# High-Density Lipoprotein Function in Cardiovascular Disease and Diabetes Mellitus

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Cardiovascular disease (CVD) is the most common cause of death worldwide.[1] Major CVD risk factors are hypertension, smoking, physical inactivity, abnormal glucose levels/diabetes mellitus, and dyslipidemia. Among these, dyslipidemia characterized by a low level of HDL-C (high-density lipoprotein cholesterol) is strongly and inversely correlated with CVD risk,[2–4] and low HDL-C levels is part of the atherogenic dyslipidemia complex associated with diabetes mellitus.[5] These observations triggered intense interest in increasing HDL-C levels for therapeutic intervention of CVD.[6,7] However, several recent lines of evidence now suggest that the association between HDL-C levels and CVD status may be indirect or more complicated than previously recognized.[7] Thus, human genetic studies demonstrate that genetically altered HDL-C levels do not necessarily translate to an altered risk of CVD.[8–12] Phase III clinical trials of drugs that elevate HDL-C, such as niacin and CETP (cholesteryl ester transfer protein) inhibitors, also have largely failed to reduce CVD events in statin-treated subjects with established CVD.[13–15] For several CETP inhibitors, improvement in CVD prevention was unsuccessful because of off-target effects (torcetrapib) or lack of efficacy (dalcetrapib and evacetrapib).[14,16–18]The exception is the recent REVEAL trial (Randomized Evaluation of the Effects of Anacetrapib Through Lipid-Modification), which included more subjects and was performed for longer than previous CETP inhibitor trials. The REVEAL demonstrated that the CETP inhibitor anacetrapib exhibited beneficial effects on cardiovascular outcomes on top of those of statin therapy, although the risk reduction was moderate[19,20] and might have been due, at least in part, to a reduction in non-HDL-C rather than elevated HDL-C.[21] Considering these cumulative observations, it remains uncertain whether increased HDL-C directly impacts atherosclerosis and the risk of cardiovascular events. It has become quite apparent that other metrics of HDL levels and functions, and specifically of HDL antiatherogenic functions, must be considered.

HDL is a heterogeneous population of particles with sizes ranging from 7 nm to 14 nm diameter. The largest HDL particles contain the most cholesterol.[22] Therefore, HDL-C levels do not always correlate with HDL particle concentration, and furthermore, do not provide information on differences in HDL subpopulations. For example, in a recent study published in *ATVB*, the effects of 2 different bariatric surgery procedures on HDL metrics were compared. Sleeve gastrectomy increased HDL-C levels more than did Roux-en-Y gastric bypass surgery, calculated as differences from baseline, but reduced HDL particle concentrations.[23] The increase in HDL-C was most likely a reflection of a relative increase in HDL particle size.[23] Scientists are now therefore increasingly considering HDL-associated metrics other than HDL-C, such as HDL particle concentration, HDL composition, and HDL function as more likely determinants of CVD risk.[24] Thus, a large body of recent research has addressed changes in HDL function and particle subpopulations in pathophysiological conditions, including CVD and diabetes mellitus, with the goal of discovering novel and reliable biomarkers and targets for the treatment or prevention of CVD. In this review, we highlight research published in *ATVB* within the past 2 years, focusing on studies addressing functions of HDL subpopulations in CVD and diabetes mellitus.

## HDL Classification and Quantification Methods

HDL particles differ in surface charge, size, and lipid and protein composition, and the ensemble of the HDL particle is determined both by its biogenesis and dynamic remodeling by a range of enzymes, including CETP, lecithin–cholesterol acyltransferase, phospholipid transfer protein, platelet-activating factor acetylhydrolase, and hepatic lipase. Thus, one possible explanation for the discrepancies between the epidemiological, genetic, and clinical trial data is that the measurement of cholesterol mass within HDL fails to capture the complexity of this highly dynamic system. Although precipitation techniques are commonly used for rapid measurements of HDL-C mass for clinical purposes, the classic method for measurements and separation of lipoprotein subfractions, including HDL, is by density gradient ultracentrifugation.[25] HDL is classified according to the analytical method used to isolate it. Based on density gradient ultracentrifugation, HDL is classified into different subfractions known as HDL2 (large buoyant particles) and HDL3 (smaller dense particles).[26] HDL subfractions can further be assessed and classified based on size into HDL2a and 2b (HDL2b larger than HDL2a), and 3a, 3b, and 3c (smallest) by nondenaturing gel electrophoresis, or by charge and size with 2-dimensional (2D) gel electrophoresis into pre-β1 and pre-β2 (larger than pre-β1), pre-α1, pre-α2, pre-α3, and pre-α4 (larger to smaller) and α1 and α2 (α1 larger than α2) HDL.[26,27]

