High-density lipoproteins (HDLs) represent a spectrum of particles that vary in their physicochemical and functional properties. It has been shown in many population studies that the concentration of HDL cholesterol (HDL-C) is inversely related to the risk of having a cardiovascular disease (CVD) event. In this paradigm, HDL-C has been considered to be a marker of the potentially cardioprotective functions of HDL. However, recent studies have suggested that the simple concentration of HDL-C may not always reflect HDL function, with growing evidence that under some circumstances HDL function may be compromised despite high concentrations of HDL-C.

The best known of the potentially antiatherogenic functions of HDLs is their ability to promote cholesterol efflux from cells, including that from macrophages in the arterial wall. Cellular cholesterol efflux is achieved by several mechanisms. One involves the interaction of phospholipid-depleted and cholesterol-deficient apolipoprotein (apo) A-I complexes (discoidal, pre-β-migrating particles [very small HDL] with the ATP-binding cassette transporter A1 (ABCA1) in a process that results in the formation of a heterogeneous population of nascent HDL particles that are discoidal in shape and contain apoA-I, phospholipids, and free cholesterol. A proportion of the free cholesterol is subsequently esterified by lecithin:cholesterol acyltransferase (LCAT); this enzyme generates a core of cholesterol esters in a process that converts HDL particles from discoidal, very small, pre-β-migrating particles into spherical, α-migrating particles [small HDL]). The interaction of spherical HDL particles with other active cellular transporters such as ABCG1 and passive diffusion of cellular cholesterol further increase the cholesterol load of HDL. However, it is often unappreciated that peripheral cholesterol efflux contributes <5% of the cholesterol content of HDL. Thus, HDL-C is an inadequate surrogate measure for the most heralded of HDL functions.

Various HDL subpopulations differ in other antiatherogenic functions that extend beyond macrophage cholesterol efflux. Small, protein-enriched, cholesterol-depleted HDL particles possess antioxidant, anti-inflammatory, cytoprotective, antithrombotic, anti-infective, and endotoxin-neutralizing activities. Structure-function analyses suggest that the simple measurement of HDL-C may not always be reflective of HDL functionality.

The challenge is to develop laboratory assays that quantify the various HDL functions that may improve CVD risk assessment and augment the evaluation of HDL-modifying therapies. Efforts to develop reproducible, cost-effective, validated assays that measure the potentially protective functions of HDL are now recognized as a major challenge for the cardiovascular field. Currently, there is no consensus concerning the HDL functions that should be targeted, nor are there standardized assays to measure HDL function as a tool to improve either CVD risk assessment or the assessment of therapeutic interventions (Figure 1). Another challenge is to validate measurements of HDL particles to be able to standardize assays of function with HDL quantification.

In this article, we review currently available measures of HDL function, explore the potential contribution of functional assays to understanding the mechanisms of atherosclerotic CVD, and describe the involvement of the proteome and lipoprotein in HDL structure-function relationships. To improve the understanding of HDL functionality, we propose a framework for future investigations addressing the validation and clinical application of HDL functional assays that may have a role as surrogates of CVD (Figure 1).

**Measures of HDL Subclasses and Compositional Determinants of HDL Functionality**

Conventionally, HDL concentration is reported in terms of the cholesterol concentration measured within the ultracentrifugally defined density range of 1.063 to 1.21 g/L. Further
There is evidence that quantification of lipoprotein functionality requires validation in studies of atherosclerosis and atherothrombotic cardiovascular events. In this model, we propose that candidate biomarkers of HDL functionality will be evaluated from specimens obtained in trials of HDL-altering therapies. After an association is established, these functional biomarkers will be prospectively evaluated in studies of atherosclerosis progression and clinical atherosclerotic cardiovascular events.

Figure 1. Validation of assays of high-density lipoprotein (HDL) functionality. The development of functional assays for HDL requires validation in studies of atherosclerosis and atherothrombotic cardiovascular events. In this model, we propose that candidate biomarkers of HDL functionality will be evaluated from specimens obtained in trials of HDL-altering therapies. After an association is established, these functional biomarkers will be prospectively evaluated in studies of atherosclerosis progression and clinical atherosclerotic cardiovascular events.

In some recent studies, HDL-P concentration has emerged as a predictor of CVD risk that may be superior to that of HDL-C in both population studies “ and randomized, clinical trials of lipid-modifying therapies. In the Multi-Ethnic Study of Atherosclerosis (MESA), low HDL-P predicted higher risk of elevated carotid intima-media thickness regardless of whether the baseline HDL-C level was high ($\geq 55$ mg/dL) or low (<$42$ mg/dL). In the Justification for the Use of Statins in Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER), HDL-P was a better marker of residual risk in statin-treated patients than chemically measured HDL-C, apoA-I, or average HDL size.

