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Reviews

The Changing Face of HDL and the Best Way to Measure It

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BACKGROUND: HDL cholesterol (HDL-C) is a commonly used lipid biomarker for assessing cardiovascular health. While a central focus has been placed on the role of HDL in the reverse cholesterol transport (RCT) process, our appreciation for the other cardioprotective properties of HDL continues to expand with further investigation into the structure and function of HDL and its specific subfractions. The development of novel assays is empowering the research community to assess different aspects of HDL function, which at some point may evolve into new diagnostic tests.

CONTENT: This review discusses our current understanding of the formation and maturation of HDL particles via RCT, as well as the newly recognized roles of HDL outside RCT. The antioxidative, antiinflammatory, antiapoptotic, antithrombotic, antiinfective, and vasoprotective effects of HDL are all discussed, as are the related methodologies for assessing these different aspects of HDL function. We elaborate on the importance of protein and lipid composition of HDL in health and disease and highlight potential new diagnostic assays based on these parameters.

SUMMARY: Although multiple epidemiologic studies have confirmed that HDL-C is a strong negative risk marker for cardiovascular disease, several clinical and experimental studies have yielded inconsistent results on the direct role of HDL-C as an antiatherogenic factor. As of yet, our increased understanding of HDL biology has not been translated into successful new therapies, but will undoubtedly depend on the development of alternative ways for measuring HDL besides its cholesterol content.

Historically, our understanding of the relationship between plasma cholesterol and cardiovascular health has benefited from investigations into the heterogeneity of lipoproteins, most notably the differentiation between the cholesterol content of HDL cholesterol (HDL-C)[3] vs the cholesterol of LDL-C. It is now well established from large-scale epidemiologic studies that increased plasma levels of HDL-C and LDL-C are associated with decreased and increased cardiovascular risk, respectively *(1)*. Consequently, HDL-C and LDL-C are routinely used as serum biomarkers for assessing an individual's cardiovascular disease (CVD) risk *(2)*.

As would be expected, our appreciation of the functions of HDL and LDL continues to evolve with further research into these macromolecular complexes and their specific subfractions. To date, much of this work has focused on how smaller LDL subfractions, rich in cholesterol, are more atherogenic. This is largely due to greater susceptibility for both lipid peroxidation and free-radical attack of polyunsaturated fatty acids, as well as a capacity for infiltrating further into smaller blood vessels and through endothelial defects *(3)*. Less progress has been made in understanding the relationship between CVD and HDL subfractions. Recent studies have questioned whether HDL-C is only a biomarker and is not mechanistically linked to cardiovascular protection *(4)*. This review will summarize the current understanding of HDL, its subfractions, and how they relate to CVD, and also discuss current and future research trajectories in the field of HDL metabolism, detection, and quantification.

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[3] Nonstandard abbreviations: HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; CVD, cardiovascular disease; RCT, reverse cholesterol transport; apo, apolipoprotein; ABCA1, ATP-binding cassette protein 1; ABCA1, ATP-binding cassette protein 1; ABCG1, ATP-binding cassette G1; SR-BI, scavenger-receptor class B, type 1; LCAT, lecithin-cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; TG, triglyceride; CHD, coronary heart disease; CAD, coronary artery disease; CEC, cholesterol efflux capacity; ACS, acute coronary syndrome; ILLUMINATE, Investigation of Lipid Level Management to Understand its Impact in Atherosclerotic Events; dal-OUTCOMES, a Study of RO4607381 in Stable Coronary Heart Disease Patients with Recent Acute Coronary Syndrome; SNPs, single nucleotide polymorphisms; MI, myocardial infarction; rHDL, reconstituted HDL; oxLDL, mildly oxidized LDL; PON1, paraoxonase subtype-1; DAMP, damage-associated pattern; NO, nitric oxide; eNOS, endothelial NO synthase; S1P, sphingosine 1-phosphate; LPS, lipopolysaccharide; TLF, trypanosome lytic factor; HRP, haptoglobin-related protein; CKD, chronic kidney disease; RA, rheumatoid arthritis; SAA, serum amyloid A; A1AT, α-1-antitrypsin; $LpPLA_2$, lipoprotein-associated phospholipase A2; NMR, nuclear magnetic resonance.

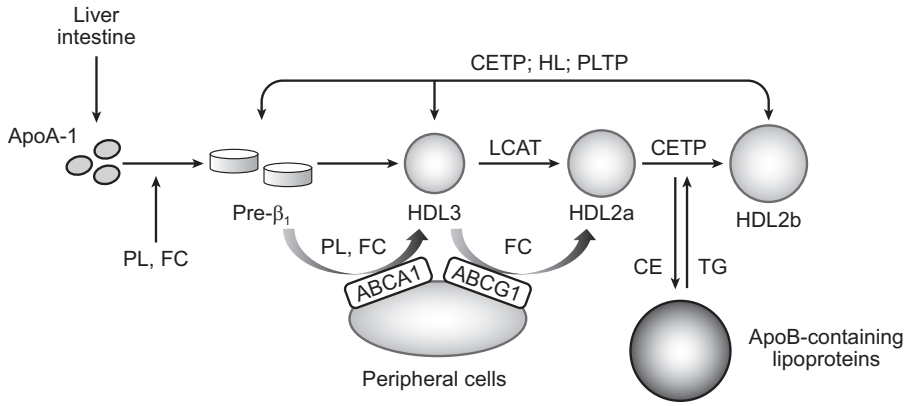

Fig. 1. Generation and interconversion of HDL subfractions.

Secreted ApoA-I associates with PL and non-esterified FC transferred from cells to HDL, via ABCA1, to form discoidal pre-β-HDLs. LCAT esterifies FC into CEs, moving into the particle core to create spherical HDL. CETP mediates HDL CE exchange onto apoB-containing lipoproteins for TG. HL hydrolyzes HDL phospholipids and TG, releasing smaller HDL and lipid-poor apoA-I. CETP, HL, and phospholipid transfer protein (PLTP) generate small pre-β_1-particles from large α-particles. Adapted with permission from Pirillo et al. *(10)*. Copyright ©2013, Karger Publishers.

