chemical inhibition of with basic lipid transport-1 (BLT-1) failed to reduce vitamin E delivery.(47) Conversely, endothelial cell uptake of HDL-vitamin E was found to be mediated by SR-BI.(43,48) Vitamin E is an essential antioxidant vitamin, although the relevance of HDL-mediated vitamin E transfer between cells is currently unknown.

Unlike vitamin E, most of vitamin D is believed to be bound to a specific serum-binding protein. The vitamin D-binding protein, however, is routinely found in

proteomic studies to be associated with HDL.(49,50) Most interestingly, vitamin D and vitamin D receptor modulators have been found to inhibit ApoA-I.(51,52)

Retinol (vitamin A) is also likely transported in various forms on HDL. Similar to vitamin D, retinol associates with a binding protein, the plasma retinol-binding protein (PRBP), which has also been reported in multiple studies to be associated with HDL.(50) HDL also transports many carotenoids, which are lipophilic precursors for vitamin A. (46) Although HDL only transports a small percentage (of total plasma mass) of nonpolar carotenoids (*e.g.*, lycopene, 17%;  $\alpha$ -carotene, 26%; and  $\beta$ -carotene, 22%), a large percentage of polar carotenoids are found on HDL (lutein, 53%; cryptoxanthin, 39%).(53)

One of the most widely studied carotenoid,  $\beta$ -carotene, has been found to inhibit atherosclerosis in hypercholesterolemic rabbits and is associated with HDL. (54) HDL has also been found to transport ubiquinone (UQ) (coenzyme Q or coenzyme Q10) and may facilitate the intercellular transfer of UQ or dietary intake delivery of UQ to recipient cells.(55) Although the role of UQ in disease is not fully understood, HDL-mediated transport of UQ has been proposed to possibly help prevent heart failure, cancer, migraines, and hypertension.

Currently, the functional relevance of the intercellular transfer of the HDL metabolome is not fully understood, nor have we likely identified all the small molecules that can be transported by HDL. A comprehensive analysis of the HDL metabolome is warranted and will likely uncover other metabolites and homeostatic networks affected by HDL.

The lipids carried by HDL can be largely categorized as being either neutral hydrophobic lipids (CE and TG), which are carried in the core of HDL, or amphipathic lipids, such as cholesterol or PL, which are on the surface of HDL. (6)

It is not cholesterol, however, but PL that quantitatively predominate in the HDL lipidome, accounting, together with sphingomyelin (SM), for 40-60 wt% of total lipid, with lesser proportions of CE (30-40%), TG (5-12%), and FC (5-10%). Structurally, individual HDL lipid classes fulfill distinct functions; PL constitute the surface lipid monolayer of HDL, whereas CE and TG form the hydrophobic lipid core. Unesterified sterols are predominantly located to the surface monolayer, partially penetrating the core.(56)

Recently, it has been realized that some of the lipids carried by HDL can be transformed into potent bioactive molecules. For example, PC, the most common PL on HDL, is highly susceptible to oxidation, which after hydrolysis generates the highly reactive and damaging lyso-PC (LPC). (57,58)

SM, another common class of lipids on HDL, is a substrate for neutral sphingomyelinase-2 (nSMase2) in the ceramide signaling pathway.(59) SM can also be converted to lysosphingolipids, namely, sphingosylphosphorylcholine and lysosulfatide, which have been found to protect against endothelial apoptosis through activation of Akt signaling. Most interestingly, the Akt-signaling cascade is responsible for HDL-lipid suppression of apoptosis, as inhibition of Akt phosphorylation of BCL-2-associated death (BAD) promoter blocked the HDL-lipid effect.(60) Recent evidence suggests that the lysosphingolipid sphingosine-1-phosphate (S1P) may mediate many actions of HDL such as vasodilation, angiogenesis and endothelial barrier function, and protection against atherosclerosis and ischemia/reperfusion injury. (61,62)

High HDL levels do not always protect against CVD and there is accumulating evidence suggesting that simply increasing the circulating HDL-C does not necessarily confer cardiovascular benefits.(8,63) This leads to the hypothesis that the HDL in some patients may be dysfunctional and its other properties and compositions like S1P might be more important than its cholesterol cargo. (65) Evidence suggests that HDL serves as an S1P signaling platform and likely mediates a multitude of cardiovascular effects, and therapeutic strategies involving HDL S1P hold much promise for the future. Recent applications of mass spectrometry technology have dramatically increased our understanding of the proteomic diversity of HDL. Depending on the method of HDL isolation, upwards of 85 proteins have been identified, and the list continues to grow. (66)