Two separate nested studies of the Veterans Affairs High-Density Lipoprotein Intervention Trial (VA-HIT) trial used 2 different analytical methods for quantifying HDL subclasses to investigate the importance of HDL subclass distribution in the prediction of CV events among coronary heart disease patients with low HDL-C levels (<$40$ mg/dL). In a case-control study, NMR-determined small HDL particle subclass levels measured at baseline and on trial were predictors of coronary heart disease events (odds ratio, 0.71; 95% confidence interval, 0.60–0.84; $P<0.0001$ for baseline measures; and odds ratio, 0.67; 95% confidence interval, 0.57–0.79; $P<0.0001$ for on-trial measures), whereas the risk associated with medium HDL particle concentration was weaker (odds ratio, 0.82; 95% confidence interval, 0.70–0.96; $P<0.02$ for baseline measures; and odds ratio, 0.82; 95% confidence interval, 0.69–0.97; $P<0.02$ for on-trial measures) and for large HDL particles nonsignificant in multivariate models that included major coronary heart disease risk factors, plasma lipids, and NMR-measured lipoprotein subclasses. In gemfibrozil-treated patients, analysis by 2-dimensional gradient gel electrophoresis indicated that elevated levels of pre-$\beta$-HDL (very small HDL particles) and low levels of $\alpha$1 HDL (very-large HDL) and $\alpha$2 HDL (large HDL) were associated with increased risk of CV events in multivariate models that adjusted for nonlipid and lipid risk factors. These conflicting findings may have resulted from differences in the study design (case-control versus cohort) and variables included in the statistical models. Other possible differences in the associations between very small HDL particles and coronary heart disease risk in VA-HIT may result from the analytical methods in which 2-dimensional gradient gel electrophoresis accurately quantifies pre-$\beta$-HDL, whereas it has not been reported whether this HDL subclass is detected by NMR. Consistent with the increased risk associated with high levels of pre-$\beta$-HDL in VA-HIT, high pre-$\beta$-1 HDL levels predict increased risk of myocardial infarction. These data suggest that impaired maturation of HDL particles increases CV risk.

From these studies, we conclude that the inclusion of a measure of atherogenic lipoproteins (LDL-P or apoB) in multivariate models is crucial in the assessment of HDL-associated risk resulting from the inverse correlation between the concentration of atherogenic lipoproteins and HDL-C and large HDL subclasses and that determination of HDL-P and individual concentrations of HDL subclasses should be considered in any clinical study that investigates HDL functionality.
**HDL Functionality**

The ability of HDL to promote efflux of cholesterol from macrophages in the artery wall is the best known of the potentially cardioprotective functions of HDL. However, HDL particles have additional properties with the potential to protect against vascular disease, some of which are related and others are unrelated to cholesterol transport and homeostasis (Table 1). This section discusses the major functional roles of HDL and the available clinical measures currently used to evaluate these functions in clinical studies.

**Cholesterol Efflux**

Reverse cholesterol transport is a term used to describe the efflux of excess cellular cholesterol from peripheral tissues and its return to the liver for excretion in the bile and ultimately the feces. It is believed to be a critical mechanism by which HDL exerts a protective effect on the development of atherosclerosis; equally, it is a critical component of the system that maintains cholesterol homeostasis. In this paradigm, cholesterol is effluxed from arterial macrophages to extracellular HDL-based acceptors through the action of active transporters and passive diffusion. After efflux to HDL, cholesterol may be esterified in the plasma by LCAT, and it is ultimately transported from HDL to the liver, either directly via the scavenger receptor BI (SR-BI) or after transfer to apoB-containing lipoproteins by the cholesteryl ester transfer protein for ultimate disposition in the feces. However, isotope kinetic studies and mass measurements of cholesterol and bile acids suggest that effective macrophage cholesterol efflux may be atheroprotective even when biliary and fecal sterol excretion is not increased. Thus, revision of earlier models of reverse cholesterol transport was recently proposed to more accurately describe the critical steps required for effective HDL-mediated atheroprotection by promotion of macrophage cholesterol efflux.

Macrophage cholesterol efflux capacity is influenced by the physicochemical properties of HDL and the interaction of these HDL subclasses with cellular transporters. As indicated in the previous section, the ABCA1 transporter interacts with cholesterol-deficient and phospholipid-depleted apoA-I complexes, whereas ABCG1 and SR-BI interact with spherical HDL particles of various sizes.

Alterations in HDL protein and lipid composition may alter cholesterol efflux. Loss of apoA-I secondary to its replacement by serum amyloid A, as occurs under proinflammatory conditions of arthritis, uremia, or psoriasis, reduces HDL-mediated cholesterol efflux. Equally, glycation and oxidation of HDL proteins may adversely affect cholesterol efflux and other antiatherogenic functions of HDL.

HDL surface lipid composition and interaction between lipid molecules have been shown to affect cholesterol efflux.

**Table 1. Major Anti-Atherosclerotic Functional Roles of HDL With Available Clinical Measures**

- Macrophage cholesterol efflux
- Anti-oxidative effects
- Anti-inflammatory effects
- Endothelial function
- Glucose homeostasis

Enrichment of HDL particles in triglycerides or depletion of phospholipid may render them deficient in their capacity to efflux cellular cholesterol via ABCA1 and SR-BI. Impaired cholesterol efflux from macrophages can also result from the accumulation in HDL of oxidized sterols, including 7-ketocholesterol. Qualitatively, the surface rigidity of HDL particles, which is partly regulated by the relative proportions of sphingomyelin and free cholesterol in the surface lipid monolayer of HDL, influences the capacity of HDL particles to serve as an acceptor of cholesterol. HDL enrichment in sphingomyelin enhances cholesterol efflux via direct interaction between sphingomyelin and cholesterol molecules, thereby counteracting the effects of diminished fluidity. In macrophage-like human THP-1 cells, cellular cholesterol efflux capacity correlated with percent weight of phosphatidylcholine and inversely correlated with percent weight of sphingomyelin. In addition, sphingomyelin inhibits LCAT activity and impairs maturation of HDL particles.