About HDL: Generation and Maturation

HDL particles are macromolecule complexes synthesized by the liver, intestine and also formed from surface components released during lipolysis of triglyceride-rich lipoproteins in the plasma. HDL particles consist of an amphipathic lipid monolayer of phospholipids and cholesterol with embedded amphipathic proteins surrounding a core of hydrophobic lipids, mostly cholesteryl esters and triglycerides *(5)*. This particular class of lipoprotein macromolecules is characterized as "high density" because of the relatively higher proportion of proteins to lipids (specifically, HDL has a density range of 1.06–1.21 g/mL) *(6, 7)*. Throughout its lifecycle, one of the primary atheroprotective mechanisms of HDL is thought to be its ability to promote the net movement of cholesterol from peripheral tissues back to the liver via the reverse cholesterol transport (RCT) pathway (Fig. 1) *(8, 9)*.

RCT begins in the liver and intestines with the synthesis of apolipoproteins (apo; primarily the apoA-I subtype). Secreted apoA-I associates with phospholipids and nonesterified free cholesterol transferred from cells to HDL via ABCA1, to form discoidal pre-β-HDL. Pre-β-HDL has a relatively low content of hydrophobic core lipids and a discoidal shape, with the term pre-β-HDL being based on its electrophoretic migration position on agarose gels *(9, 10)*. Because of their small size (<10 nm), pre-β-HDL particles are readily filtered through capillaries and enter the interstitial space, where they interact with parenchymal cells to recruit nonesterified free cholesterol and phospholipids to form larger discoidal particles. These larger discoidal particles show α migration on agarose and 2D-gels, and are often designated as α4 particles *(9)*. Nonesterified free cholesterol and phospholipids are transferred from cells onto HDL via the adenosine-triphosphate-binding cassette protein A1 (ABCA1) plasma membrane transporter *(5, 11)*. The exact mechanism by which ABCA1 promotes efflux of excess cellular cholesterol is not known, but likely involves creation of a specialized plasma membrane domain from which apoA-I and other exchangeable apos remove cholesterol and phospholipids via a detergent-like extraction process *(5)*. As further described below, other transporters and receptors [adenosine-triphosphate-binding cassette protein G1 (ABCG1) and scavenger-receptor class B, type 1 (SR-BI)], as well as the physical desorption of cholesterol through aqueous diffusion), also likely contribute to the efflux of cellular cholesterol *(5)*.

A key event in the RCT pathway is the esterification of cholesterol by lecithin-cholesterol acyltransferase (LCAT) *(12)*. Because of its increased hydrophobicity, cholesteryl ester partitions into the core of HDL and transforms the discoidal forms of HDL into spherical α-HDL (α1–3) *(9)*, the predominant form of HDL found in the circulation *(10)*. When HDL is fractionated by density, the small, dense forms of HDL, known as HDL_3, are converted into larger and less-dense forms, called HDL_2, by LCAT. Large HDL_2 can be further remodeled by cholesteryl ester transfer protein (CETP), which exchanges HDL-associated cholesteryl esters for triglycerides (TGs) from TG-rich lipoproteins (e.g., very-low- VLDL and LDL) *(13, 14)*. Additionally, VLDL can transfer associated apoC and apoE, as well as phospholipid, to HDL via the phospholipid-transfer protein *(11)*. Phospholipids and TGs associated with larger HDL sub-

fractions can be hydrolyzed to form smaller HDL particles via hepatic lipase or endothelial lipase, and are either reintegrated into the RCT pathway, or are cleared by the liver and kidney *(9, 10)*. Finally, the major route for HDL-C delivery is via the CETP-mediated transfer of cholesteryl esters to apoB-containing lipoproteins (LDL, VLDL, and intermediate-density lipoprotein), which deliver LDL-C to the liver via the LDL receptors *(15)*.

The generation of different HDL subfractions throughout the RCT pathway is demonstrative of the size, compositional, and functional diversity of HDL. In addition to altering the size of the HDL subfractions, integration of cholesteryl esters and TGs fundamentally impacts the cardioprotective properties of HDL *(9, 10)*. Further, the makeup of HDL subfractions is dynamic, as different lipolytic enzymes, lipid transporters, and apo exchange mechanisms (with adjacent circulating lipoproteins and tissues) contribute to the formation and remodeling of HDL subfractions *(16)*. This also illustrates how simply measuring plasma HDL-C concentrations may not fully capture the impact of HDL on cholesterol flux between tissues, or its larger effect on CVD.

The Evolving Role of HDL: RCT and Beyond

Initial evidence supporting an inverse correlation between plasma HDL-C concentration and cardiovascular risk dates back to the mid-1970s, when Miller, et al. *(17)* observed a strong negative correlation between HDL-C concentrations and ischemic heart disease. The Framingham study expanded on this observation, identifying an association between low serum HDL-C and both the incidence of coronary heart disease (CHD) and overall mortality *(18, 19)*. Since the Framingham study, several large-scale population observational studies have provided additional support for the relationship between low concentrations of HDL-C (often quantified as between 15–40 mg/dL in serum) and an increased risk for ischemic heart disease *(20–22)*, coronary artery disease (CAD) *(23, 24)*, CHD *(24–27)*, and stroke *(28)*. These early landmark findings directed the next several decades of research into the specific contributions of HDL to cardiovascular health.

