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REVIEW

Dysfunctional HDL Diagnostic Metrics for Cardiovascular Disease Risk Stratification: Are we Ready to Implement in Clinics?

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Abstract

Epidemiological studies have revealed that patients with higher levels of high-density lipoprotein cholesterol (HDL-C) were more resistant to cardiovascular diseases (CVD), and yet targeting HDL for CVD prevention, risk assessment, and pharmacological management has not proven to be very effective. The mechanistic investigations have demonstrated that HDL exerts anti-atherogenic functions via mediating reverse cholesterol transport, antioxidant action, anti-inflammatory activity, and anti-thrombotic activity. Contrary to expectations, however, adverse cardiovascular events were reported in clinical trials of drugs that raised HDL levels. This has sparked a debate between HDL quantity and quality. Patients with atherosclerotic CVD are associated with dysfunctional HDL, and the degree of HDL dysfunction is correlated with the severity of the disease, independent of HDL-C levels. This growing body of evidence has underscored the need for integrating HDL functional assays in clinical practice for CVD risk management. Because HDL exerts diverse athero-protective functions, there is no single method for capturing HDL functionality. This review critically evaluates the various techniques currently being used for monitoring HDL functionality and discusses key structural changes in HDL indicative of dysfunctional HDL and the technical challenges that need to be addressed to enable the integration of HDL function-based metrics in clinical practice for CVD risk estimation and the development of newer therapies targeting HDL function.

Keywords Dysfunctional HDL · LDL oxidation · HDL Antioxidant Capacity · Paraoxonase-1 · Cardiovascular Risk

Introduction

Decades of epidemiological research have established that elevated plasma levels of low-density lipoprotein cholesterol (LDL-C) (> 100 mg/dl) significantly increase the risk of atherosclerotic cardiovascular disease (CVD). Mechanistic studies have uncovered the causal role of oxidized LDL particles in the initiation and progression of atherosclerosis. Collectively, this evidence has promoted the discovery and development of lipid-lowering therapies such as statin and non-statin drugs (for example, PCSK9 inhibitors, ezetimibe) for clinical management of high-risk patients (e.g., type 2 diabetes mellitus, metabolic syndrome) for CVD (myocardial infarction, atherosclerotic CVD) development [1]. The success of lipid-lowering drug trials for mitigating CVD risk was possible largely because of the establishment of LDL-C as a robust predictive biomarker of CVD risk and a reliable surrogate marker of prognosis for use in drug intervention trials. In contrast to the LDL-C story, although several seminal studies, including Framingham's heart study, have revealed that individuals with higher levels of plasma high-density lipoprotein cholesterol (HDL-C) are more protected from CVD [2], to date, there has not been much success in targeting HDL for the prevention, risk assessment, and pharmacological management of CVD. A meta-analysis involving four studies has reported that for every 1 mg increase in HDL-C levels, there is a 2–3% reduction in CVD risk [3]. Earlier studies have illustrated that the cardioprotective effect of HDL is primarily mediated by HDL-mediated reverse cholesterol transport (RCT), which refers to the process of acquiring cholesterol by HDL from extrahepatic tissues and then transporting it to the liver for further metabolism to bile acids and elimination through

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feces. Besides RCT function, HDL exerts powerful antioxidant, anti-inflammatory, and anti-thrombotic effects, which collectively contribute to cardio-protective functions.

Despite the well-established association between higher HDL-C levels and reduced CVD risk, interventions targeting HDL-C elevation (e.g., fibrates, niacin, and cholesterol ester transfer protein (CETP) inhibitors) have not consistently and significantly demonstrated cardiovascular benefits. Notably, a phase 3 randomized clinical trial (ILLUMINATE [4]) of torcetrapib (a CETP inhibitor) increased HDL-C levels by 70% and decreased LDL-C levels by 25% compared to placebo; however, patients in the torcetrapib group were associated with greater death and adverse cardiovascular events compared to those in the placebo group. Although the exact mechanisms behind these surprising effects are yet to be investigated, a recent study suggests that Torcetrapib may increase the levels of HDL subtype harboring apolipoprotein C3, which is known to increase the risk of coronary artery disease [4, 5]**.** Few studies have reported a positive relationship between very high HDL-C levels and CVD mortality [6]. Hence, not all plasma HDL particles are functionally similar; this argument is supported by many studies [7]. HDL isolated from patients during the acute inflammatory phase exhibited pro-inflammatory responses compared to HDL isolated from healthy controls [8]. HDL isolated from patients with type 2 diabetes mellitus exhibited poor endothelial vaso-protective function compared to HDL isolated from control non-diabetic subjects [9, 10]. Furthermore, a growing body of evidence has shown that HDL functions (e.g., RCT, antioxidant, and anti-inflammatory capacity) are better predictors of cardiovascular outcomes than HDL-C levels [10]. Collectively, evidence has emphasized the need to assess HDL functionality rather than HDL-C quantity for CVD risk assessment and patient selection for HDL-directed therapies [10, 11]. This review provides critical insights into commonly used methods for characterizing dysfunctional HDL; discusses structural alterations linked with dysfunctional HDL, and the technical challenges associated with specific HDL functional assays, which have restricted their utility and integration in routine clinical practice for CVD risk assessment and in the discovery of newer HDL-targeted therapies.