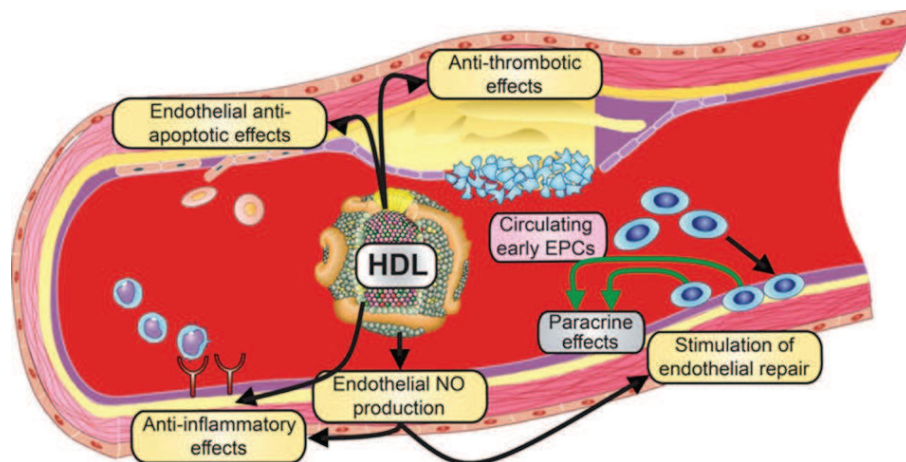
HDL carries surprising constituents, such as members of the complement pathway, protease inhibitors involved in hemostasis, acute-phase response proteins, immune function mediators, and even metal-binding proteins. This compositional diversity fits well with hundreds of studies demonstrating a wide functional pleiotrophy, including

roles in lipid transport, oxidation, inflammation, hemostasis, and immunity. The recent studies of the HDL proteome have revealed that besides the “classic” proteins on HDL, such as ApoA-I, that largely serve a structural role in HDL or a role in its metabolism, HDL cargo includes other proteins that may have distinct biological effects and could account for some of the other functions of HDL besides cholesterol transport. Recently, we discovered that HDL also transports small RNAs, including miRNA, tRNA-derived RNA fragments (tRF), and RNase P-derived RNA fragments. (67) Outside of cholesterol efflux, HDL has many other beneficial functions, and unique miRNA, lipid, protein, and small molecule components likely mediate each, if not all of these functions, including cytoprotective, anti-thrombotic, anti-infectious, vasodilatory, and many other functions to be discovered.

### Biological Activities of HDL Subpopulations

HDL possesses several antiatherogenic activities which involve cholesterol efflux from cells as well as anti-oxidative, anti-inflammatory, cytoprotective, vasodilatory, anti-thrombotic and anti-infectious activities.(68,69) The efflux of cholesterol from a variety of cell types, including macrophages, to HDLs in the extracellular space is mediated by four distinct processes.(70). These are: i) the efflux of cholesterol to ApoA-I from cells expressing ABCA1 (71,72); ii) the efflux of cholesterol to HDLs from cells expressing ABCG1 (73,74); iii) the bidirectional exchange of cholesterol between HDLs and cell membranes expressing sSR-B1 (75,76); and iv) passive aqueous diffusion of cholesterol from cell membranes to HDLs (75).

Several miRNAs, for example, miR33, miR122, and miR144, decrease the production of ABCA1 and hence reduce cholesterol efflux.(77-79) Thus, cholesterol



**Figure 3. Proposed direct vascular protective and potentially antiatherogenic effects of normal HDL.(5)** (Adapted with permission from American Heart Association).

efflux is determined by the extracellular concentration and composition of HDL particles as well as by the activity of ABC transporters. By finetuning cellular cholesterol homeostasis, cholesterol efflux by ApoA-I / ABCA1- and HDL-mediated efflux in concert with ABCG1 exerts important regulatory steps on many cellular functions, including proliferation and mobilization of hematopoietic stem cells (79). ABCG4-mediated cholesterol efflux to HDL regulates megakaryocyte proliferation.(80) Cholesterol efflux also regulates the inflammatory responses of monocytes and macrophages (81), expansion of lymphocytes (82), nitric oxide (NO) production by endothelial NO synthase (eNOS) (83), and insulin secretion from pancreatic  $\beta$ -cells (84).