A key denominator for assessing HDL function may be the molar concentration of HDL particles in blood as it reflects the quantity of HDL particles independent of its composition

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(ie, a small cholesterol-poor HDL particle is equivalent to a large, cholesterol-rich particle). Indeed, clinical studies suggest that HDL particle concentration may provide information on CVD status independent of HDL-C.[28,29] Two principal methods have been described for quantifying HDL particles in human plasma, one based on nuclear magnetic resonance (NMR)[30,31] and the other based on ion mobility analysis,[32] classifying HDL into large, medium, and small subfractions. One potential limitation of the NMR method is that it is based on measurements of distinctive NMR signals arising from the HDL particle lipids with assumptions made about the physical properties of HDL lipids and HDL volume. The HDL particle concentration is derived from deconvolution of a complex overlapping NMR signal of all lipoprotein classes (including VLDL [very-low-density lipoprotein] and LDL) and has not yet been formally biochemically validated.[33] To quantify HDL by ion mobility analysis, HDL particles are separated on the basis of their differential mobility in a carrier gas and directly counted using laser scattering.[32,34] The ion mobility analysis method is further calibrated using both nanoparticles and recombinant HDL, yielding molar concentrations of HDL in line with estimated stoichiometry of apoA-I (apolipoprotein A-I) on HDL in contrast to NMR.[34] Size exclusion fast protein liquid chromatography or high-performance liquid chromatography systems can then be used separate lipoproteins according to particle size in solution. Furthermore, structure and composition analyses of HDL particles (proteomic/lipidomic methods and immunoaffinity chromatography), provide additional assessment of HDL particles.

Because of the use of different methods to classify, isolate, measure, and normalize HDL, it is difficult to compare studies and experimental results centered on analyzing HDL functions. Thus, to assess the issue of HDL function as a potential therapeutic target, standardized robust, simple, and universal analytical methods will be required in the future.

## HDL Subpopulations and Cholesterol Efflux Capacity

The cardioprotective nature of HDL has been primarily attributed to its role in promoting reverse cholesterol transport, the net movement of cholesterol from peripheral tissues to the liver for excretion through the bile. Cholesterol efflux from peripheral cells, such as macrophages in lesions of atherosclerosis, involves the cholesterol transporters ABCA1 (ATP-binding cassette transporter A1) and ABCG1 (ATP-binding cassette transporter G1), which are highly induced when macrophages accumulate excess cholesterol. Cholesterol export to lipid-poor apolipoproteins[35] and to small dense HDL[36] by ABCA1 is a key first step in cholesterol efflux from macrophages. ABCG1, which seems to act at least in part intracellularly by redistributing sterols away from the endoplasmic reticulum membrane,[37] then mediates the transport of cellular cholesterol to larger more mature lipidated HDL particles.[38,39] A recent study published in *ATVB* demonstrates that a similar transporter, ABCA8, can also mediate cholesterol efflux to apoA-I.[40] However, ABCA8 does not seem to mediate reverse cholesterol transport from macrophages. Instead, ABCA8 acts in the liver, perhaps by interacting with ABCA1[40] to stimulate HDL biogenesis. Here, the serine/threonine-protein kinase Pim-1 protects ABCA1 from lysosomal degradation, thereby maintaining plasma HDL-C levels.[41]