Assessment of HDL-Mediated Reverse Cholesterol Transport

Reverse Cholesterol Transport Process by HDL

Apolipoprotein A1 (ApoA1) is the predominant apolipoprotein, constituting almost 70% of protein mass in HDL, which plays a central role in the RCT process. After synthesis, ApoA1 undergoes early lipidation (acquisition of phospholipids and cholesterol) predominantly in the liver and small intestine to form nascent discoidal HDL particles [12]. After initial lipidation, the bulk of cholesterol content in HDL particles is acquired from peripheral tissues, such as skeletal muscles, adipose tissue, and skin, through interaction with the transmembrane lipid transporter, ABCA1[12]. Excess cholesterol accumulation in target peripheral cells increases the expression of ABCA1[12], which aids the export of cholesterol and phospholipids to ApoA1. Mature HDL particles also acquire cholesterol from macrophages through interactions with another transmembrane lipid transporter, ABCG1 [13]. The acquired free cholesterol is esterified by HDL-bound lecithin-cholesterol acyl transferase (LCAT), and the resulting cholesterol ester is propelled inside the hollow hydrophobic core to form mature HDL particles (Fig. 1).

In circulation, mature HDL particles constantly exchange lipid molecules (phospholipids, cholesterol, and/or triglycerides) from apolipoprotein B (apoB)-containing lipoproteins (chylomicron, VLDL, and LDL), which are facilitated by lipid transferring proteins, phospholipid transfer protein (PLTP), and cholesterol ester transport protein (CETP). PLTP remodels HDL particles into smaller and larger sizes by non-specific exchange of lipid molecules [14] with other lipoproteins, predominantly LDL. Overexpression of PLTP increased HDL-C levels [15], whereas loss of the PLTP gene reduced HDL-C levels [16] in mice. CETP facilitates the transfer of cholesterol esters from HDL to apoB100-containing lipoproteins (VLDL, LDL, and IDL) in the exchange of triglycerides [17], resulting in cholesterol depletion and triglyceride enrichment in HDL particles. Mature HDL-C particles are selectively internalized by hepatocytes via scavenger receptor class B type 1 (SR-B1) (Fig. 1). In mice, selective overexpression of SR-B1 in hepatocytes markedly enhances HDL particle uptake, depletes plasma HDL-C levels, and increases biliary cholesterol [18]. In contrast, SR-B1-deficient mice showed increased plasma HDL-C levels without any significant changes in circulatory ApoA1 levels [19].

Association of HDL Cholesterol Efflux Capacity and CVD risk

The capacity of HDL to efflux cholesterol from target cells, referred to as the HDL cholesterol efflux capacity (CEC), is the rate-limiting step that determines the extent of RCT performed by HDL. Hence, HDL-CEC is an indirect measure of HDL RCT function. Several studies have reported poor HDL-CEC in patients with cardiovascular disease, type 2 diabetes mellitus, metabolic syndrome, and autoimmune diseases. *Khera* et al. [20] reported a strong inverse relationship between HDL-CEC and the presence of atherosclerotic cardiovascular disease in a population-based