When comparing cholesterol efflux properties of HDL subpopulations, it is essential to keep in mind the concentration basis employed for such comparison. Thus, on the basis of PL content, small, dense HDLs more potently promote cholesterol efflux, whereas on a particle number basis, large HDLs are more effective.(85,86)

HDLs possess antioxidant properties that have the capacity to inhibit the proatherogenic oxidative modification of LDL.(87) The mechanism by which HDLs inhibit oxidation is uncertain. The most extensively studied aspect of this process involves activity of paraoxonase-1 (PON-1), which is transported in the plasma as a component of the HDL fraction.(88) ApoA-I (89), ApoA-II (89), ApoA-IV (90,91) and ApoE (92) all have antioxidant properties *in vitro*. Which of these apolipoproteins, if any, is also antioxidant *in vivo* is not known. HDL particles are also heterogeneous in their capacity to protect LDL from oxidative damage induced by one-electron oxidants such as free radicals. A non-uniform distribution of apolipoproteins, enzymes and lipids across the HDL particle spectrum could underlie this observation. There is evidence that the antioxidant activity of HDL 3 is superior to that of HDL 2.(42) It has also been reported that PL modulate the antioxidant properties of HDLs.(93)

Inflammation plays a pivotal role in both the genesis and the instability of atherosclerotic plaques. HDLs have the capacity to inhibit this inflammation by multiple mechanisms. They inhibit the binding of monocytes to cultured endothelial cells *in vitro*.(87) They also reduce the cytokine induced expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin in cultured endothelial cells in a concentration dependent manner.(18) The ability of HDLs to inhibit inflammation in endothelial cells *in vitro* varies according to the PL composition of the particles (94), with their constituent apolipoproteins exhibiting much lower specificity (95).

Protein that is induced both *in vitro* (96) and *in vivo* (97) by rHDLs is the antioxidant protein 3  $\beta$ -hydroxysteroid- $\Delta$ 24 reductase (also known as 24-dehydrocholesterol reductase or DHCR24). Silencing DHCR24 expression in endothelial cells not only increases nuclear factor  $\kappa$ B (NF- $\kappa$ B) activation and VCAM-1 protein levels in both nonactivated and TNF- $\alpha$ -activated endothelial cells, but it is also associated with a loss of the anti-inflammatory effects of rHDLs.(96)

The potential heterogeneity of HDL anti-inflammatory activity remains poorly characterised. Small, dense, protein-rich HDL3 has been reported to be superior to large, light, lipid-rich HDL2 in terms of capacity to inhibit VCAM-1 expression in endothelial cells on the basis of both ApoA-I and total cholesterol (98), consistent with the potent antioxidative activity of small, dense HDL3 (99). HDL from healthy subjects can exert several protective effects in the vasculature and, in particular, on endothelial cells. Of note, HDL from healthy subjects stimulates NO release from human aortic endothelial cells in culture and increases the expression of eNOS.(100-103) HDLs display cytoprotective activity, an effect attributed to ApoA-I, ApoE and HDL-associated lysosphingolipids and observed as a prevention of endothelial cell apoptosis elicited by growth factor withdrawal and other agents.(104) This antiatherogenic activity is paralleled by the ability of HDL to stimulate endothelial cell migration and survival, an effect potentially mediated by S1P.(105) HDLs inhibit thrombosis by attenuating expression of tissue factor and selectins, by downregulating thrombin generation *via* the protein C pathway, and by directly and indirectly blunting platelet activation.(19) Inhibition of platelet activation is also dependent on SR-B1.(106,107) HDLs enhance endothelial repair by two distinct mechanisms. They stimulate endothelial cell migration from healthy to damaged endothelium *via* the SR-B1-mediated activation of Rac GTPase in a process involving activation of Src kinases, phosphatidylinositol 3 kinase (PI3K), and p44/42 mitogen-activated protein kinases.(21) In addition, HDLs can promote endothelial repair *in vivo* by enhancing the engraftment of endothelial progenitor cells into areas of damaged endothelium.(22)