In 2 large studies,[42,43] an impairment in HDL's cholesterol efflux capacity was found to be a stronger predictor of prevalent and incident coronary artery disease than was HDL-C. HDL's cholesterol efflux capacity was also found to be decreased in subjects with type 2 diabetes mellitus.[43] Furthermore, in a separate 10-year follow-up study, HDL's cholesterol efflux capacity, quantified using a fluorescent substrate assay, was strongly and inversely associated with future cardiovascular events in a large population free of CVD at baseline, whereas HDL-C levels exhibited no such association.[44] Recently, Ogura et al[45] came to a similar conclusion by studying patients with familial hypercholesterolemia, who are at high risk for premature atherosclerotic CVD even after statin therapy. They found that cholesterol efflux capacity was independently and inversely associated with the presence of atherosclerotic CVD in heterozygous familial hypercholesterolemia even after adjusting for HDL-C levels.[45] In another recent study published in *ATVB*, Lin et al[46] demonstrated that endogenous cholesterol excretion, as a measure of reverse cholesterol transport, was negatively associated with carotid intima-media thickness in 86 nondiabetic adults, an association that remained significant after adjusting for HDL-C levels. Taken together, these observations suggest that the ability of serum HDL to remove cholesterol from peripheral tissues is a promising metric for assessing the cardioprotective effects of HDL, and that HDL's functionality may be more important than HDL-C levels in predicting CVD risk.

In contrast, in the JUPITER trial (Justification for the Use of Statins in Prevention: An Intervention Trial Evaluating Rosuvastatin), serum HDL's cholesterol efflux capacity was not associated with incident CVD in individuals at baseline.[47] Instead, HDL particle number, measured by NMR, was the strongest of 4 HDL-related biomarkers (cholesterol efflux capacity, HDL-C, apoA-I, and HDL particle number) as an inverse predictor of incident events and biomarker of residual risk. Therefore, the question remains whether serum HDL's cholesterol efflux capacity is a better clinically relevant measure of HDL functions than other biomarkers. Recently, Koekemoer et al[48] quantified serum HDL's cholesterol efflux capacity of 1988 individuals from the GRAPHIC (Genetic Regulation of Arterial Pressure of Humans in the Community) cohort comprising individuals from 2 generations. They found that cholesterol efflux capacity is a stable trait over time in an individual, but a trait that is only modestly heritable between generations.[48] This study also indicated that cholesterol efflux capacity can be determined by multiple specific clinical, serum, and HDL parameters beyond HDL-C, for example, age, systolic blood pressure, triglycerides, phospholipids, lipoprotein(a), HDL particle number, HDL particle size, and apolipoprotein A-II levels.[48] To date, it is unknown whether HDL's cholesterol efflux capacity is a universally better predictor than HDL-C for CVD events.[49] To understand this issue, we need to better define HDL and its metabolism.

The heterogeneous population of HDL particles may collectively contain >95 different proteins.[50,51] Each HDL subspecies can have distinct functions and may be metabolized through different pathways in humans. For example, Du et al[36]

used reconstructed HDL and endogenous human HDL isolated by ultracentrifugation to demonstrate that small dense HDLs (HDL3b and HDL3c), not medium and large HDLs (HDL2), are the most efficient lipidated mediators of cholesterol efflux by the ABCA1 pathway, similar to that of lipid-free apoA-I, whereas ABCG1 has a negligible role in mediating cholesterol efflux to HDL3b and HDL3c subfractions. Furthermore, a recent *ATVB* study showed that HDL subspecies may be metabolized within discrete and stable sizes in humans, suggesting that stepwise size expansion and contraction as major pathways for HDL metabolism may be less important than believed previously.[22] Four standard HDL size subfractions were each secreted into plasma and circulated mainly within the secreted size for 1 to 4 days before they were removed from circulation. Thus, subjects with high HDL-C (larger HDL particles) had an increased secretion of large HDL and faster clearance of very small HDL, and subjects with lower HDL-C had a faster clearance of large and very large HDL. In this context, Xu et al[52] showed that the cholesterol and phospholipids of HDL and the structural apoA-I protein are cleared by independent mechanisms that do not involve holo-particle uptake. Interesting questions relate to how different HDL therapies affect these HDL subspecies and whether they provide clues to understanding mechanisms of CVD.

Niacin and CETP inhibitors raise HDL-C levels through different mechanisms. Do they also have different effects on HDL's cholesterol efflux capacity? Niacin raises plasma HDL-C by decreasing the fractional clearance rate of HDL-apoA-I without affecting its synthetic rate.[53–55] Ronsein et al[56] recently analyzed the impact of niacin and statin therapies on HDL-related biomarkers. These researchers reported that niacin markedly increased HDL-C levels, as expected, but failed to improve ABCA1-specific cholesterol efflux.[56] Accordingly, HDL particle analysis revealed that niacin selectively raised levels of large HDL instead of boosting levels of small HDL particles, which are superior to large HDL in promoting ABCA1-specific cholesterol efflux. This study and others[57] provided a possible mechanistic explanation for why niacin failed to improve HDL's atheroprotective properties in humans.