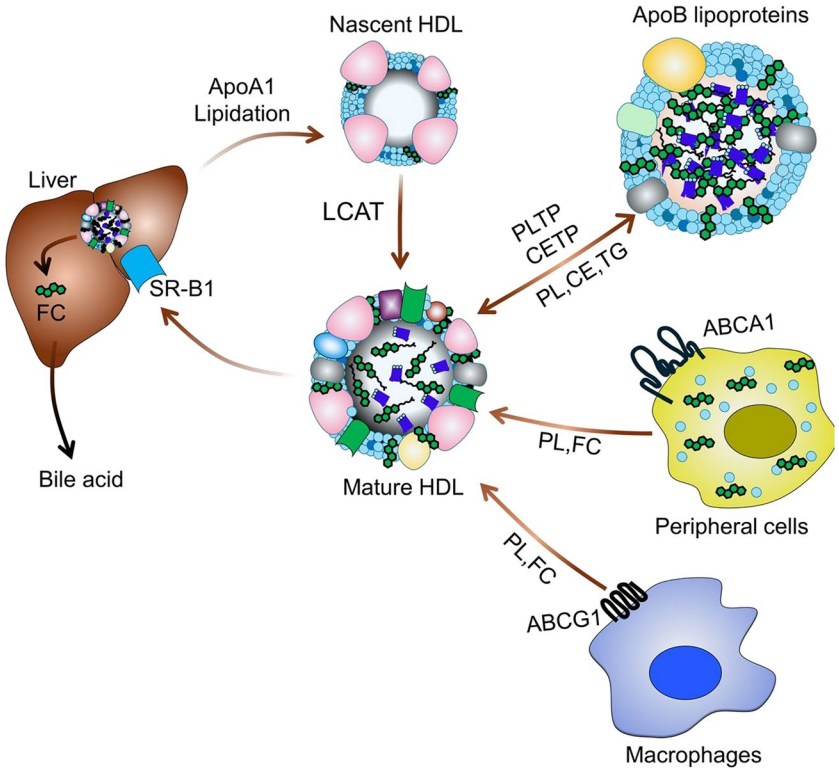

Fig. 1 Schematics illustrating HDL metabolism and transport. Lipid-free ApoA1 undergoes early lipidation predominantly in the liver to form nascent discoidal HDL particles, which acquire cholesterol from peripheral cells and macrophages via ABCA1 and ABCG1 transporters, respectively, and form the mature HDL. Circulatory mature HDL exchanges triglycerides for free cholesterol from apoB100 lipoprotein, which is catalyzed by phospholipid transfer protein (PLTP), and cholesterol ester transport protein (CETP). Through the SR-B1 receptor, mature HDL delivers cholesterol to the liver cells, where it is utilized to synthesize bile acids. (FC, free cholesterol; PL, phospholipid; CE, cholesterol ester; TG, triglycerides)

cohort. *Rohatgi* et al. [21] study, which measured HDL-CEC at baseline and followed them for an average of 9.4 yr for cardiac events, found that those with low HDL-CEC had a greater risk of developing atherosclerotic cardiovascular events. In contrast, the HDL cholesterol levels were less powered to predict cardiovascular events and showed a weak correlation with HDL-CEC [21]. Another prospective study (PREVEND, n = 8267) found an inverse association between baseline HDL-CEC and the incidence of CVD events, regardless of HDL-C or apoA-I levels [22]. Obesity, hypertension, hsCRP, and triglyceride levels all showed negative effects on HDL-CEC [22]. In a multiethnic population study, HDL-CEC was found to be negatively associated with metabolic syndrome [23]. HDL isolated from diabetes patients showed considerably lower CEC than HDL from non-diabetics [24, 25]. However, few studies have also found no significant differences in HDL-CEC between diabetics and non-diabetic subjects [26, 27]. *Emmens* et al. [28] evaluated HDL-CEC in heart failure patients at baseline and 9 months later and found that HDL-CEC declined significantly over time. The same study revealed that higher baseline HDL-CEC was related to a decreased risk of all-cause mortality among patients with heart failure. In the Young Finns study of healthy young individuals (24–39 yr old), baseline HDL-CEC was correlated with traditional CVD risk factors and subclinical atherosclerotic alterations after 6 years of follow-up [29]. The study reported that HDL-CEC increased with HDL cholesterol increase in the cohort. Individuals in the top quartile for total cholesterol, HDL cholesterol, triglycerides, and ApoA1 displayed significantly higher HDL-CEC than those in the lowest quartile. Furthermore, the study found an inverse association between baseline HDL-CEC and several subclinical parameters of atherosclerosis assessed after 6 years, implying that patients with high HDL-CEC were better protected from developing atherosclerosis [29]. A modest but significant increase in HDL-CEC was observed from baseline following a year of rosuvastatin treatment in the nested-case control study of the JUPITER primary prevention trial, which compared rosuvastatin to placebo. Moreover, in the rosuvastatin arm, HDL-CEC positively correlated with HDL cholesterol, HDL particle number, and ApoA1 [30]. A 5-year PREDIMED trial of high-risk CVD individuals (n = 7447) found that a Mediterranean diet supplemented with extra virgin olive oil or mixed nuts significantly reduced cardiovascular events compared to a low-fat diet. HDL-CEC was evaluated at baseline and one year after the intervention in a randomly selected subset of subjects. In contrast to the low-fat diet group, participants in the Mediterranean diet group showed a significant increase in HDL-CEC from baseline [31]. Collectively, the accumulated evidence suggests that monitoring CEC of HDL is a useful parameter for stratifying and managing patients at high CVD risk.

Mechanisms Contributing to Impaired HDL-CEC

Oxidative modification (Fig. 2) of ApoA1 is the main underlying mechanism contributing to defective HDL-CEC. Myeloperoxidase (MPO), secreted by phagocytes, is the major source of oxidants in the plasma and subendothelial space that modifies ApoA1 through chlorination and nitration of tyrosine residues and oxidation of amino acid residues such as methionine, tyrosine, or tryptophan [32, 33]. Oxidative modification of Tyr-166 and Met-148 amino acid residues in ApoA1 affects the LCAT binding site and results in the loss of cholesterol acceptor function [34]. Besides MPO, malondialdehyde, a lipid peroxidation product, also oxidatively modifies ApoA1 and impairs ApoA1's ability to stimulate ABCA1 to induce cholesterol efflux from peripheral cells [35]. ApoA1 glycation also impairs CEC function [36]. An inverse association between the glycation of ApoA1 at the lys133 residue and ABCA1-mediated CEC was observed in T2DM patients [37].