Given what is known about the nature of different HDL subfractions and the RCT pathway, the key question remains as to whether this information is relevant to the pathogenesis of CVD. In considering this question, it is important to note that—despite the strong correlation thought to exist between lower plasma HDL-C concentrations and increased cardiovascular risk—subsequent findings from several clinical trials investigating treatment with HDL-C–elevating therapies have yielded inconsistent, and sometimes paradoxical, results (Table 1) *(27, 29–41)*. Specifically, although some trials investigating HDL-raising pharmacotherapies such as gemfibrozil *(27, 29, 30)*, statins *(31, 32)*, and combinations of statins and niacin *(33)* have been efficacious in reducing risk of cardiovascular events, other trials have failed to demonstrate a relationship with raising HDL-C and decreasing cardiovascular risk *(15, 34, 35, 39, 41–43)*. Further, most of these drugs also lower LDL-C and or TG concentrations *(2)*, making it difficult to attribute any unique benefit of HDL-C arising from any of the positive studies.

The most recent class of HDL-C–raising drugs that has been investigated, but so far has not shown a clinical benefit, is CETP inhibitors. CETP inhibitors act to increase HDL-C concentrations by preventing the CETP-catalyzed exchange of TGs in apoB-containing lipoproteins for HDL cholesteryl esters. This results in the retention of cholesterol in spherical HDL particles and an observed increase and decrease in HDL-C and LDL-C, respectively *(44)*. The rationale for inhibiting CETP to increase HDL-C dates back to the early 1990s, when genetic studies in Japanese subjects found that individuals with CETP deficiency had increased HDL concentrations *(45, 46)*. Subsequent clinical studies have evaluated the HDL-raising properties of CETP inhibitors *(15, 36, 47–49)*. For example, treatment with 300 mg evacetrapib raised HDL-C concentrations by >80% in healthy volunteers, and increased total HDL-C and non-ABCA1–specific cholesterol efflux capacity (CEC) in individuals with mild dyslipidemia *(36, 47–49)*. In patients with acute coronary syndrome (ACS), treatment with dalcetrapib, another CETP inhibitor, also significantly raised HDL-C, principally via an increase in ABCA1-mediated cholesterol efflux *(15, 49)*.

Despite the early successes of CETP inhibitors, no candidate compounds have demonstrated efficacy. In the case of torcetrapib, the first CETP inhibitor tested in a large-scale clinical Investigation of Lipid Level Management to Understand its Impact in Atherosclerotic Events (ILLUMINATE) trial, the clinical trial was terminated early because of off-target adverse events (increased serum aldosterone and blood pressure levels) *(50)*. The phase 3 study of evacetrapib in High-Risk Vascular Disease (ACCELERATE) trial investigating evacetrapib, and the Study of RO4607381 in Stable Coronary Heart Disease Patients with Recent Acute Coronary Syndrome (dal-OUTCOMES) investigating dalcetrapib, were also ended early due to lack of efficacy with respect to clinical cardiovascular outcomes *(15, 51)*. Newer generations of CETP inhibitors, such as anacetrapib and TA-8995, have demonstrated favorable lipid effects and tolerability in early-stage clinical trials, while investigations into CVD outcomes (e.g., DEFINE and REVEAL trials) remain ongoing *(52–54)*.

As most of the cholesterol delivered to the liver is derived from the CETP-derived apoB-containing lipoproteins and not HDL, the rationale for expecting RCT

Table 1. Representative list of clinical trials investigating HDL-C–elevating pharmacotherapies on cardiovascular risk outcomes.

Drug administered	Findings of studies supporting negative association of HDL and cardiovascular risk	Findings of studies that do not support a negative association of HDL and cardiovascular risk
Fibrates	Veterans Affairs HDL Intervention Trial • In patients with CHD, gemfibrozil treatment raised numbers of HDL particles and small HDL subclass particles, and were significant, independent, predictors of CHD events at 5-year follow-up [Otvos et al. *(27)*; Rubins et al. *(29)*] • In patients with CHD and low HDL cholesterol, gemfibrozil significantly reduced stroke incidence at 5-year follow-up [Robins et al. *(30)*] Other studies • In men who had CAD and undergone coronary bypass surgery, treatment with gemfibrozil significantly increased HDL_3 levels and was associated with protection against angiographic progression in native coronary lesions [Syvänne et al. *(37)*].	Bezafibrate Infarction Prevention Study • In patients with previous MI, treatment with bezafibrate significantly increased HDL-C, but did not significantly reduce risk of MI or sudden death [BIP Study *(34)*] Other studies • In patients with low baseline HDL-C, treatment with fibrates elevated HDL-C, but did not confer an additional CVD risk reduction at up to 8 years of follow-up [Nicholls et al. *(36)*]
Statins	AFCAPS/TexCAPS Trial[a] • In patients with low HDL-C and no CVD, treatment with lovastatin significantly reduced the risk for the first acute major coronary event at 5-year follow up [Downs et al. *(31)*] GREACE Study • In patients with CHD, treatment with atorvastatin significantly increased HDL-C levels and reduced risk of coronary events at up to 2 years follow-up [Athyros et al. *(32)*] Other studies • Patients who newly started statin therapy after acute MI and had on-treatment increases in HDL-C level (≅85% of participants), had fewer adverse cardiovascular events reported at 6–9 months of follow-up [Ota et al. *(38)*].	JUPITER Trial • In patients with no history of CVD or diabetes, patients treated with rosuvastatin had no significant relationship between HDL-C levels and vascular risk [Ridker et al. *(42)*] • Treatment with rosuvastatin increased both HDL-C, HDL particle number and size. Only the on-treatment increase in HDL particle number had a significant association with CVD [Mora et al. *(39)*] Metaanalysis • In a meta-analysis of 8 statin trials, increases in HDL-C levels were not associated with reduced cardiovasculuar risk, while a rise in apoA-I level was [Boekholdt et al. *(40)*]
Statins + Niacin	Other studies • In patients with coronary disease and low HDL-C levels, treatment with simvastatin plus niacin significantly increased HDL_2 levels and were associated with a reduced risk of cardiovascular events or progression of coronary stenosis [Brown et al. *(33)*].	AIM-HIGH Trial • In patients treated with simvastatin and extended-release niacin, addition of niacin therapy was not associated with a change in ischemic stroke risk, CHD, nonfatal MI, or ACS hospitalization at 36-month follow-up [Boden et al. *(35)*; Teo et al. *(41)*].