Clinically, ex vivo assays have been used to assess the capacity of individual patient serum and HDL specimens to remove cholesterol from cultured cholesterol-loaded macrophages. At present, in vivo quantification of macrophage reverse cholesterol transport can be determined only in animal models; however, studies are underway to develop methods for use in humans. The J774 mouse macrophage cell line has been used extensively for ex vivo cholesterol efflux studies. Typically, the cells are lipid loaded and treated with cAMP or a liver X receptor agonist to increase the magnitude of cholesterol efflux, particularly via the ABCA1 pathway. Human macrophage THP-1 cells provide an alternative to the J774 model, which features a slightly different expression pattern of major proteins that are involved in cholesterol efflux and may be more relevant for human atherosclerosis. SR-BI–mediated cholesterol efflux may be determined with the Fu5AH hepatoma cell line. From studies of macrophage cholesterol efflux with the J774 cell line, serum specimens having similar HDL-C or apoA-I levels can exhibit significant differences in fractional efflux. A comparison of the contribution of efflux pathways of high- and low-efficiency HDL demonstrated that the increased cholesterol efflux observed in the higher-efficiency sera was attributed largely to greater efflux via the ABCA1 pathway.

A recent study reported that the capacity of individual patient serum to stimulate cholesterol efflux from J774 macrophages has a strong inverse association with angiographically quantified coronary artery disease (CAD) that is independent of HDL-C or apoA-I levels. The efficiency of cholesterol efflux in CAD patients was most strongly associated with HDL-C, however, it accounted for only 26% of the reported variation. The role of cholesterol efflux in apoB-depleted serum as a predictor of cardiovascular risk remains controversial. A more recent study confirmed the finding that increased cholesterol efflux activity in apoB-depleted serum is associated with reduced risk of prevalent CAD. Unexpectedly, however, higher cholesterol efflux activity was also associated with an increase in prospective (3 years) risk of myocardial infarction, stroke, and death. Regardless of the cellular model, elevated experimental between-assay variability (coefficients of variation close to 10%–15%) has been reported compared with <4% for analytic measurements. Such variability results from the...
very nature of the cell culture approach used, requires normalization on the basis of the efflux capacity of a serum pool run with each assay, and has been an impediment to the development of these assays for use in clinical practice.

Future use of the measurement of HDL-mediated efflux in CVD risk assessment will require integration of advanced vascular imaging in human studies that quantify the volume and composition of atherosclerotic plaques and atherosclerotic CVD events (Figure 1).2 Guidance in the development of new HDL-targeted therapies for humans will require screening of large numbers of serum specimens, and the use of radiolabeled cholesterol for large-scale screening is generally not practical. Thus, fluorescent dipyrromethene boron difluoride cholesterol34 may serve as a substitute for the labeled cholesterol, which would allow the development of a fluorescence-based high-throughput efflux assay.

Endothelial Function

HDL particles have direct effects on endothelial function that are considered antiatherosclerotic and antithrombogenic. Specifically, HDL particles isolated from healthy individuals induce the expression of endothelial nitric oxide (NO) synthase (eNOS) and synthesis of NO by endothelial cells, inhibit adhesion molecule expression,35 promote endothelial cell migration contributing to endothelial repair,36 and attenuate tissue factor expression.35

ApoA-I appears to be critically involved in the effects of HDL on the endothelium.72 The protective effects of HDL on eNOS production and endothelial repair may be partly dependent on processes that involve SR-BI.38 In a process dependent on apoA-I–dependent binding to SR-BI in endothelial cells, SR-BI initiates a signaling cascade that involves PDZK1-dependent activation of the Src family kinases PI3K and AKt, which phosphorylate eNOS at Ser177, increasing enzyme activity.39,40 Akt-activating phosphorylation (AKt-Ser473) and eNOS-activating phosphorylation (eNOS-Ser1177) diminish LOX-1 activation and inhibit protein kinase Cβ-II activation of AKt and eNOS phosphorylation events. Reduced protection from endothelial apoptosis in CAD and acute coronary syndrome patients was associated with lower HDL content and HDL-mediated endothelial function.38 However, in the absence of oxidized LDL, only low HDL concentrations (10–50 µg/mL) improved endothelial function and endothelial progenitor cell survival, whereas high concentrations of HDL (400–800 µg/mL) paradoxically increased endothelial progenitor cell senescence and increased angiogenesis through activation of the Rho-associated kinase pathway.