Methods for Monitoring HDL-CEC

Most studies have used a monocyte (THP-1) or macrophage cell line (J774.1 or RAW 264.7) to perform CEC assays [38]. Macrophages were cultured in 10% serum-containing media with radiolabeled cholesterol for 24 h-48 h in the presence of a cell-permeable cAMP analog, 4-chlorophenylthio-cyclic adenosine monophosphate. After labeling, the cells were washed, equilibrated with serum-free media, and subsequently incubated with or without cholesterol efflux acceptors (ApoA1, purified HDL, and apoB-depleted serum or plasma) in the presence of an acyl-coenzyme A cholesterol acyltransferase inhibitor for an additional 2 h-6 h. CEC is expressed as radioactive cholesterol levels in cell-free media relative to the total radioactive cholesterol in the cells and media. Although the radioactive CEC assay is widely used, a newer fluorescently labeled cholesterol-based CEC assay has been developed that offers advantages such as better safety and easy automation for high-throughput testing [38, 39].

Few researchers have developed cell-free assays that provide indirect measurements of HDL-CEC function. During the HDL-elicited cholesterol efflux process, reversible spontaneous on- and off-movement of lipid-poor ApoA1 from HDL particles occurs, which is a rate-limiting step during the cholesterol efflux process [40]. Based on this principle, *Borja* et al. developed a method to monitor the extent of exchange of exogenously added, spin-labeled ApoA1 with plasma HDL-associated ApoA1 using electron paramagnetic resonance spectroscopy as an indirect measure of HDL-CEC [41]. The researchers reported that HDL-ApoA1 exchange was markedly impaired in patients with atherosclerotic CVD compared to that in control subjects [41]. Along with cholesterol, ApoA1 also facilitates the efflux of phospholipids during the HDL-elicited cholesterol efflux process. This is the principle behind the development of a cell-free HDL-specific phospholipid efflux assay by *Sato* et al. [42]. This

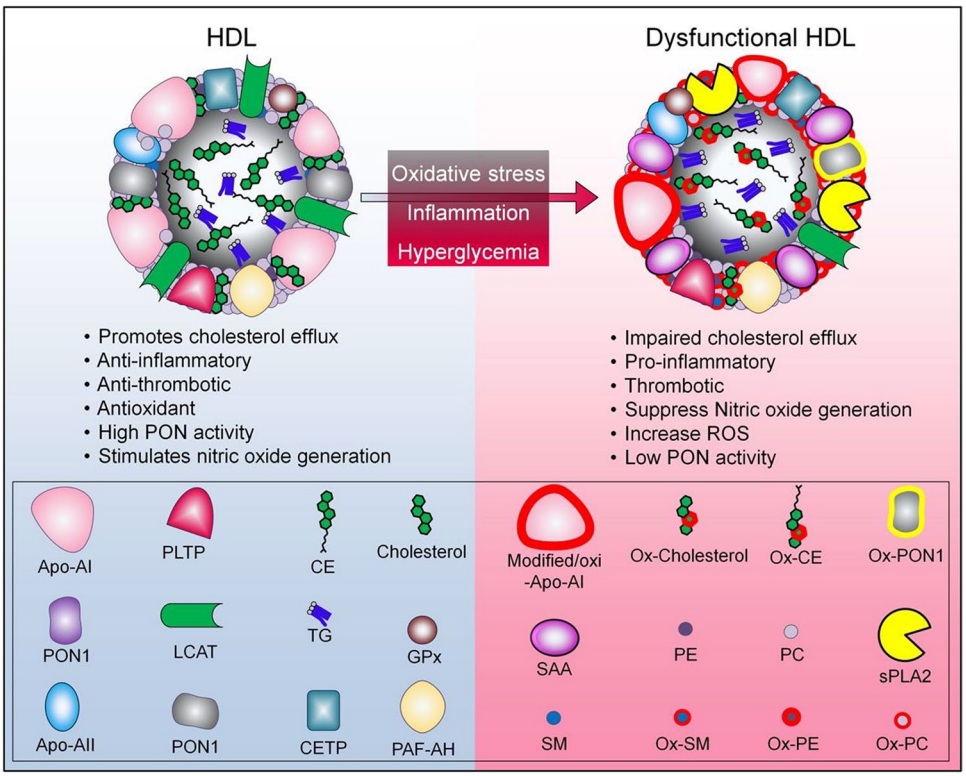

Fig. 2 Schematics illustrating dysfunctional HDL with structural alterations. The changes in HDL structure and function induced by chronic oxidative stress, inflammation, and hyperglycemia are depicted. CE, cholesterol ester; TG, triglyceride; PE, phosphatidylethanolamine; Ox-PE, oxidized phosphatidylethanolamine; SM, sphingomyelin; Ox-SM, oxidized sphingomyelin; Ox-PC, oxidized phosphatidylcholine

method monitors the extent of exchange of exogenously added fluorescently labeled phosphatidylethanolamine with plasma HDL as a surrogate measure of CEC. The authors found that HDL phospholipid efflux capacity was significantly reduced in patients with CVD compared to control subjects, and the performance of the assay was comparable to cell-based traditional HDL-CEC assay.

Challenges and Potential Solutions

There are several limitations to CEC assay, which hamper its clinical utility in its current form. There is no consensus on the macrophage cell line, precipitation method for preparation of HDL-enriched fraction, treatment time with cholesterol efflux acceptor (HDL-enriched fraction), and data interpretation and representation. Furthermore, the assay is laborious, time-consuming (takes 48 h to 72 h), involves multiple steps, and requires a dedicated cell culture facility and expertise in cell culture. In contrast, the newly developed cell-free assays are easy to perform and can be easily automated; however, they need more systematic validation in clinical settings and a standardized approach for data analysis and interpretation.