[a] AFCAPS/TexCAPS, Air Force/Texas Coronary Atherosclerosis Prevention Study; AIM-HIGH, Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides: Impact on Global Health.

improvements under CETP inhibition has remained unclear *(15)*. This observation that CETP inhibitors are not effective despite the significant reduction of plasma LDL-C levels is concerning, given the established benefits of LDL-C reduction with statins.

Further evidence of the lack of support for the efficacy of HDL-C–elevating drugs for the prevention of CVD has been found in recent Mendelian randomization studies, which are observational studies investigating possible causal linkages of genetic polymorphisms with biomarkers like HDL-C, as well as how genetic polymorphisms impact disease *(55, 56)*. For example, in a study of white individuals from Copenhagen, Denmark, lower plasma concentrations of HDL-C due to heterozygous

loss-of-function mutations in the ATP binding cassette subfamily A member 1 (*ABCA1*)[4] gene were not associated with increased risk of ischemic heart disease, likely because of a concomitant decrease in LDL-C *(55)*. Similarly, in a Mendelian randomization study, a lecithin-cholesterol acyltransferase (*LCAT*) single nucleotide polymorphism (SNP) associated with decreased plasma HDL-C, in the general population, was not associated with increased risk of myocardial infarction (MI), despite low plasma HDL-C concentrations being robustly associated with increased risk of MI *(56)*. These results suggest that low plasma HDL-C concentrations from genetic factors, which account for only approximately 50% of the variation of HDL-C concentrations, do not directly cause MI, particularly when the LDL-C concentration is not increased. Similar to the *ABCA1* study, the decrease in HDL-C due to the *LCAT* SNP would also decrease LDL-C via the CETP pathway *(56)*, further complicating the interpretation of these results regarding the role of low plasma HDL concentration and CVD risk.

Recent metaanalyses of genome-wide association studies identified numerous genes associated with regulation of plasma HDL-C, as well as genes associated with total cholesterol, LDL-C, and TG plasma concentrations *(57, 58)*. While several gene SNPs associated with HDL-C were found to be associated with CAD, it was difficult to assign causality. Other traits, such as diabetes, and increased TG and LDL-C concentrations, were also associated with the same genes, confounding interpretation of the results. Plasma HDL-C concentrations are also known to be affected significantly by diet, lifestyle, other diseases, and certain pharmacotherapies *(2)*, further complicating analysis of genome-wide studies. For example, certain β blockers have been found to reduce plasma HDL-C *(59)*. Importantly, most plasma HDL-C is generated by liver, so HDL-C plasma concentrations do not necessarily reflect the level of cholesterol efflux from arterial macrophages *(10)*.

Patients with genetic mutations in apolipoprotein A1 (*APOA1*), the gene coding for the main protein component of HDL, frequently suffer from CHD *(60–62)*. In addition, a single infusion of reconstituted HDL (rHDL) made from apoA-I and phospholipids markedly reduced lipid content in femoral artery plaque in patients with peripheral vascular disease *(63)*. CSL112 (Commonwealth Serum Laboratories. Inc.), a new formulation of human apoA-I being developed to reduce the incidence of ACS cardiovascular events, is both well tolerated and efficacious in increasing apoA-I levels, and increased cholesterol in plasma compared with placebo *(64)*. While future phase 3 studies are necessary to elaborate on CSL112 efficacy in reducing cardiovascular events, results thus far are promising.

Alternative Measures of HDL

Given the disappointment so far in developing new HDL-C–targeted drugs for treatment of CVD, there has been great interest in determining whether another HDL metric may better capture its potential antiatherogenic effects. A functional assay assessing HDL-promoted cholesterol efflux has garnered interest, and may be a convenient way to assess HDL function in RCT. In this section, we also discuss other potential functions of HDL and novel qualitative and quantitative assays. HDL is known to bind over 80 different types of proteins and transports more than 100 different species of lipids, so it likely has other functions outside RCT *(65, 66)*. Finally, the function of HDL is highly associated with its composition, so we also discuss methodologies for identifying HDL-associated proteins, lipids, and physical properties.

CHOLESTEROL EFFLUX EFFECTS

A common methodology for assessing the antiatherogenic functionality of HDL is measuring CEC, or the ability of HDL to initiate the RCT pathway by accepting cholesterol from lipid-laden macrophages *(67, 68)*. The methodology for determining CEC, first validated in a large clinical trial by Khera, et al., *(67)* involves quantification of total cholesterol efflux from macrophages with apoB-depleted serum, and demonstrated CEC as a strong inverse predictor of CAD status independent of HDL-C concentration.

Several prospective and retrospective studies have addressed CEC, and yielded similar inverse associations with CEC and decreased incidence of cardiovascular events *(69–71)*. In the Dallas Heart Study, healthy subjects in the top quartile vs the lowest quartile of CEC had a 67% reduced CV risk in a fully adjusted statistical model *(69)*. In an analysis comparing patients with incident CHD vs healthy controls, CEC was significantly and inversely associated with incident CHD events *(70)*. HDL CEC is also significantly lower in patients with both coronary disease and increased HDL-C compared with healthy controls *(71)*, suggesting that HDL may be dysfunctional in some patients with markedly increased HDL-C concentrations. These studies underscore the importance of continuing research into better understanding the role of HDL-C efflux and for standardizing the CEC assay. Future studies should also evaluate in greater depth the specific contributions of cholesterol transport pathways, specific cell types, HDL subfractions, and genetic predispositions in moving towards the

[4] Human genes: *ABCA1*, ATP binding cassette subfamily A member 1; *LCAT*, lecithin-cholesterol acyltransferase; *APOA1*, apolipoprotein A1.

development of a more robust and predictive assay for measuring cholesterol efflux.