Effects of HDL on endothelial cells can be measured in vitro in cell culture, with eNOS activation and NO production as main outcomes. Endothelial cell viability has been measured with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, which analyzes the effect of HDL on oxidized LDL–mediated impairment in cell viability.52 Few data are available on the association between HDL-C values and HDL-mediated endothelial function. The ability of normolipemic HDL to counteract the inhibition of endothelium-dependent relaxation induced by oxidized LDL in rabbit aorta rings was lost in patients with type 1 and type 2 diabetes mellitus and dyslipidemic, abdominally obese individuals.73,74 The beneficial effects of HDL on endothelial NO production, superoxide generation, NADPH oxidase activity, and endothelium-dependent vasodilation in aortic rings are equally impaired in HDL from patients with type 2 diabetes mellitus.46 However, both patients with type 2 diabetes mellitus and obese subjects exhibited reduced HDL-C levels in these studies. Only patients with type 1 diabetes mellitus revealed dissociation between HDL-C levels, which were normal, and HDL function.53 Moreover, HDL from quasi-normo-high density lipoproteinemia patients with stable CAD or with acute coronary syndrome was largely unable to stimulate endothelial NO production.49

Clinical measures of endothelial function include noninvasive measures of brachial artery flow-mediated dilatation by B-mode ultrasound55,56 and invasively with measures of coronary arterial endothelium-dependent and endothelium-independent vascular function.57,58 In a prospective study, both endothelium-dependent and endothelium-independent vasoreactivity predicted long-term atherosclerosis progression and CVD events.58

The use of standardized protocols and computer-assisted flow analysis software effectively reduced the coefficient of variation for this method to <2.0%.55 In the Vascular Effects and Safety of Dalcetrapib in Patients With or at Risk of Coronary Heart
Disease (dalcetrapib [dal]-VESSEL) trial, flow-mediated dilation was used to investigate the effect of dalcetrapib, a cholesteryl ester transfer protein inhibitor, on endothelial function. Changes in coronary arterial vascular function can be measured with endothelium-dependent responses to acetylcholine and endothelium-independent responses after infusion of nitroprusside or adenosine. The cold pressor test integrates flow-dependent vasodilation resulting from endothelial and smooth muscle cell responses to sympathetic activation, and these responses correlate with endothelium-dependent responses to acetylcholine. Other measures include the measurement of endothelial cell–mediated endothelial progenitor cell repair.

**Anti-Inflammatory and Antioxidant Effects**

HDL particles are heterogeneous in their capacity to protect LDL against oxidative modification as occurs from 1- and 2-electron oxygen free radicals. Attenuation of LDL oxidation reduced activation of redox-sensitive transcription factors; thus, the antioxidant properties of HDL contribute to reduced oxidative stress and inflammation. Other anti-inflammatory properties of HDL encompass suppression of macrophage inflammatory cytokine production and inhibition of the expression of endothelial cell adhesion molecules that promote entry of monocytes and neutrophils into arteries (Figure 2). Anti-inflammatory properties of HDL have been linked to its ability to promote cellular sterol efflux from cells and from mechanisms unrelated to cholesterol efflux.

ApoA-I plays a central role in HDL-mediated protection from oxidative damage, acting via inactivation of LOOHs, because its Met residues 112 and 148 can reduce LOOH into redox-inactive lipid hydroxides, thereby terminating chain reactions of lipid peroxidation. Other HDL apolipoproteins, including apoA-II, apoA-IV, apoA-V, apoE, apoI, and apoM, may equally contribute to HDL-mediated protection of LDL from free radical–induced oxidation. Enzymatic components contributing to antioxidative properties of HDL include PON1, platelet acting factor–acyl hydrolylase, and LCAT, all of which are able to hydrolyze proinflammatory short-chain oxidized phospholipids. In addition, HDL carries glutathione selenoperoxidase, which can reduce LOOH to the corresponding hydroxides and thereby detoxify them.

Protein components whose accumulation can be involved in the loss of antioxidative and anti-inflammatory activities of HDL include complement C3 protein and serum amyloid A. Loss of antioxidative activity of HDL after acute-phase induction occurs concomitantly with decreases in HDL-associated PON1 and platelet acting factor–acyl hydrolylase activities. Enrichment of the HDL lipidome with sphingomyelin and saturated fatty acids elevates the rigidity of the phospholipid surface monolayer of HDL. Such structural anomalies may