CETP inhibitors increase HDL-C by preventing transfer of cholesteryl esters from HDL to other lipoprotein particles, but it is unclear how they affect HDL functions. Recently, Zhang et al[58] developed a CETP knockout rabbit by zinc finger nuclease-mediated gene targeting to clarify the pathophysiological functions of CETP in atherosclerosis. This study supported a beneficial role of CETP inhibition, because deletion of CETP protected against cholesterol diet–induced atherosclerosis concomitant with an increase in HDL-C and an increase in cholesterol efflux capacity, as compared with the wild-type controls.[58] However, like in the REVEAL trial, plasma levels of total cholesterol were reduced by CETP inhibition, making it difficult to conclude that improved HDL function was responsible for the anti-atherosclerotic effects. Reyes-Soffer et al[59] recently investigated the effects of anacetrapib on HDL subpopulations in human subjects in the presence or absence of statin therapy. Similar to effects of niacin, this group observed an increase in HDL-C and apoA-I levels because of a reduction in the fractional clearance rate of HDL apoA-I, and a concomitant shift in HDL subspecies from smaller to larger HDL particles.[59] However, unlike niacin therapy, the absolute mass of apoA-I in pre-β particles increased,[59] which supports the idea that inhibition of CETP results in improved cholesterol efflux capacity.[57,60]

The concept that diabetes mellitus might impair HDL's cholesterol efflux capacity was addressed by Apro et al.[61] They showed that HDL isolated by ultracentrifugation from plasma and interstitial fluid from suction blisters from subjects with diabetes mellitus exhibited reduced cholesterol efflux capacity when normalized to HDL-C content, as compared with HDL from subjects without diabetes mellitus, and that the defect in cholesterol efflux was more pronounced in interstitial fluid than in plasma. The results also demonstrated that HDL from subjects with diabetes mellitus on average was smaller (determined by gel filtration fast protein liquid chromatography) than HDL from controls, and that these subjects had reduced levels of apoA-I in both plasma and interstitial fluid. The measurements in interstitial fluid are important because cholesterol efflux occurs in peripheral tissues, such as lesions of atherosclerosis, rather than in circulation. A recent study by Mehta et al[62] supports the concept that reactive oxygen species, which are thought to be increased in diabetes mellitus, can impact HDL function. They demonstrated that hemopexin, a heme scavenger, results in reduced reactive oxygen species, and in reduced proinflammatory actions and increased cholesterol efflux capacity of HDL, concomitant with reduced atherosclerosis.

These studies demonstrate that HDL is a heterogeneous population of particles that can be divided into multiple subspecies, and that HDL measures other than HDL-C need to be considered. Each of the HDL subspecies may have distinct functions and may be metabolized partly through distinct pathways. Further studies are needed to elucidate the role of each HDL subspecies and possibilities to take advantage of this new knowledge for development of CVD therapeutics.

## Direct Endothelial Cell Effects of HDL in CVD and Diabetes Mellitus

In addition to promoting cholesterol efflux from macrophages, HDL can exert antiatherosclerotic effects in endothelial cells, such as provide direct stimulation of endothelial nitric oxide (NO) production (a vasoprotective molecule), anti-inflammatory effects, and antioxidant effects.[63] Several different mechanisms have been proposed to account for the endothelial NO-stimulating capacity of HDL. Early studies suggested that HDL acts by preventing the detrimental effects of oxidized LDL on endothelial NO-synthase (eNOS), whereas a subsequent study by Yuhanna et al[64] suggested that HDL can bind to endothelial SR-BI (scavenger receptor class B type 1) and thereby directly stimulate eNOS-mediated NO production through a process that involves eNOS stimulation in plasmalemmal caveolae. Mechanistically, binding of HDL to SR-BI initially leads to tyrosine kinase Src-mediated activation of phosphoinositide (PI) 3-kinase (PI3K), which in turn activates Akt and Erk pathways.[65] The activation of endothelial Akt has been shown to stimulate phosphorylation of eNOS at serine residue 1177, known to be an important

regulatory mechanism leading to eNOS activation.[65] HDL also can preserve endothelial function by improving ABCG1-mediated efflux of oxysterols, thereby preventing inhibitory interactions of eNOS with caveolin-1, and restoring eNOS activity in cholesterol-loaded endothelial cells.[66]