ANTIOXIDATIVE EFFECTS

Low levels of HDL have been associated with increased oxidative stress in healthy individuals *(72)*. Conversely, higher levels of HDL can prevent the development of atherosclerotic lesions in the arterial wall by inhibiting the mildly oxidized LDL (oxLDL) formation *(73)*. Small, dense HDL subfractions and several HDL-associated enzymes [e.g., paraoxonase 1 (PON1), and platelet activating factor acetylhydrolase] are believed to contribute to HDL's overall antioxidative activity *(74–77)*. Evaluations of the antioxidative activity of HDL subfractions identified a positive association with LDL lipid hydroperoxides inactivation, and a positive association of antioxidative activity with subfraction density (HDL_{2b}<HDL_{2a}<HDL_{3a}<HDL_{3b}<HDL_{3c}) *(74)*. In addition to being an acceptor of free cholesterol, HDL also receives lipid hydroperoxides from oxLDL via CETP transfer, leading to rapid clearance from the circulation *(77)*. Together, these actions of HDL serve to reduce oxidative stress and prevent atherosclerotic damage.

Navab and colleagues *(78)* were the first to develop a novel and rapid cell-free assay to determine the ability of HDL to prevent the inactivation or formation of oxidized phospholipids. This fluorospectroscopic assay quantified the fluorescent signal generated by 2 oxidized phospholipids; oxLDL and oxidized L-α-1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine. A similar fluorometric biochemical assay based on the oxidation of dihydrorhodamine 123 by HDL was developed by Kelesidis, et al. *(79)*, featuring methodology that allowed for differentiation of HDL oxidative potential in different persons and a high-throughput implementation. Other techniques are available to quantify oxidized phospholipids, including immunoassays, separation techniques, and mass spectrometry assays *(80)*. Methodology for detecting and quantifying oxidized phospholipids is advancing rapidly, and will be continually refined as mass spectroscopy techniques improve.

ANTIINFLAMMATORY EFFECTS

An essential process in the development of atherosclerosis is inflammation-mediated adhesion and migration of immune cells into endothelial vessel walls, which subsequently fosters plaque formation. In macrophages, HDL induces activating transcription factor 3, suppressing macrophage activation and proinflammatory cytokine production *(81)*. These proinflammatory mediators can lead to significant endothelial cell damage and premature cell death *(82)*. Recent data have suggested that HDL may both lose its protective effects and gain pathogenic properties in certain disease states by acting as a damage-associated pattern (DAMP) molecule to stimulate scavenger receptors involved in innate immune activation *(83)*. For example, HDL from patients with systemic lupus erythematosus suppresses macrophage-activating transcription factor 3 activation by engaging the scavenger lectin-like oxidized LDL-receptor 1 *(84)*.

Direct effects of HDL on the function and proliferation of myeloid cells, including monocytes, macrophages, and monocyte-derived dendritic cells, have resulted in the suppression of cytokine and chemokine production, down-regulation of costimulatory molecules, and inhibition of antigen presentation *(85–89)*. In vitro studies have demonstrated that HDL inhibits endothelial adhesion molecules, including vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and E-selectin *(90, 91)*. This antiinflammatory activity of HDL varies in different subfractions, with the smaller, denser HDL subfractions (HDL_{3b} and HDL_{3c}) inhibiting the production of proinflammatory oxidized phospholipids to a greater degree than larger HDL subpopulations *(92)*. This HDL subfraction-specific effect may be important in protecting various myeloid cells' structure and function from being compromised by the cytokine response.

ANTIAPOPTOTIC EFFECTS

Endothelial cell apoptosis is a hallmark of CVD and is involved in the formation of atherosclerotic plaques and oxLDL signaling. HDL and its subfractions offer important cytoprotection for endothelial cells in response to different proapoptotic stimuli. Attenuation of endothelial cell apoptosis by HDL involves inhibition of death receptor, mitochondrial, and endoplasmic reticulum signaling pathways *(93–96)*. Tumor necrosis factor α-induced apoptosis via death-receptor signaling is blocked by HDL via inhibition of CPP32-like protease activity *(97)*. Inhibition of oxLDL-induced apoptotic signaling by HDL involves multiple apoptotic pathways.

Although all HDL subfractions can inhibit caspase-dependent mitochondrial pathway apoptosis to some degree, HDL_{3c} and lipid-free apoA-I have greater antiapoptotic activity than HDL_{2b} or lipid-free apoA-II, respectively *(93, 94, 98–100)*. These antiapoptotic mechanisms of HDL may be important in atherosclerotic conditions where endothelial injury promotes cell death *(77)*.

ANTITHROMBOTIC EFFECTS

Interactions between HDL and procoagulant mediators may also be important in maintaining endothelial cell hemostasis. In experiments with human endothelial cells, rHDL limits the development of the procoagulant state via several mechanisms. Incubation with rHDL in vitro causes a dose-dependent inhibition of platelet activity *(101)* and downregulates tissue factor expression induced by thrombin *(102)*. Similar results were seen in

vivo, with infusion of rHDL significantly reducing activation of coagulation, fibrinolysis, and platelet aggregation associated with endotoxemia *(103)*.

HDL also inhibits platelet aggregation through the prostacyclin signaling pathway, where HDL and apoA-I promoted prostacyclinsynthesis by increasing cyclooxygenase-2 expression *(91, 104–107)*. Both HDL and apoA-I in endothelial cells appear to activate cell signaling by SR-BI, which upregulates expression of endothelial nitric oxide (NO) synthase (eNOS) *(108)*. Activation of both of these pathways leads to inhibition of platelet activation and aggregation *(109)*.

There is also an association between levels of HDL and sphingosine 1-phosphate (S1P) that contributes to the antithrombotic effects of HDL. Like HDL, S1P stimulates eNOS activation and prostacyclin synthesis via cyclooxygenase-2 *(110)*. Because patients with type 2 diabetes exhibit increased levels of S1P compared with controls, managing HDL levels in these patients may be important *(111)*. Indeed, infusion of rHDL in these patients, and healthy volunteers, has been shown to significantly reduce platelet aggregation *(101, 112)*.