**Figure 2.** Proposed models for anti-inflammatory effects of high-density lipoprotein (HDL). In model A, as proposed by Suzuki and colleagues, HDL inhibits TLR4/TRIF signaling, resulting in decreased synthesis of type 1 interferons such as interferon-β. This effect appears to be independent of cholesterol efflux and expression of ABCA1/G1. Effects of HDL could potentially be mediated via CD14 or TRAM. In model B, as proposed by Chung and colleagues and Yvan-Charvel and colleagues, cholesterol efflux mediated by ABCA1 and ABCG1 leads to disruption of cholesterol–enriched plasma membrane microdomains and decreased signaling via TLR4 (and TLR2/3), possibly as a result of decreased amounts of MD2-TLR4 complexes at the cell surface. ABCA1 indicates ATP-binding cassette transporter ABCA1; ABCG1, ATP-binding cassette subfamily G member 1; CD14, cluster of differentiation 14; HDL-L, large high-density lipoprotein; HDL-M, medium high-density lipoprotein; HDL-S, small high-density lipoprotein; IkBα, nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor, α; IL-1β, interleukin-1β; IL-6, interleukin-6; IP-10, interferon-γ–induced protein 10; IRF3, interferon regulatory factor 3; JAK2, Janus kinase 2; LPS, lipopolysaccharide; MD2-TLR4, myeloid differentiation 2–Toll like receptor 4; MyD88, myeloid differentiation primary response protein 88; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; STAT3, signal transducer and activator of transcription 3; TLR4, Toll-like receptor 4; TNFa, tumor necrosis factor-α; TTP, tristetraprolin.
Impair the capacity of HDL to acquire LDL-derived oxidized lipids and to protect LDL from free radical–induced oxidative damage.72 Evaluation of antioxidant effects of HDL has encompassed several functional methods that evaluate the efficiency of HDL particles to protect LDL against oxidative modification. LDL lipid peroxidation by free radicals the presence of HDL proceeds in a 2-step process that involves a slow rate of conjugated diene accumulation ascribed to the presence of antioxidants including those in HDL and a second rapid phase that is principally dependent on the antioxidative functionality of HDL. Conventionally, rates of LDL oxidation have been measured by the formation of conjugated dienes, which is monitored as the change in absorbance of the sample.74 However, this method is nonspecific because multiple factors affect diene conjugation, including the source of oxidant, the fraction of polyunsaturated fatty acids in the fatty acids of LDL, the presence of other biomolecules that adsorb at the same wavelength, endogenous antioxidants, metal chelators present in the system, and the level of contaminating metal ions that promote further lipid oxidation. On the other hand, this conjugated diene assay provides an evaluation of the integrated antioxidant potential of a given HDL particle in that it reflects the sum of the content of several oxidizable lipid components. An alternative method for evaluating the antioxidant effects of HDL is the cell-free assay. The cell-free assay or the HDL-oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (OxPAPC) assay measures the ability of plasma HDL to reduce formation of oxidized phospholipids.75-77 The addition of the fluorochrome 2′,7′-dichlorofluorescein produces a fluorescent signal that depends on the concentration of OxPAPC in vitro. This approach, however, also suffers from interferences related to the source of oxidant, the presence of metal chelators, and the level of contaminating transition metal ions.

In human studies, the monocyte chemotaxis assay73 and an endothelial cell assay76 represent in vitro models for assessing the anti-inflammatory effects of HDL particles. The monocyte chemotaxis assay assesses the effects of standardized LDL added to a coculture of human vascular endothelial and smooth muscle cells to promote monocyte entry into the coculture. The impact of test HDL on the monocyte chemotaxis assay can be compared with this reference measurement, and the resulting inflammatory index can contrast HDL that reduces monocyte chemotaxis assay (anti-inflammatory; index <1.0) from HDL that paradoxically enhances monocyte chemotaxis assay (proinflammatory; index >1.0).75,76,79 An alternative approach for quantifying the anti-inflammatory response to HDL involves measuring the cytokine response in lipopolysaccharide-activated macrophages.80 The endothelial cell assay investigates the effects of HDL on cell surface adhesion molecule expression by cultured human umbilical vein endothelial cells activated by proinflammatory agents.78

Immunomodulatory Effects

HDL and the ABC transporters act at the level of hematopoietic stem cells (HSCs) to suppress HSC proliferation and the production of monocytes and neutrophils.83 These studies have shown that ABCA1, ABCG1, and apoE are highly expressed in HSCs.82-84 ApoE is found in a proteoglycan-bound pool on the surface of HSCs, where it interacts with ABCA1/G1 to promote cholesterol efflux and to control levels and signaling of the interleukin-3/granulocyte macrophage colony-stimulating factor receptor.85 In the absence of apoE or ABCA1/G1, there is excessive proliferation of HSCs, especially after challenge with the Western-type diet. This proliferation of HSCs and the resulting monocytosis and neutrophilia can be suppressed by pharmacological treatments with liver X receptor activators,83 whereas other studies have shown that leukocyte infiltration into the arterial wall is inhibited by reconstituted HDL infusion.86,87 Recent studies in mice with macrophage-specific knockout of ABCA1/G1 have shown HSC mobilization and extramedullary hematopoiesis in the spleen that are independent of HSC proliferation, with suppression of these processes by increased HDL.88 The mechanism is related to increased production of interleukin-23 by splenic macrophages driving granulocyte colony-stimulating factor production and increased HSC release from the bone marrow. HDL acts directly on HSCs to control proliferation and monocyte production but also at the level of macrophages to suppress inflammatory responses, including those that lead to increased release of HSCs from the bone marrow. This process is also activated after myocardial infarction in mice, leading to extramedullary hematopoiesis in the spleen and increased monocyte production and entry into plaques, ultimately resulting in accelerated atherosclerosis.89,90 The ability of HDL to suppress monocytosis, neutrophilia, monocyte activation, and macrophage inflammation; stem cell mobilization; and extramedullary hematopoiesis appear to represent key antiatherogenic properties. The suppression of granulocyte colony-stimulating factor, monocytosis, and neutrophilia may also provide biomarkers for the assessment of the in vivo efficacy of different HDL-raising therapies.85,88 Anti-inflammatory effects of HDL that are unrelated to cholesterol efflux involve inhibition of tumor necrosis factor-α-stimulated endothelial superoxide production and NADPH oxidase activity77 induction of the antioxidant and anti-inflammatory protein 3-β-hydroxysteroid-delta 24 reductase (DHCR24) (Figure 3).91 Activation of human coronary artery endothelial cells with tumor necrosis factor-α induces expression of the adhesion molecules vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and E-selectin.