HDL must be transported through endothelial cell barriers to reach atherosclerotic lesion macrophages and other peripheral tissues. Velagapudi et al[67] recently used microscopy-based high-content screening of 141 kinase inhibiting drugs to demonstrate that VEGFR2 (vascular endothelial growth factor receptor 2) is required for transendothelial transport of HDL, but not LDL. Furthermore, VEGF-A was found to be required for the localization of SR-BI in the plasma membrane of endothelial cells. These results indicate that VEGF-A may play an important regulatory role in the vascular protective effects of HDL.[67]

Stimulation of eNOS-mediated NO production is also induced by binding of HDL-associated sphingosine-1-phosphate (S1P) to the lysophospholipid receptor $S1P_3$, which is expressed in endothelial cells and may partially mediate HDL- and lysophospholipid-induced vasodilation.[68] Studies have revealed that apoM (apolipoprotein M), a minor apolipoprotein on HDL, is a carrier and a modulator of S1P.[68] A recent report defines apoM-bound S1P as a key component of HDL, which is partially responsible for several HDL-associated protective functions in the endothelium, including anti-inflammatory effects, regulation of adhesion molecule expression, leukocyte–endothelial adhesion, and endothelial barrier function.[68] However, it is important to note that not all S1P in HDL is associated with apoM, as HDL from apoM-deficient mice still carries appreciable amounts of S1P and exerts similar biological activities as HDL from control mice.[69]

Notably, current evidence suggests that vascular effects of HDL can be highly heterogeneous and may be altered in patients with CVD and diabetes mellitus. Thus, when normalized based on HDL protein content, HDL preparations from subjects with diabetes mellitus failed to stimulate endothelial cell NO production and to promote endothelial repair when compared with HDL from subjects without diabetes mellitus.[70] Accordingly, subjects with type 2 diabetes mellitus were shown to have reduced levels of S1P in HDL,[71] and HDL from normoglycemic patients with metabolic syndrome, before onset of diabetes mellitus, exhibited a reduced ability to activate eNOS; a defect explained by S1P depletion of HDL.[72]

To understand the functional disparity of different HDL subfractions, Frej et al[73] performed an interesting study on HDL isolated from women with type 1 diabetes mellitus and matched control subjects. They showed that buoyant HDL is less anti-inflammatory in endothelial cells, as compared with dense HDL. They also demonstrated that type 1 diabetic subjects have similar levels of S1P and apoM in total HDL as compared with controls, but that the HDL-associated apoM/S1P complex shifts from dense to buoyant HDL particles in type 1 diabetes mellitus, where the complex is less able to suppress expression of the adhesion molecule VCAM-1 (vascular cell adhesion molecule 1).[73] These reduced anti-inflammatory effects of apoM/S1P-containing buoyant HDL were explained by differential activation of the $S1P_1$ receptor, leading to differential Akt and Erk activation depending on the density and size of the HDL particle.[73] Recently, it was shown that CETP can shift the distribution of S1P and apoM from HDL to apoB-containing lipoproteins, on which S1P might exert more potent bioactivities.[74] In this regard, Wu et al[75,76] showed that increasing HDL-C levels by inhibiting CETP activity with the anacetrapib analog des-fluoro-anacetrapib reduced intimal thickening and regenerated functional endothelium in New Zealand White rabbits after balloon injury in an SR-B1-dependent and PI3K/Akt-dependent manner. It will be interesting to determine the effects of CETP inhibitors on the S1P/apoM complex in different lipoproteins, and its functional repercussions.

Together, these studies suggest that the biological functions of HDL in the endothelium are heterogeneous, depend on signal transduction mediated by different receptors and alterations in cellular membranes, and might be altered in patients with CVD and diabetes mellitus. Recent and future mechanistic and clinical observations may have profound implications for the understanding of direct vasoprotective effects of HDL and how these relate to HDL's cholesterol efflux capacities.