VASOPROTECTIVE AND MYOCARDIALLY PROTECTIVE EFFECTS

In addition to preventing vascular damage from inflammation, apoptosis and thrombosis, HDL also demonstrates direct vasoprotective effects on endothelial cells by stimulating production of the potent vasodilator NO by eNOS. Stimulation of eNOS activity has been demonstrated using rHDL in both in vitro and in humans *(113, 114)*. This activation of eNOS is mediated by the SR-BI signaling pathway, involving sequential activation of Src tyrosine kinase, PI-3K, Akt kinase, and Erk1/2 MAPK leading to phosphorylation (and thus activation) of eNOS at Ser-1179 *(115)*.

Improper functioning can compromise the ability of HDL to promote NO-induced vasodilation. Dysfunctional HDL engages lectin-like oxidized LDL-receptor 1, inducing reactive oxygen species generation and suppression of endothelial NO production *(113)*. Cardioprotection by HDL is also observed in the myocardium, where it can act as a tumor necrosis factor α scavenger, removing the cytokine before it is able to induce ischemic tissue damage *(116–118)*. Together, HDL activity in the endothelium and myocardium demonstrate the ability of HDL to provide protection in multiple cell types involved in maintaining vascular homeostasis.

ANTIINFECTIOUS EFFECTS

HDL demonstrates antiinfectious properties through inhibition of bacterial antigens. One notably pathogenic factor affected by HDL is lipopolysaccharide (LPS), which is an essential component of the bacterial cell wall released into the blood following bacterial cell reproduction, lysis, or death *(119)*. LPS is a potent promoter of inflammation in response to bacterial infection *(120)* and is a commonly used experimental reagent for inducing inflammation in vivo. HDL acts to sequester and neutralize LPS, thus inhibiting its ability to induce proinflammatory signaling in macrophages *(121–123)*. Once bound to LPS, HDL promotes LPS clearance through SR-BI *(124–126)*. Together, neutralization and clearance of LPS by HDL serves as the primary method of LPS detoxification in the body.

The innate immune response to trypanosome microbes is also affected by HDL. Trypanosome lytic factor (TLF), an HDL subfraction containing apoA-I, apoL-1, and haptoglobin-related protein (HRP), is responsible for the innate killing of susceptible trypanosome parasites *(127–130)*. The HRP component of TLF binds with high affinity to the cell-surface receptor of the parasite, promoting endocytosis and trafficking of the TLF–parasite particle complex to the lysosome *(131–134)*. Once contained within the acidified lysosome of the trypanosome, the apoL-1 component of TLF mediates lysosomal rupture and spilling of lysosomal contents into the cytoplasm, effectively killing the trypanosome. This HDL particle thus mediates the direct killing of some species of trypanosome parasites.

COMPOSITION AND ROLE OF HDL-ASSOCIATED PROTEINS

The wide variety of potential HDL functions parallels the heterogeneous nature of its protein composition. HDL-associated proteins can be divided into several major subgroups, which include apolipoproteins, enzymes, lipid transfer proteins, acute-phase response proteins, complement components, proteinase inhibitors, and other components *(65)*. The stoichiometry and distribution of each of these proteins on HDL subfractions is not well understood. In addition, how these proteins physically bind to HDL is also not known in most cases, but presumably involves either a weak association with lipids on HDL, or a protein–protein interaction with an apolipoprotein, which provides the structural framework for HDL. To date, 14 different apolipoprotein subtypes have been identified on HDL, the most abundant being apoA-I, accounting for 70% of total HDL protein. Studies employing fast protein liquid chromatography for separating HDL by size into subfractions have found that several of the most abundant plasma proteins, including albumin, haptoglobin, and α-2-macroblobulin, are all at least partially associated with HDL in all apoA-I–containing fractions *(135)*. Enzymes associated with HDL particles, such as LCAT, paraoxonases, and lipid transfer proteins, catalyze several integral processes relevant to the function of HDL *(65)*.

Several systemic processes—including general health state, disease presence, or pharmacotherapy treatment—can have a profound effect on the protein composition of HDL. The expression patterns and antiatherogenic func-

tionality of HDL-associated proteins may, therefore, vary in disease states associated with systemic inflammation, including CAD, chronic kidney disease (CKD), end-stage renal disease, rheumatoid arthritis (RA), and psoriasis (Table 2) *(111, 136–160)*. Each of these diseases involves modulating expression of apolipoprotein subfractions, most often due to replacement by acute-phase response proteins like serum amyloid A (SAA) and complement component 3. Increased levels of the acute-phase response protease inhibitor α-1-antitrypsin (A1AT) are also believed to be involved in the pathogenesis of acute MI *(161, 162)*. Integration of SAA, complement component 3, and A1AT into HDL particles limits its antiinflammatory functionality and thus may contribute to the pathogenesis of vascular disease *(163, 164)*.

HDL fractions containing lower levels of apoA-I are observed in CKD, RA, and psoriasis, with SAA replacing apoA-I on HDL particles under inflammatory conditions *(137, 138, 165–168)*. Patients with psoriasis have decreased apoA-I and apoM and increased apoA-II and SAA levels *(169)*. Changes in the apoA-IV composition of HDL are dependent on the disease state, with increases in apoA-IV observed in renal diseases (CKD and end-stage renal disease), and decreases in apoA-IV with ACS *(139, 140, 170)*. Patients with end-stage renal disease displayed profound alterations in the HDL proteome, with upregulation of SAA, apoC-II, apoC-III, and apoA-IV, as well as downregulation of apoA-I and apoA-II *(165, 166)*.

HDL-associated enzymatic activity is also modulated by inflammatory diseases. PON1 activity is substantially lower in patients with CAD, ACS, CKD, and RA, whereas lipoprotein-associated phospholipase A_2 ($LpPLA_2$) activity is increased in CKD and psoriasis *(89, 137, 160, 165, 171–175)*. Dysregulating the activity of PON1 and $LpPLA_2$ in this fashion not only compromises the antioxidative activity of HDL, but also promotes the formation of atherosclerotic plaques.