Assessment of HDL-mediated Antioxidant Activity

Antioxidant Functions of HDL

HDL prevents the accumulation of Ox-LDL particles in plasma and subendothelial spaces by removing the oxidized lipid species on the LDL surface through two main processes. First, oxidized phospholipids generated in LDL are transferred to HDL, which may be facilitated by ApoA1, CETP, and/or PLTP. The oxidized lipid molecules acquired by HDL particles may then be delivered to hepatocytes via SR-B1 for degradation. This process represents a safe passage for the removal of reactive lipid peroxidation byproducts generated on lipoproteins. Second, the oxidized lipid molecules acquired by HDL undergo reduction by HDL-associated antioxidant proteins. Paroxonase 1 (PON1), glutathione-peroxidase, LCAT, and PAF-acetylhydrolase are the antioxidant enzymes anchored to the HDL surface that are involved in the degradation of oxidized lipid molecules acquired by HDL [43]. Although PON1 is a minor component, accumulated evidence suggests that HDL antioxidant activity is largely influenced by PON1 activity. PON1 catalyzes the hydrolysis of a wide range of substrates, including lipid peroxidation byproducts such as 5-hydroxy-eicosatetraenoic acid 1,5-lactone, and 4-hydroxy-docosahexaenoic acid [44]. In the presence of recombinant PON1, the oxidation of HDL by copper is markedly delayed, and the content of lipid peroxides on HDL is significantly reduced, which was reversed in the presence of PON1 chemical inhibitor [45]. PON1-enriched HDL showed enhanced resistance to copper-induced oxidation compared to HDL free of PON1 [45]. PON1 also shows the ability to directly decompose hydrogen peroxide, lipid hydroperoxides, and oxidized phospholipids [45]. HDL particles isolated from PON1-deficient mice were more prone to oxidation and showed less capability to protect LDL from oxidation in vitro model systems [46]. Furthermore, PON-deficient mice were more susceptible to atherosclerosis compared to wild-type littermates when fed a high-fat and high-cholesterol diet [46]. Conversely, HDL from PON1 overexpressing mice showed enhanced resistance to oxidation compared to HDL from wild-type mice [47]. LCAT functions as chain chain-breaking antioxidant by catalyzing the hydrolysis of the oxidized acyl chain of oxidized phospholipids. Glutathione peroxidase anchored to HDL helps in the degradation of lipid hydroperoxides on the surface of HDL and LDL. PAF-AH, associated with HDL, exerts antioxidant activity by hydrolyzing the oxidized fragments of phospholipids present on the surface of HDL [43]. HDL also protected LDL from glycation, which was primarily mediated by the inhibition of LDL oxidation [48]. Besides repressing the oxidation of LDL, HDL3 also improves intracellular antioxidant defenses. The apoptotic effects of Ox-LDL were reversed by HDL3 through inhibition of TRAF3IP2 expression in endothelial cells [49]. Furthermore, HDL and ApoA1 were able to reduce the oxidative stress induced in macrophages by inhibiting NOX2 protein expression and increasing superoxide dismutase (SOD) expression [49].

Association of Impaired HDL Antioxidant Function and CVD Risk

Dysfunctional HDL particles not only show poor capacity to inhibit LDL oxidation but also exert pro-inflammatory effects. Therefore, the assays used to measure HDL antioxidant activity also estimate the 'HDL inflammatory index' (HII). The antioxidative capacity of HDL to inhibit LDL oxidation was significantly reduced in patients with acute coronary artery disease as compared to patients with stable coronary artery disease [50]. In the same study, the HDL antioxidant capacity positively correlated with HDL-C levels and negatively correlated with body mass index and triglycerides. In another study of patients with premature myocardial infarction (MI) (< 40 yr), compared to age- and gender-match controls, the HDL antioxidant activity during the acute phase of MI was significantly lower, which persisted even after one year during the stable phase [51]. While in the control group, the HDL antioxidant function positively correlated with all the sizes of HDL particles, in the patient with acute MI, the HDL antioxidant function

was associated positively only with small and very small HDL particles [51]. Furthermore, the association between HDL antioxidant function and HDL cholesterol was modest in the acute MI patient group but strong in the control group. In general, the HDL antioxidant function was significantly lower in subjects with high BMI, hypertension, diabetics, and current smokers [51]. In diabetic subjects, the antioxidant activity of HDL3a and HDL3b was significantly compromised as compared to non-diabetic control subjects and showed a negative correlation with plasma 8-isoprostane levels [52]. Similarly, HDL from human subjects with metabolic syndrome showed significantly decreased antioxidative capacity to protect LDL from oxidation compared to control subjects [53]. In a multicenter case–control study, the HDL antioxidant capacity correlated positively with HDL-C levels and was considerably lower in patients with acute myocardial infarction than in controls. In a case–control study, HDL antioxidant activity was found to be significantly lower in patients with ischemic cardiomyopathy than in control participants [54]. In the same study, HDL antioxidant function correlated positively with HDL cholesterol and negatively with BMI and triglycerides in patients with cardiomyopathy. In a nested case–control study of the JUPITER trial, rosuvastatin treatment for one year significantly increased the HDL antioxidant function compared to placebo [50].