Studies conducted in mice have shown that suppression of the cholesterol efflux promoted by ABCA1 and ABCG1 in bone marrow cells leads to hematopoietic stem cell proliferation and mobilization.(24) Importantly, HDL particles can progressively lose normal biological activities and acquire altered properties as a result of alterations in HDL composition, structure and metabolism, which are characteristic of dyslipidemia, insulin resistance, inflammation, infection and CVD.(68)

## HDL Endothelial Dysfunction and Atherosclerosis

HDL has the potential to protect against atherosclerosis by multiple mechanisms, including the removal of cholesterol from macrophages in the arterial wall in the first step of the reverse cholesterol transport pathway.(108) HDL also inhibit vascular inflammation and oxidation, enhance endothelial repair, and improve endothelial function. (97,109) Endothelial injury and dysfunction (ED) represent a link between cardiovascular risk factors promoting hypertension and atherosclerosis, the leading cause of death in western populations. HDL is considered anti-atherogenic and known to prevent ED.(110)

HDL-C has direct effects on numerous cell types that influence cardiovascular and metabolic health. These include endothelial cells, vascular smooth-muscle cells, leukocytes, platelets, adipocytes, skeletal muscle myocytes, and pancreatic  $\beta$  cells. The effects of HDL or ApoA-I, its major apolipoprotein, occur through the modulation of intracellular calcium, oxygen-derived free-radical production, numerous kinases, and enzymes, including eNOS. ApoA-I and HDL also influence gene expression, particularly genes encoding mediators of inflammation in vascular cells. In many paradigms, the change in intracellular signaling occurs as a result of cholesterol efflux, with the cholesterol acceptor methyl- $\beta$ -cyclodextrin often invoking responses identical to HDL or ApoA-I. The ABCA1, ABCG1 and SRBI frequently participate in the cellular responses.(111)

HDL has been suggested to protect endothelial cell functions by prevention of oxidation of LDL and its adverse endothelial effects. Moreover, HDL from healthy subjects can directly stimulate endothelial cell production of NO and anti-inflammatory, anti-apoptotic, and anti-thrombotic effects as well as endothelial repair processes (101,112-114). Hessler, Robertson, and Chisolm (115) reported early on that HDL protects against LDL-induced cytotoxicity on endothelial cells. HDL was demonstrated to prevent copper-induced LDL oxidation or LDL oxidation by cultured endothelial cells.(116-118) HDL is a major carrier of lipid peroxidation products (119-120) which are thought to play a role in the initiation and progression of atherosclerotic vascular disease.(121) HDL can directly inhibit oxidation of LDL via transfer of oxidation products from LDL to HDL.(119) A study by Navab *et al.*(122) demonstrated that ApoA-I binds to and removes lipid hydroperoxides of LDL *in vitro* and *in vivo*.

ApoE has been shown to have allele-specific anti-oxidant activity.(123) ApoE-II can stimulate endothelial

NO release and has anti-inflammatory activities.(124) In contrast, ApoE-IV has been described as pro-inflammatory. (125) It has also been reported that HDL-associated Apo-J can inhibit LDL oxidation by artery wall cells.(126) In addition, ApoA-IV has been demonstrated to exert anti-oxidant, anti-inflammatory, and anti-atherosclerotic actions *in vivo*.(91,127,128)

Notably, HDL also carries antioxidant enzymes that may be involved in prevention of lipid oxidation or degradation of lipid hydroperoxides such as PON1, LCAT, and platelet-activating factor acetylhydrolase (PAF-AH). In particular, PON1 has been suggested to be an important regulator of the potential anti-atherogenic capacity of HDL. (129,130) endothelial NO (eNO) plays a crucial role in the regulation of vascular tone and structure. eNOS-derived NO has been shown to exert a variety of atheroprotective effects in the vasculature, such as anti-inflammatory and anti-thrombotic effects.(131) Reduced eNO bioavailability has therefore been suggested to promote initiation and progression of atherosclerosis (131). Accumulating evidence suggests that HDL can directly stimulate eNOS-mediated NO production *via* endothelial SR-BI.(101)