COMPOSITION AND ROLE OF HDL-ASSOCIATED LIPIDS

The HDL-associated lipidome consists of 2 subcategories: surface amphipathic lipids (phospholipids and sphingolipids) and neutral lipids found in the hydrophobic core. Of the 10 subtypes of phospholipids currently identified, phosphatidylcholine is the most abundant *(65)*. Sphingomyelin is the most abundant of the 7 types of sphingolipids, constituting approximately 5–6 mol % of total lipids. Another important sphingolipid of note is S1P, a lipid preferentially enriched in small, dense HDL particles which, as previously discussed, plays an important signaling role in the antithrombotic nature of HDL *(176)*. Other vasoprotective functions of S1P include promoting endothelial barrier function, mediating RCT, and inhibiting monocyte adhesion and LDL oxidation *(177, 178)*. Neutral lipids include free cholesterol, cholesteryl esters, small amounts of other sterols, and TGs. Free cholesterol is the form of cholesterol effluxed to nascent HDL by ABCA1 *(65)*. Esterification of free cholesterol in HDL by LCAT results in mature, spherical α-HDL particles, which carry most of the HDL-C in plasma. TG is present in HDL at low levels owing to the CETP reaction, and high levels of TGs can destabilize HDL particles.

S1P levels have been correlated to specific CVDs. A highly significant inverse relationship exists between HDL-containing S1P levels and the occurrence of ischemic heart disease *(179)*. S1P levels were also reduced in patients with acute MI and stable CAD, with preclinical rodent assays demonstrating that administering S1P in vivo can correct HDL dysfunction in CAD *(180, 181)*. These data suggest that S1P has an active involvement in CVD pathogenesis and that future assays quantifying S1P content of HDL could be useful as a cardiovascular risk biomarker.

Future HDL Assays Based on Protein and Lipid Composition

When considering the known functions of HDL, it becomes clear that each is driven by some structurally bioactive protein or lipid component of the HDL particle. While the research assays for measuring HDL functionality described above can provide evidence of the ability of HDL to perform specific cardioprotective functions, these assays can be difficult to standardize for routine diagnostic testing. It, therefore, may be more feasible to develop future compositional assays based on the measurement of specific HDL-associated proteins or lipids with established functional importance.

Besides the HDL-C assays, the only other composition-based assay that is routinely available as a diagnostic test is for apoA-I. Except when measured in conjunction with apoB and used as a ratio, apoA-I does not add any value over our current risk markers for predicting CVD *(182)*. Whether the measurement of the other protein or lipid components of HDL will be valuable as a diagnostic test is currently not known.

A major complication of this effort is that there are over 80 proteins and hundreds of lipids associated with HDL *(65)*. Because some of these components may work together synergistically, this complicates the choice of which protein or lipids should be targeted. This is exemplified by HDL carrying the TLF complex as described above *(128–130)*. When combined on HDL, the specific combination of TLF complex proteins performs a very specific function in the innate immunity that would be nearly impossible to predict knowing only the individual components of the HDL particle. One approach

Table 2. Dysfunction of HDL-C, HDL subfractions, and associated enzymes in disease.

Areas of dysfunction	HDL subfractions	Apolipoprotein activity	HDL-associated enzymes
Type 2 diabetes/metabolic syndrome	• $HDL_{3b}$and HDL_{3c} antioxidative activities are diminshed in people with diabetes [Nobécourt et al. *(142)*] • The endothelial-protective effects of HDL are impaired in people with diabetes/metabolic syndrome [Sorrentino et al. *(141)*]	• The antiinflammatory properties of HDL and apoA-I are diminished by glycation [Nobécourt et al. *(142)*; Nobecourt et al. *(143)*; Curtiss and Witztum *(195)*]	• S1P levels elevated in people with diabetes [Tong et al. *(111)*] • PON1, $LpPLA_2$, and LCAT activity is decreased in people with diabetes [Nobécourt et al. *(142)*; Hedrick et al. *(145)*; *(146)*; Sanchez-Quesada et al. *(196)*; Ghanei et al. *(197)*] • Decreases in HDL_2 in people with type 2 diabetes are associated with increased C-peptide activity [Baynes et al. *(147)*].
Rheumatoid arthritis	• In patients with high levels of inflammation, the antioxidative activity of small, dense HDL_3 particles is diminished [Gómez Rosso et al. *(148)*] • Women with RA, but not men, have reduced HDL_2 and HDL_3 levels [Arts et al. *(150)*].	• SAA replaces apoA-I in HDL-C in patients with RA [Eren et al. *(161)*] • Patients with RA have lower levels of apoA-I, and higher levels of apoC-III [Knowlton et al. *(149)*].	• PON1 activity is lower in patients with RA [Watanabe et al. *(138)*] • C-reactive protein and SAA are elevated in patients with RA [Gómez Rosso et al. *(148)*].
Psoriasis	• Cholesterol efflux in patients with psoriasis is impaired due to the compositional changes with apos [Holzer et al. *(137)*] • HDL_3 functionality is diminished in patients with psoriasis [He et al. *(151)*].	• Patients with psoriasis, even with only modest inflammation, have decreased apoA-I and apoM levels, as well as elevated apoA-II and SAA levels [Holzer et al. *(137)*; He et al. *(151)*]	• $LpPLA_2$ activity is increased in patients with psoriasis [Holzer et al. *(137)*] • PON1 activity is decreased in patients with psoriasis [He et al. *(151)*].
Chronic kidney disease	• Plasma HDL-C levels are lower in patients with CKD than healthy controls [Calabresi et al. *(152)*] • The antiinflammatory effects of HDL in reducing the production of ROS is decreased in patients with CKD [Moore and Fisher *(153)*].	• ApoA-I levels are decreased, while apoA-II and SAA increased in patients with CKD [Kronenberg *(170)*] • ApoA-IV levels are elevated in patients with CKD [Kronenberg *(170)*].	• $LpPLA_2$ activity is increased in patients with CKD [Holzer et al. *(137)*] • LCAT concentration and activity was decreased in patients with CKD [Vaisar et al. *(139)*].
Acute coronary syndrome	• HDL functionality in mitigating oxidative stress and promoting cholesterol efflux is impaired in patients with ACS [Bounafaa et al. *(154)*] • HDL from patients with ACS has compromised ability to stimulate eNOS [Gomaraschi et al. *(156)*].	• ApoA-IV was decreased and apoA-V was increased in patients with ACS [Bounafaa et al. *(154)*; Huang et al. *(157)*]	• PON1 activity is decreased in patients with ACS [Alwaili et al. *(140)*; Bounafaa et al. *(154)*] • The ratio of myeloperoxidase activity to PON1 is significantly higher in patients with ACS [Emami Razavi et al. *(155)*].