**Figure 3.** Potentially protective properties of high-density lipoprotein (HDL) unrelated to cholesterol transport. HDL particles have antioxidant, anti-inflammatory, antiapoptotic, and antithrombotic properties and enhance endothelial function in processes apparently unrelated to cholesterol metabolism. The mechanisms of these effects include HDL-mediated increases in endothelial nitric oxide (NO) and 3-β-hydroxysteroid-delta 24 reductase (DHCR24) and inhibition of tissue factor.
However, if the cells are preincubated with HDL particles, the expression of these adhesion molecules is greatly inhibited. This HDL-mediated anti-inflammatory effect remains apparent even if the HDL particles have been removed from the incubation several hours before activation of the cells with tumor necrosis factor-α. Similar findings were reported in vivo in a rabbit model of vascular inflammation. Administration of HDL particles 24 hours before the induction of vascular inflammation, a time when most of the injected HDL particles have been cleared from the plasma, again suggests that the injected HDL particles induced the expression of an anti-inflammatory factor. This factor, subsequently identified as DHCR24 or seladin-1, has been shown to scavenge hydrogen peroxide and to possess potent antioxidant and anti-inflammatory properties that are independent of the role of DHCR24 in cholesterol synthesis.

The ability of HDL particles to induce DHCR24 is independent of cholesterol metabolism in that the effect remains apparent in cells in which efflux has been blocked by silencing the genes for the ABCA1 and ABCG1 transporters. Furthermore, neither the promotion of cholesterol efflux by cyclodextrin nor the inhibition of cholesterol synthesis with sihnvastatin has any effect on the level of DHCR24 in cells. Induction of DHCR24 by HDL particles, however, is dependent on the activity of SR-B1, a receptor known to bind HDL particles.

Other anti-inflammatory pathways that may be independent of cholesterol efflux include the ability of HDL to suppress Toll-like receptor-4/TIR domain-containing adaptor protein inducing interferon/interferon regulatory factor 3–dependent signaling. In addition, apoA-I has been reported to signal via ABCA1 to activate Janus kinase 2/signal transducer and activator of transcription 3 signaling with consequent suppression of inflammatory responses.

Antioxidative effects of HDL are thought to primarily involve inhibition of LDL oxidation in the arterial intima. HDL particles, particularly small, dense, protein-rich HDL3, may provide potent protection of LDL from oxidative damage by free radicals, resulting in the reduced accumulation of proinflammatory oxidized lipids, primarily lipid hydroperoxides, but also short-chain oxidized phospholipids. HDL-mediated inactivation involves initial transfer of lipid hydroperoxides from LDL to HDL, which is governed by the rigidity of the surface monolayer of HDL, and subsequent reduction of lipid hydroperoxides by redox-active Met residues of apoA-I with the formation of redox-inactive lipid hydroperoxides and methionine sulfoxides. HDL-associated enzymes may in turn contribute to the hydrolytic inactivation of short-chain oxidized phospholipids.

**HDL Particle Heterogeneity and Its Role in Antioxidant/Anti-Inflammatory Effects**

The antioxidant function of HDL is associated with distinct proteomic and lipidomic profiles of the various HDL subfractions. Of the ultracentrifugally defined HDL subfractions, dense HDL3 (small HDL) is the most effective in protecting LDL against oxidation on a per-particle basis as defined by apoA-I content.

Evaluation of the proteome and lipidome may provide insights into specific HDL functions. Recent proteomics studies suggest that different HDL subpopulations carry distinct proteins that carry out specific roles in lipoprotein metabolism and functions that are distinct from those implicated directly in lipoprotein metabolism.

**Heterogeneity of HDL Proteome and Its Potential Role in HDL Functionality**

It is well established that HDL in human plasma comprises a complex mixture of particle subpopulations, which are distinct in their structure, composition, metabolism, and functionality. A comprehensive mass spectrometry–based proteomic analysis of HDL demonstrated that HDL carries a complex protein cargo. For example, these investigators identified more acute-phase response proteins in HDL than proteins implicated in lipid metabolism. This finding supports the idea that HDL is involved in inflammation biology. Second, 2 protein families—protease inhibitors and regulators of complement activation—were identified that were not previously known to reside in HDL. These observations strongly support the view that the physiological role of HDL centers on lipid metabolism, inflammation, and the immune response.