HDL binding to SR-BI initiates tyrosine kinase Src-mediated activation of PI3K, which in turn activates Akt and MAPK/extracellular signal-regulated kinase (Erk) pathway.(132) Activation of endothelial Akt by HDL stimulates phosphorylation of eNOS at serine residue 1177 (112,132), which is known to be an important regulatory mechanism leading to eNOS activation.(133) HDL is thought to contribute to atheroprotection as an anti-inflammatory through, for example, antioxidant properties of its enzymatic and non-enzymatic components, the ability to remove normal and toxic lipid species from cells, and the dampening of Toll-like receptor (TLR) signaling by regulating plasma membrane cholesterol content.(134-136) The anti-inflammatory effects of HDL included changes in the balance between M1 and M2 macrophages. (28) How HDL contributes to a reduction in M1 or an enhancement in M2 polarization may depend on a number of its properties. As noted, there is growing evidence that it or ApoA-I may limit TLR responsiveness to inflammatory stimuli (by regulating plasma membrane cholesterol content and microenvironments), and there is more recent evidence indicating that it can promote the phosphorylation of STAT6, an integral signaling component of the M2 polarization pathway.(135-140) Furthermore HDL reduced eNO availability via TLR-2 leading to impaired endothelial repair, and increased proinflammatory activation. These data demonstrate how symmetric dimethylarginine (SDMA) can modify the HDL particle to mimic a damage-associated



molecular pattern that activates TLR-2 *via* TLR-1- or TLR-6-coreceptor-independent pathway, linking abnormal HDL to innate immunity, ED, and hypertension.(110)

In pathological studies of coronary atherosclerotic plaque erosion, thrombi were observed in direct contact with the intima in areas with absent endothelium likely promoting disease progression.(141) Endothelial cell apoptosis has therefore been suggested to contribute importantly to the pathophysiology of coronary disease. (142-144) The capacity of HDL to attenuate endothelial cell apoptosis may therefore represent a potentially important anti-atherogenic property of HDL.(60,145-147)

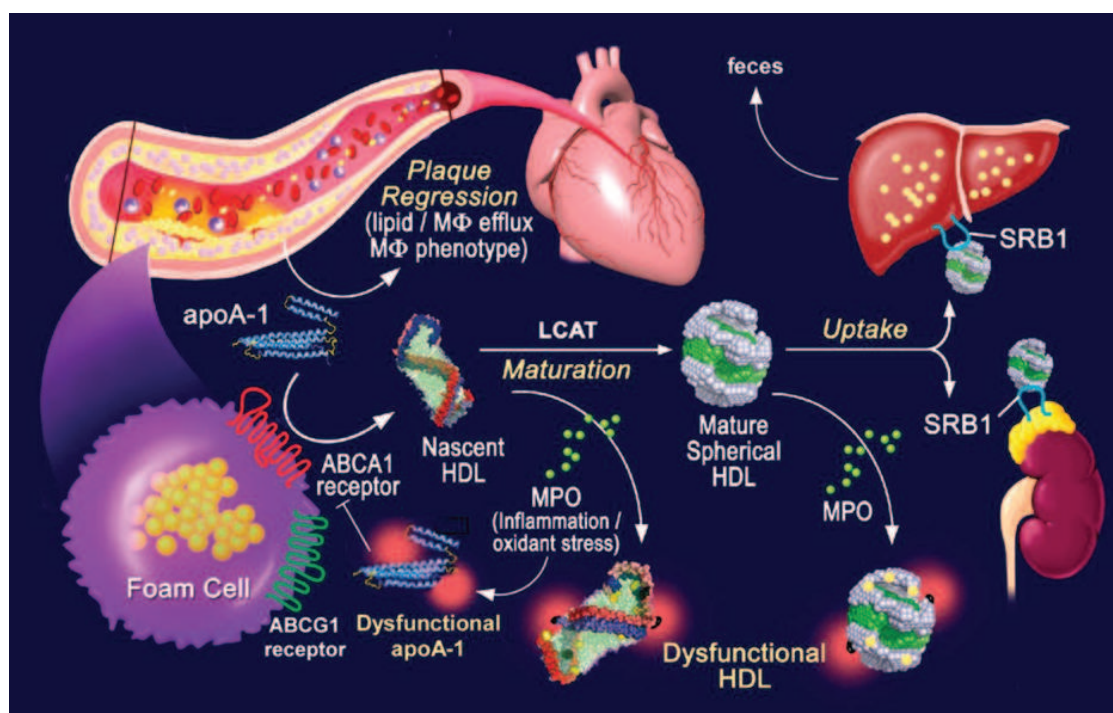
### Inflammation Alters HDL Composition and Function