Mechanisms Mitigating Antioxidant Capacity of HDL

Oxidation and glycation of ApoA1 diminish its antioxidant activity and transform it into a proinflammatory molecule [32, 55]. PON1 anchored to HDL is also susceptible to glycation and the glycated PON1 showed poor ability to reduce lipid hydroperoxides [56]. The antioxidant activity of HDL is markedly influenced by its lipidome and proteome. The higher surface rigidity of HDL negatively impacts its ability to transfer hydroperoxides from LDL to HDL [57]. The surface rigidity is determined by the sphingomyelin: phosphocholine ratio, and free cholesterol content. HDL3, which is deficient in sphingomyelin, shows greater surface phospholipid monolayer fluidity and superior efficiency in mediating the transfer of hydroperoxides from LDL to HDL3 compared to HDL2 [58]. An increase in cholesterol and triglyceride content increases HDL surface rigidity and, concurrently, reduces its antioxidant properties, whereas an increase in sphingosine-1-phosphate and phosphatidylserine is found to increase the capacity of HDL to inhibit LDL oxidation [43]. Alterations in lipid composition may also result in ApoA1 destabilization and its removal from HDL during lipolysis. During acute and chronic inflammation, the plasma levels of acute phase proteins, SAA and CRP, which preferentially bind to HDL, are markedly elevated. These acute phase proteins are found to displace ApoA1 and HDL-associated antioxidant enzymes, PON1 and PAF-acetylhydrolase from HDL particles [59], a process that markedly impairs the antioxidant capacity of HDL.

Methods to Assess HDL Antioxidant Activity

Inhibition of Oxidized LDL (Ox-LDL)

Most studies have assessed HDL function by two approaches: HDL capacity to inhibit LDL oxidation, and HDL capacity to reduce oxidized lipid content in Ox-LDL. Although both cell-free and cell-based assays have been used to monitor HDL antioxidant activity, because of their ease of performance, cell-free assays are widely used. To measure the ability of HDL to inhibit LDL oxidation, the LDL is exposed to a free radical generator (CuSO4 or AAPH) without or with purified HDL or apoB-depleted serum in the presence of a redox-sensitive dye, 2′,7′-dichlorodihydrofluorescein diacetate (DCF) that becomes fluorescent upon oxidation. To assess the HDL capacity to reduce oxidized lipid content in LDL, Ox-LDL is incubated with or without purified HDL or apoB-depleted serum for a specific period, and the content of oxidized lipids is assessed by change in DCF fluorescence at the end of incubation. The HDL antioxidant activity (also referred to as the serum HDL oxidative index or HDL inflammatory index) is calculated by the ratio or subtraction between the DCF florescence in the presence of HDL and the DCF florescence without HDL. The ratio < 1 indicates the pro-oxidant activity and the ratio of > 1 indicates the antioxidant activity of HDL. The DCF assay may also be employed for evaluating oxidized lipid content in HDL by incubating the DCF with isolated HDL [60]. Few studies have assessed the oxidized lipid content in LDL by measuring conjugated dienes [60, 61].

Challenges and Potential Solutions

Although the DCF assay is easy to perform and cost-effective in assessing HDL antioxidant activity in clinical samples, non-specific oxidation of DCF by the free radical generator itself and other oxidants such as metals (Fe and Cu), oxidized fatty acids, and ceruloplasmin [62] present in the apoB-depleted serum or plasma is a major limitation of the assay. The use of redox-sensitive probes that specifically react with reactive byproducts of lipid peroxidation and not with other prooxidants present in the sample could improve the specificity of the assay. Purified HDL by ultracentrifugation method is the most suitable test sample for assessing intrinsic HDL antioxidant activity. However, isolation of HDL by ultracentrifugation in a clinical laboratory is not feasible as it is laborious and lengthy (takes ~ 48 h). In contrast,

apoB-depleted serum, which represents the HDL fraction, is the most suitable clinical sample for analyzing the HDL antioxidant oxidant capacity in clinical settings. However, HDL fraction is also composed of other plasma antioxidant components, including antioxidant enzymes, glutathione, vitamin E, vitamin C, and albumin, which may yield falsely positive results. Nevertheless, the apoB-depleted serum provides a close approximation of the HDL antioxidant activity of an individual. Because HDL cholesterol does not necessarily correlate with HDL particle numbers, normalizing antioxidant activity estimates in the cell-free assays with the number of HDL particles in the sample may provide a more accurate estimate of the antioxidant capacity of HDL of an individual.