Proteomic analysis of 5 major HDL subpopulations isolated from normolipidemic subjects by isopycnic density gradient ultracentrifugation identified 5 distinct patterns of distribution of individual protein components across the HDL density subfractions. The most interesting of these distributions identified small, dense HDL3c as a particle subpopulation that predominantly carries 7 proteins, notably apoJ, apoL-1, apoF, PON1/3, phospholipid transfer protein, and platelet activating factor–acyetyl hydroxylase (also known as lipoprotein-associated phospholipase A1). Activities of HDL-associated enzymes (PON1, platelet activating factor–acyetyl hydroxylase, LCAT) are elevated equally in small, dense HDL3c. Importantly, these associations can be confirmed in part with an alternative approach to subfractionation of HDL particles involving size exclusion chromatography.

Tandem mass spectrometry was used to test the hypothesis that aggressive lipid therapy with atorvastatin and niacin modifies the HDL proteome in humans with established CAD. This approach identified 4 proteins in the HDL3 subfraction whose relative abundance appeared to change as a result of treatment: apoE, apoJ, apoF, and phospholipid transfer protein. Levels of apoE fell whereas levels of apoJ, apoF, and phospholipid transfer protein rose. Immunochemical studies confirmed that combination therapy with niacin and statin reduced the levels of apoE in HDL, in an independent group of different subjects. These observations suggest that when CAD subjects are treated with combination therapy, the HDL proteome is remodelled to more closely resemble that of HDL of healthy control subjects.

The low abundance of the majority of HDL-associated proteins of <1 molecule per HDL particle suggests further internal heterogeneity of ultracentrifugally isolated HDL subfractions. This conclusion is consistent with the isolation of a unique particle containing the trypanosome lytic factor in the HDL3 density range, which is composed of apoA-I, apoL-, and haptoglobin-related protein. Specific protein–protein interactions appear to determine the formation of such lipoprotein complexes in the circulation. In support of such a mechanism, PLTP in human plasma resides on lipid-poor complexes dominated by apoJ and proteins implicated in host defense and inflammation.
Limitations of HDL proteomic analyses involve its semiquantitative character, the critical dependence of the results on the methodology of HDL isolation and purification, the nature of the starting biological material (serum or plasma), and equally the mass spectrometric technology used for protein analysis and quantification. For example, HDL particles isolated from serum and plasma samples differ in their content of several proteins, including complement C3, which is elevated in serum-derived HDL. However, recent methods have been developed to analyze the HDL proteome that use isotope dilution and are quantitative. Another issue is the coelution of high-molecular-weight plasma proteins with HDL that contaminates specimens isolated by size-exclusion chromatography by fast protein liquid chromatography. For this reason, ultracentrifugation remains the predominant isolation method to study the HDL proteome.

Heterogeneity of HDL Lipidome and Its Potential Role in HDL Functionality

In addition to differences in bound proteins, HDL subpopulations may differ in their lipid content. Indeed, particle contents of phospholipids, free cholesterol, cholesteryl ester, triglycerides, and total fatty acids progressively decrease with increased hydrated density and particle size from large, lipiddense HDL2b to small, lipid-poor HDL3c.

The proportion of sphingomyelin relative to total lipids decreases progressively in parallel with HDL density and size from 12.8% in HDL2b to 6.2% in HDL3c. The distinctly low sphingomyelin content in HDL3c suggests that this pool is not in equilibrium with that of other HDL subpopulations, which is consistent with the slow rate of exchange of sphingomyelin between lipoproteins and cell membranes. The low sphingomyelin-to-phosphatidylcholine ratio may reflect a distinct cellular origin or origins of small HDL as suggested by the low sphingomyelin content of small nascent HDL particles secreted by J774 macrophages, which originate from the exofacial leaflet of the plasma membrane.

Similar to sphingomyelin, the proportion of free cholesterol relative to total lipids decreases 2-fold from HDL2b to HDL3c. As a result, the ratio of cholesteryl ester to free cholesterol increases significantly with HDL density, supporting the contention that small HDL constitutes a major site of cholesteryl esterification within the HDL particle spectrum. Elevated LCAT activity and a diminished ratio of sphingomyelin to phosphatidylcholine in HDL3c are consistent with this proposal because sphingomyelin functions as a physiological inhibitor of LCAT. Because both sphingomyelin and free cholesterol increase the rigidity of liquid-crystal lipids, their low abundance in small, dense HDL suggests reduced rigidity of the surface lipids in this subpopulation.

Differences between HDL subpopulations in the abundance of cholesteryl ester and free cholesterol result in the enrichment of total cholesterol in large, light HDL. It is essential to emphasize in this regard that routine clinical measurement of plasma HDL-C primarily reflects levels of large, cholesterol-rich HDL and frequently lacks sensitivity to detect small, cholesterol-poor particles with important physiological functions. Furthermore, because HDL-C simply quantifies the amount of cholesterol contained within the HDL lipoprotein fraction, this measurement does not necessarily correlate with the number of HDL particles or their particle profile. The marked complexity of the HDL particle profile is therefore inadequately reflected by a single measurement of its cargo of cholesterol, that is, HDL-C.