## HDL Regulation by Epigenetic Mechanisms, microRNAs, and Transcription Factors

Several studies demonstrate that genetic variations that associate with altered HDL-C levels do not strongly associate with altered CVD risk.[77,78] However, alterations in epigenetic mechanisms, microRNAs, and transcription factors have recently been shown to impact HDL function. Thus, epigenetic DNA methylation at different loci associates with HDL-C levels, but its role in HDL functions was not clear.[79] Recently, Sayols-Baixeras et al designed an epigenome-wide association study on individuals from the REGICOR study (Registre Gironi del Cor) to investigate the association between DNA methylation, HDL's cholesterol efflux capacity, and HDL's inflammatory index.[80] They identified 5 methylation sites in *HOXA3*, *PEX5*, *PER3*, and *CMIP* (2 sites) associated with HDL's cholesterol efflux capacity and 1 methylation site in *GABRR1* associated with HDL inflammatory index.[80] Further study is needed to validate these associations and to uncover their functional significance in humans.

Studies over the past several years have identified microRNAs as important regulators of HDL-C metabolism.[81,82] MicroRNAs, small noncoding RNAs, control most of the steps of the reverse cholesterol transport pathway, including HDL biogenesis, cellular cholesterol efflux, and hepatic HDL-C uptake.[83] In particular, miR-33a/b and miR-27b have been found to act as regulatory hubs that repress genes involved in lipid metabolism.[84–86] MicroRNA-33a/b targets genes involved in cholesterol metabolism and insulin signaling, including ABCA1 and ABCG1, the endolysosomal transport protein NPC1 (Niemann-Pick C1) and IRS2 (insulin receptor substrate 2).[86,87] MicroRNA-27b regulates the expression of several key lipid metabolism genes, including ANGPTL3 (angiopoietin-like 3), which increases triglycerides and HDL-C.[84] Interestingly, HDL isolated from microRNA-33-deficient mice exhibits an increased ability to suppress expression of matrix metalloproteinase 9 in macrophages and monocyte chemotactic protein-1 in vascular smooth muscle cells, suggesting

that HDL function is altered by miR-33.[88] In a recent study by Ouimet et al[89], OSBPL6 (oxysterol-binding protein-like 6) was identified as a novel target gene in the miR-33/miR27b network that contributes to cholesterol homeostasis and HDL's cholesterol efflux capacity. Knockdown of OSBPL6 in mice resulted in aberrant clustering of endosomes and accumulation of free cholesterol in these structures, which resulted in reduced cholesterol esterification in the endoplasmic reticulum. In contrast, overexpression of OSBPL6 enhanced cholesterol trafficking and cholesterol efflux in macrophages and hepatocytes.[89] Hepatic expression of OSBPL6 was positively correlated with plasma levels of HDL-C in humans, although the correlation was relatively weak.[89]

Last, recent studies in mice demonstrate that the transcription factors CREB–H (cAMP-responsive element-binding protein H)[90] and iodothyronine deiodinase 1[91] can regulate apoA-I expression. In particular, Liu et al[91] found that hepatic insulin signaling plays a significant role in the regulation of *Apoa1* gene expression through the regulation of iodothyronine deiodinase 1. This study suggested a new pathway linking hepatic insulin signaling, iodothyronine deiodinase 1, and apoA-I, which may be relevant to the increased risk of CVD in subjects with metabolic syndrome or type 2 diabetes mellitus.

Thus, HDL and HDL functions are modulated by a large number of mechanisms. Future studies are needed to explore these mechanisms as possible treatment strategies.

## Summary

Recent articles published in *ATVB* and elsewhere have highlighted the heterogeneity of HDL and the effects of different HDL subpopulations in cells as they relate to CVD risk. A relatively new focus of research is to understand the links among HDL composition and HDL functions, including cholesterol efflux capacity, endothelial anti-inflammatory effects, and effects in other cells. The molecular mechanisms leading to the generation of HDL with optimal cardioprotective effects, and the possibility of the presence of dysfunctional HDL, need to be further studied and validated in large clinical studies in subjects with cardiovascular disease and diabetes mellitus.

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