Assessment of HDL-mediated Anti-inflammatory Function

Anti-inflammatory Effects of HDL

HDL exerts anti-inflammatory effects by direct and indirect mechanisms. The indirect mechanism primarily involves reducing the pro-atherogenic Ox-LDL levels in plasma and subendothelial space. Accumulation of Ox-LDL in the subendothelial space activates the endothelium to express adhesion molecules (e.g., vascular cell adhesion molecule (VCAM-1), intercellular adhesion molecule-1), cytokines, and chemokines, which enable the migration of circulatory monocytes into the local subendothelial spaces [63]. Subsequently, the accumulated Ox-LDL is phagocytized by macrophages via the surface receptor CD36 resulting in the formation of pro-inflammatory and pro-atherogenic foamy macrophages. Prevention of monocyte influx into subendothelial space by inhibiting chemokine secretion or blocking chemokine receptors retards the development of atherosclerosis in mouse models [64]. The HDL-associated PON1 and PAF-AH inhibit the influx of monocytes into subendothelial spaces by reducing the deposition of Ox-LDL. In an in vitro system, recombinant PON1 inhibited monocyte adherence to endothelial cells induced by oxidized phospholipids, largely by degrading the oxidized lipid moiety [65]. In line with in vitro evidence, PON1-deficient mice were associated with a two-fold increase in leukocyte adherence to vascular walls compared to wild-type mice [66] indicating the crucial role of HDL-associated PON1 in mitigating vascular inflammation. HDL may directly suppress pro-inflammatory responses in the vascular wall by multiple mechanisms. HDL stimulates nitric oxide (NO) production in the endothelium by activating endothelial nitric oxidase synthase (eNOS) through various endothelial cell receptors, SR-BI, ABCA1, and ABCG1 [67]. HDL also increases eNOS protein abundance in endothelial cells by increasing its stability [68]. Endothelial NO exerts anti-inflammatory effects by dampening NF-κB signaling [69]. Furthermore, HDL attenuates monocyte activation by reducing CD11b expression [70] and inhibits inflammatory M1 macrophage polarization through redistribution of caveolin acting via ERK1/2 and STAT3 signaling pathways [71]. HDL is also found to repress Toll-like receptor 4-induced cytokine expression in macrophages through the induction of the transcriptional repressor ATF3 [72]. HDL sequesters and neutralizes bacterial lipopolysaccharides in plasma with the help of anchored lipid-binding proteins. HDL-associated sphingosine-1-phosphate promotes the proliferation of endothelial progenitor cells by activating the STAT3 pathway [73].

Association of Impaired HDL Anti-inflammatory Capacity and CVD Risk

Dullaart et al. [74] investigated the anti-inflammatory capacity of HDL isolated from individuals with acute MI versus those with non-cardiac chest pain. Patients with acute MI had significantly lower HDL anti-inflammatory capacity compared to patients with non-cardiac chest pain. At baseline, both groups had comparable levels of HDL cholesterol, ApoA1, total cholesterol, and LDL cholesterol. After a median follow-up of 1210 days, the risk of new CVD events was substantially higher in patients with poor HDL anti-inflammatory capacity than patients with stronger HDL anti-inflammatory capacity at baseline [74]. In the PREVEND study (Prevention of Renal and Vascular End Stage Disease), a nested case–control study of patients revealed that incident CVD mortality had an inverse relationship with the anti-inflammatory capacity of HDL, and this relationship remained even after normalizing with HDL cholesterol [75]. High HDL cholesterol levels, however, were not associated with reduced CVD deaths. Furthermore, high triglycerides and high BMI had an adverse effect on HDL's anti-inflammatory capacity. Also, there was no relationship between anti-inflammatory capacity and CEC of HDL in the cohort [75]. *Ebtehaj* et al. [76] investigated the anti-inflammatory capacity of HDL isolated from diabetic and non-diabetic individuals. The researchers found that diabetics had much lower HDL anti-inflammatory capacity than non-diabetics, which was linked to hyperglycemia, reduced PON1 activity, and low-grade chronic inflammation [76]. Likewise, *Morgantini* et al. reported significantly reduced HDL anti-inflammatory capacity in diabetics compared to non-diabetic individuals [60]. *Holzwirth* et al. reported that HDL anti-inflammatory capacity is significantly impaired in patients with arterial fibrillation compared to the non-arterial fibrillation cohort [76]. However, the HDL anti-inflammatory capacity improved in patients at follow-up after catheter ablation [76]. Stroke

patients with unfavorable outcomes at 3 months were associated with poor anti-inflammatory and antioxidant capacity of HDL compared to patients who showed favorable outcomes. However, the lipid profile (HDL-cholesterol, total cholesterol, LDL/VLDL, and triglycerides) was comparable between the patients' group at 3 months after ischemic stroke [77]. *Patel* et al. demonstrated that infusion of recombinant HDL (rHDL) in patients with diabetes markedly raised HDL levels compared to baseline levels [78]. More importantly, the plasma HDL fraction isolated from diabetes patients after infusion showed a significant enhancement in anti-inflammatory capacity and CEC function compared to placebo (pre-infusion) [78].