Recent studies provided convincing evidence that inflammation generates dysfunctional or even pro-atherogenic forms of HDL by promoting PL depletion and enrichment with pro-inflammatory proteins such as serum amyloid A (SAA), ApoC-III or complement component 3 (C3).(148-151) HDL loses potential anti-atherosclerotic properties in patients with chronic inflammatory disorders such as the anti-PL syndrome (152), systemic lupus erythematosus and rheumatoid arthritis (153), scleroderma (154), the metabolic syndrome (155), diabetes (156,157), and coronary disease (100,158,159). Notably, in a study of

189 patients with chronic kidney disease on hemodialysis, an impaired anti-inflammatory capacity of HDL was correlated with a poor clinical outcome.(160)

HDL from coronary artery disease (CAD) subjects is dysfunctional. In fact, HDL from many CAD patients was actually proinflammatory, thus increasing monocyte chemotaxis in response to LDL, unlike the HDL from healthy controls that reduced monocyte chemotaxis. There are many possible alterations between this dysfunctional HDL and normal functional HDL.(161) These pro-inflammatory HDL particles have been termed “dysfunctional” HDL. The heterogeneity of the vascular effects of HDL may be attributed to changes in the HDL-associated proteome and lipids, *i.e.*, post-translational protein modifications and changes in the amount and type of proteins and lipids bound to the HDL particle. In particular, HDL is susceptible to oxidation / modification *in vitro* by a variety of oxidants, such as metal ions, peroxy and hydroxyl radicals, aldehydes, various myeloperoxidase (MPO)-generated oxidants, lipoxygenase, phospholipase A2, elastase, nonenzymatic glycation, and homocysteinylolation.(162)

Inflammation induces major changes in HDL levels and composition. Mediators of inflammation such as tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-6 induce expression of serum amyloid A (163) and sPLA2-IIA (164), which dramatically alter HDL apolipoprotein content and levels, respectively. Acute phase SAA in the plasma is associated with HDL, where it can comprise the major



**Figure 4. HDL dysfunction impairs reverse cholesterol transport.**(171) (Adapted with permission from American Heart Association).

apolipoprotein.(165) The increase in sPLA2-IIA activity results in hydrolysis of HDL surface PL and a decrease in HDL particle size.(166) The presence of SAA on HDL holds the potential to impact both the CE transfer and the ApoA-I liberating ability of CETP. sPLA2-IIA could also impact the latter action of CETP as ApoA-I was shown to dissociate more readily from CETP-remodeled reconstituted HDL after hydrolysis by bee venom phospholipase A2.(167)

Post-translational modification of ApoA-I can directly lead to HDL dysfunction. HDL from diabetic subjects can have glycated ApoA-I with altered lipid-binding activity, and incubation of HDL with glucose impaired its anti-inflammatory and antioxidant activities.(168,169)

One of the best studied modifications of ApoA-I is mediated by MPO, a leukocyte-derived heme protein abundant in neutrophils, monocytes, and a subset of tissue macrophages. Part of the innate immune host defense system, MPO uses  $H_2O_2$  to generate an array of reactive oxidant and free radical species that are antimicrobial, such as hypochlorous acid (HOCl). These same species can also foster spurious oxidative injury to normal tissues as well, such as within atherosclerotic plaque, where MPO has been shown to promote both protein modifications and initiate lipid peroxidation. Once released from activated leukocytes, in the circulation and within lesions, MPO has been shown to bind to HDL. This tight binding, which has been mapped to helix 8 region of ApoA-I, likely accounts for the selective oxidative targeting of ApoA-I within HDL for modification by MPO-generated oxidants.(170) These and other recent observations support a role for MPO serving as an enzymatic catalyst for site-specific modification of ApoA-I and HDL, leading to functional impairment within the artery wall. Indeed, HDL isolated from human atherosclerotic plaque has been shown to coimmunoprecipitate with MPO.(171)

Subsequent studies have revealed that MPO-induced modification of ApoA-I/HDL inhibits additional HDL functions. For example, oxidative modification of ApoA-I Tyr166 through MPO-catalyzed nitrating and chlorinating pathways is linked to functional impairment of HDL binding to LCAT activation and activity.(172) Similarly, oxidation of ApoA-I Met148 also impairs LCAT activation (173). HDL exposure to MPO or HOCl results in the loss of anti-apoptotic and anti-inflammatory activities of HDL, and specifically the loss of SR-B1-binding activity, while increasing its proinflammatory activities, such as endothelial cell NF- $\kappa$ B activation and VCAM-1 expression.(156)

Taken together, plasma HDL-C levels are likely not an appropriate marker of vascular effects of HDLs, and therefore do not represent a reliable therapeutic target. Inflammation is proposed to impair HDL function and RCT.