Continued on page 205

Table 2. Dysfunction of HDL-C, HDL subfractions, and associated enzymes in disease. *(Continued from page 204)*

Areas of dysfunction	HDL subfractions	Apolipoprotein activity	HDL-associated enzymes
Coronary artery disease	• Patients with lower HDL cholesterol levels have a higher rate of CAD than those with higher HDL [Whayne et al. *(136)*] • Serum levels of HDL_2 are reduced in patients with CAD due to an enhanced exhange process between enlarged VLDL pool and HDL [Calabresi et al. *(159)*].	• ApoB levels are elevated in patients with CAD [Whayne et al. *(136)*] • HDL from patients with CAD is enriched with apoE [Vaisar et al. *(139)*].	• Plasma HDL $LpPLA_2$ concentrations is higher in patients with CAD than healthy controls [Bostan et al. *(158)*] • Patients with CAD have reduced PON1 activity [Kuchta et al. *(160)*].

might be to develop a multiplex panel for simultaneous measurement of multiple components on HDL that may be involved in individual aspects of its function. In considering such an assay, one would expect significant increases in complexity and cost.

HDL Assays Based on Physical Properties

HDL was initially defined based on its density after density-gradient ultracentrifugation, while other assays assessing physical properties of HDL have also recently been developed *(9)*. Some of these assays may be amenable to routine diagnostic testing and relate to HDL function, and hence CVD risk. The best example of this is the use of nuclear magnetic resonance (NMR) spectroscopy for measuring HDL particle number, which has been shown in several large studies to be superior to HDL-C in predicting future cardiovascular events *(4, 183, 184)*. NMR can also be used to quantify specific HDL subfractions, such as apoB-containing lipoproteins, as well as LDL and HDL particle size and distribution, which may also be predictive of increased mortality in a wide range of disease states *(185–190)*. Correlations between specific HDL subfractions particle concentration and other cardiovascular measures have also been identified using NMR. For example, after adjusting for HDL-C, particle concentration of small and medium size HDL was inversely associated with internal carotid intima media thickening and PON1 activity in the Multi Ethnic Study of Atherosclerosis study population *(188)*, and inversely associated with coronary artery calcification in a cross-sectional population-based study in Jerusalem *(191)*. Additionally, by discordance analysis, lipoprotein particle counts have been shown to better correlate with CVD than lipoprotein cholesterol content in patients with diabetes mellitus and metabolic syndrome. Taken together, these studies demonstrate the versatility of NMR as a promising new technique for expanding our understanding of the interrelationship between lipoprotein metabolism and CVD and for identifying new.

Other physical separation tests, such as capillary isotachophoresis and high-performance liquid chromatography *(4)* have also been used to charge- or size-fractionate HDL. Recently, a mass spectrometry-based test called ion mobility analysis has been developed as an alternative way to measure HDL particle number, as well as HDL composition and size subfractions *(190, 192)*. Finally, a test based on electron paramagnetic resonance spectroscopy has also been described for measuring the integration of apoA-I into HDL in samples, which in small-scale studies appears to be related to HDL function and CVD risk *(4, 189)*. In summary, these new physiochemical techniques for characterizing HDL thus show promise and may provide an alternative way for measuring the cardioprotective effects of HDL.

Future Perspectives

Recent findings from a wide variety of clinical and experimental studies have clearly demonstrated that HDL-C is neither a driver for CVD, nor a suitable biomarker for drug development, despite the fact that HDL-C is a useful negative risk factor for estimating CVD risk. In terms of evaluating cardiovascular risk based on HDL, elucidating the specific contributions of its individual apolipoproteins and the other proteins that reside on it is paramount and may help explain the divergence in the observations on how HDL-C relates to CVD risk. Likewise, a more detailed understanding of HDL lipidomics may lead to better lipid markers than the cholesterol content of HDL. HDL functionality metrics, such as those discussed above, also need to be further developed and validated in large-scale clinical trials to determine their utility to predict CVD progression, as well as for biomarkers for drug development.

Although most of the efforts on HDL-based drug development have been focused on improving HDL levels and function, it should not be forgotten that, in the context of disease, HDL can be converted into noxious particles reminiscent of DAMPs and can engage pattern-recognition scavenger receptors, triggering pathogenic activation of innate immunity *(193, 194)*. Specific blockade of these pathogenic processes might offer new, innovative approaches for drug discovery targeting HDL and possibly yield new diagnostic tests for measuring dysfunctional HDL.

In summary, despite our increased understanding of HDL metabolism and its regulation, this knowledge has not yet been translated into the development of successful HDL-targeted therapies to decrease cardiovascular morbidity and mortality. Improved methodologies for assessing HDL functionality, composition, and physiochemical properties should aid in this effort. It is also important to note that the majority of the methodologies discussed herein are research assays for drug development, and not diagnostic tests routinely performed in a clinical setting. Future research could clarify the physiological and diagnostic role of these alternative measures of HDL, which could eventually replace HDL-C as a routine cardiovascular risk biomarker.

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