Compositional differences between HDL subpopulations may equally include those with minor bioactive lipid components. Thus, the abundance of S1P per HDL particle is asymmetrical across the HDL spectrum, with preferential enrichment in HDL3 (40–50 mmol/mol HDL) compared with HDL2 particles (15–20 mmol/mol). Enrichment of small HDL3 in S1P can be enhanced by apoM, a specific protein transporter for S1P, which is associated predominantly with small, dense HDL.

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<td>• Lack of insight into the complexity of authentic HDL particle subspecies; clear definition of composition, structure and functionality of individual subspecies lacking.</td>
</tr>
<tr>
<td>• Lack of coherence across proteomic analyses of HDL when compared on the basis of the same clinical state.</td>
</tr>
<tr>
<td>• Insufficient clinical and biological phenotyping of study subjects (eg, stable CHD versus ACS versus post myocardial infarction).</td>
</tr>
<tr>
<td>• Wide variation in degree of inflammation and stage of clinical recovery across studies.</td>
</tr>
<tr>
<td>• Failure to consider dynamic aspects of the inflammatory process.</td>
</tr>
<tr>
<td>• Need for in vivo turnover studies of HDL proteins and lipid components in protein-defined HDL subspecies in inflammatory states.</td>
</tr>
<tr>
<td>Unresolved issues</td>
</tr>
<tr>
<td>• Can we implicate HDL-associated functions to unique HDL subspecies as defined by their proteome?</td>
</tr>
<tr>
<td>• Can distinct functions of HDL subspecies be accounted for by specific protein or lipid components?</td>
</tr>
<tr>
<td>• If the HDL proteome and lipidome are perturbed by metabolic/inflammatory background, can we relate such changes to alteration in function?</td>
</tr>
<tr>
<td>• Can we pharmacologically target altered HDL proteome, lipidome and function in cardiometabolic disease states, and in consequence, normalize them?</td>
</tr>
<tr>
<td>• Is such normalization accompanied by reduced cardiovascular risk?</td>
</tr>
<tr>
<td>Methodological approaches</td>
</tr>
<tr>
<td>1) New highly resolutive, non-denaturing reproducible preparative methods for fractionation of HDL subspecies on the basis of specific associations of protein components;</td>
</tr>
<tr>
<td>2) Standardization and calibration of MS/MS methodologies for HDL proteome and lipidome analyses at an international level</td>
</tr>
</tbody>
</table>

ACS indicates acute coronary syndromes; CHD, coronary heart disease; HDL, high-density lipoprotein; and MS/MS, tandem mass spectrometry.
Current limitations for the use of proteomics and lipidomics as surrogate markers of HDL functionality are described in Table 2. These studies have included small numbers of subjects and have used an analytic technology (mass spectrometry and tandem mass spectrometry) that is not widely available. However, it is important to note that 85 proteins have appeared in at least 3 different studies (from independent laboratories) and that these represent the best current estimate of the HDL proteome.104 Thus, it is critical to increase the number of investigations in this area and to provide analytical methods that use a high-throughput method for quantification of functionally relevant HDL components that is sensitive and specific.

Conclusions
The spectrum of biological activities of HDL particles has immediate relevance to understanding key mechanisms implicated in the pathophysiology of atherosclerosis. Nearly 50 years ago, Glomset and Wright105 emphasized the fundamental importance of HDL particles as the preferred substrate for LOCAT, which provided the basis for the proposed role of HDL in mediating reverse cholesterol transport.106 The accumulation of cholesteryl esters results in the formation of large, mature HDL particles that transport their cholesterol cargo to the liver for eventual fecal elimination. These concepts emphasized HDL-C as a biomarker of this critical aspect of HDL functionality. However, many structure-function analyses of HDL have identified small, cholesterol-depleted HDL particles as more effective than the large, cholesterol-enriched HDL particles in mediating the biological functions of HDL. For example, lipid-free apoA-I and apoE, but not the HDL holoparticle, are the most effective ligands for the ABCA1 transporter that promotes the cellular excretion of cholesterol.2 It has also been demonstrated that small, dense, spherical HDL particles are more effective in mediating the antioxidant, anti-inflammatory, antiapoptotic, and anti-inflammatory properties of HDL.1,3

Despite the extensive cellular biology on the protective role of protein-rich and cholesterol-depleted HDL particle subpopulations, there continues to be an emphasis on HDL-C in genome-wide association studies107 and nearly all clinical trials of lipid-modifying therapies.108-111 Low HDL-C levels may represent either a reduced number of HDL particles or a biomarker for excess numbers of apoB-containing particles.6,7 Importantly, the cholesterol content itself of HDL particles is not atheroprotective. Thus, HDL-C should not be considered a surrogate marker of HDL functionality. Assessment of the direct contributions of HDL particles to CVD prevention requires investigation of HDL-related biomarkers that are more tightly associated with critical atherothrombotic effects of HDL than with HDL-C, as shown in VA-HIT.8

However, it is important to extend the lessons learned from static HDL measures to functional assays that may provide important insights into the multifarious antiatherogenic effects of HDL. The clinical application of these new functional studies will require concomitant development of validated, reproducible, and cost-effective measures of key HDL functions.

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References


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Translation of High-Density Lipoprotein Function Into Clinical Practice: Current Prospects and Future Challenges


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