This may be of pathophysiological significance because attenuation of RCT might contribute to atherosclerosis in chronic inflammatory states, including metabolic syndrome and type 2 Diabetes Mellitus.(161)

Tests for HDL function or biomarkers associated with dysfunctional HDL may be useful for identifying subjects at risk for CAD. For example, plasma MPO levels are positively associated with CAD and the risk of a subsequent major adverse cardiac event.(174,175) Similarly, the level of ApoA-I chlorotyrosine, detected by mass spectrometry, is also a predictor of CVD. Although this assay may be for a potential means of quantifying dysfunctional HDL levels, it is not suitable for routine clinical use.(170)

### Novel Therapies Focused on HDL Particle

The hypothesis that HDL-C directly confers biological protection against atherosclerosis has, as of yet, never been proved and, as a consequence, HDL-C has been argued to be merely a CVD biomarker rather than an active player in atherogenesis. This notion has gained support because therapies with an established HDL-C increasing effect were shown not to result in the anticipated decrease in CVD risk. In particular, 3 large outcome trials were prematurely terminated for futility (Athero-thrombosis Intervention in Metabolic Syndrome with Low HDL/High TG: Impact on Global Health Out-comes [AIM-HIGH] trial (8), Heart Protection Study 2-Treatment of HDL to Reduce the Incidence of Vascular Events [HPS2-THRIVE] study (176), the Dalcetrapib CVD Outcome Study [DAL-OUTCOMES] (9)), despite significant HDL-C increases. In addition, data from Mendelian randomization studies have shown that common variants that raise HDL-C levels are not associated with a proportionally lower CVD risk.

Simply increasing the amount of circulating HDL-cholesterol does not reduce the risk of coronary heart disease (CHD) events, CHD deaths, or total deaths.(177) Heinecke (178) has noted that HDL-cholesterol does not define the proteins associated with HDL and suggests that the HDL proteome is a marker, and perhaps a mediator, of CHD. Zheng *et al.* (179) reported that ApoA-I, the major protein in HDL, is a selective target for myeloperoxidase-catalyzed oxidation, which results in impairment of the ability of HDL to promote cholesterol efflux. Singh *et al.* (170) suggested that HDL could be a therapeutic target by modifying its lipid and protein cargo to improve its anti-inflammatory properties.

Although it may be premature to suggest at this time, the noncholesterol cargo of HDL may ultimately

be more closely linked to its beneficial anti-atherogenic properties and may confer many of HDL's benefits outside of CVD. In support of this theory, HDL particle number has recently been found to be a better indicator of CVD risk than HDL-C levels.(180) Although this may be due to the increased ability of more HDL particles for removing excess cholesterol, increasing HDL particle numbers may also affect other functions of HDL and its ability to transport other cargo. Therapeutic strategies to increase HDL particle number may turn out to be better in reducing cardiovascular events than simply increasing HDL-C. One method that has been reported to modify the lipid and protein cargo of HDL involves treatment with apolipoprotein mimetic peptides. (181)

## Conclusion

There has been a recent move toward the view that an accurate assessment of HDL function is likely to provide a more informative assessment of cardiovascular risk than simply measuring HDL-C levels. This has led to considerable progress in recent years in understanding known and identifying new cardioprotective functions of HDLs. There is also a growing awareness that simply measuring HDL-C levels may be less informative than a direct assessment of specific HDL functions. To complicate matters even further, it is also possible that at least some of the known functions of HDL may be specific to particular subsets of HDL particles.

Perhaps it is time for us to change the way we look at HDL and move from a cholesterol-based to a particle-based world. This requires considerable ongoing validation and testing in the clinical trial setting; however, by doing this, we may ultimately get one step closer to a setting in which functional HDL takes center stage. It may be time to rethink the HDL-C hypothesis, not necessarily to replace it, but to expand the role of HDL beyond that of cholesterol to incorporate some of the recent findings on the importance of the other cargo of HDL in CVD.

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