Methods for Evaluating HDL Anti-inflammatory Capacity

The anti-inflammatory capacity of HDL in human cohort studies has been evaluated by three main methods: monocyte chemotaxis assay, monocyte adhesion assay, and expression of adhesion molecules in endothelial cells. In the monocyte chemotaxis assay, the primary aortic endothelial cell cultures are maintained in lipoprotein-deficient serum media and then exposed to purified Ox-LDL in the presence or absence of a test HDL sample for a period of up to 24 h. Subsequently, the cell-free culture medium is collected and added to the lower compartment of the Boyden chamber plate. The freshly derived human blood monocytes (or monocyte cell lines (THP-1)) suspended in serum-free media are placed in the upper compartment of the plates. After incubation, the chamber is disassembled, and the number of monocytes migrated to the lower compartment is enumerated microscopically or by measuring cellular content such as ATP levels. HDL anti-inflammatory activity is calculated as a ratio of the value obtained in the absence of HDL with the value obtained in the presence of HDL. A value < 1.0 indicates HDL anti-inflammatory activity; values > 1.0 indicate the pro-inflammatory function of HDL.

In monocyte adhesion assays, the endothelial cell cultures are incubated with Ox-LDL in the absence or presence of a test HDL sample. After incubation, the cells are washed to remove the lipoproteins (LDL and HDL) and then incubated with fluorescently labeled peripheral monocytes for 30–60 min. Subsequently, the monocyte suspension is removed, and the endothelial cells are vigorously washed to remove non-adherent monocytes. The adherent monocytes are enumerated microscopically. The reduction in the number of adherent monocytes to endothelial cells indicates HDL anti-inflammatory activity [79].

The anti-inflammatory capacity of HDL is also measured by its ability to inhibit the expression of VCAM1 in endothelial cells [75]. The endothelial cell cultures are pre-incubated in the absence or presence of a test HDL sample (purified HDL or apoB-depleted serum) and then stimulated with TNFα. The mRNA expression of VCAM1 in endothelial cells is measured by Real-time polymerase chain reaction. The reduction in TNFα-induced VCAM1 expression in endothelial cells in the presence of HDL compared to the absence of HDL indicates the anti-inflammatory activity of HDL.

Challenges and Potential Solutions

The major challenges in integrating these inflammatory assays in clinical practice are: a requirement of primary cultures of aortic endothelial cells, and fresh blood-derived monocytes; batch-to-batch variations associated with primary cell cultures; intense labor and high cost of primary cell cultures maintenance; day-to-day variations in readout and data normalization**.** The use of immortalized cells as an alternative for primary cultures of endothelial cells and/or monocytes could improve the performance of the assay and reduce the cost of maintenance as well as day-to-day and batch-to-batch variations.

Analysis of Structural Components to Evaluate HDL Functionality

Paraoxonase 1 (PON1) Activity

Because circulatory PON1 is predominantly associated with HDL particles and contributes significantly to HDL antioxidant capacity, plasma PON1 activity is considered a surrogate marker of HDL antioxidant function [45, 80]. The PON1 enzyme primarily acts as a lactonase and an arylesterase, hydrolyzing various substrates, including lipid hydroperoxides [81]. Several case–control studies have found that circulatory PON1 activity is significantly reduced in patients with MI, atherosclerotic CVD, diabetes, and dyslipidemia compared to controls [81]. Furthermore, a few prospective studies have found that low blood PON1 activity predicts the risk of CVD in high-risk populations [82, 83], which was dependent on HDL cholesterol levels. Genetic polymorphism in *PON1* alters its enzymatic activity and is found to be an independent determinant of CVD risk [84]. The rate of hydrolysis of paraoxon to p-nitrophenol is monitored to evaluate PON lactonase activity [45]. Plasma PON arylesterase activity is assessed by monitoring the hydrolysis of phenyl acetate [45]. More recently, a nanoparticle-gated electrokinetic membrane sensor platform has been developed for the quantification of HDL-associated PON1, which does not require pre-sampling processing and has been demonstrated to be more sensitive compared to enzymatic activity in distinguishing healthy subjects from patients with coronary artery diseases [85].

Measurement of Native ApoA1, Modified ApoA1 or ApoB/ApoA Levels

Several studies have analyzed plasma levels of native ApoA1 as a measure of HDL levels and found it to be a better marker in CVD risk stratification than HDL-C levels. The ratio of ApoB to ApoA1 was more sensitive than ApoA1 levels in CVD risk prediction [86–88]. Since the oxidative modification of ApoA1 disrupts HDL function and makes it more immunogenic, some studies have monitored the levels of modified ApoA1 [89, 90] by immunoassay and the circulatory antibodies against modified ApoA1 as an indicator of dysfunctional HDL [91]. The plasma levels of native or modified ApoA1 were measured by standard enzyme-linked immunosorbent assay (ELISA) [86]or turbidometric methods [91].

Challenges and Potential Solutions

PON1 activity reflects HDL function, however, there is inadequate human data to suggest what levels of PON1 activity constitute normal HDL function. More prospective longitudinal studies are required to ascertain normal versus abnormal values of PON1 activity and whether the lactonase or arylesterase activity of HDL is a better predictor of the anti-atherogenic function of HDL. Furthermore, as the PON1 enzyme anchored to HDL particles is predominantly responsible for HDL antioxidant capacity, combining serum PON1 enzyme activity with HDL antioxidant capacity determined by cell-free assay may provide a comprehensive estimate of an individual's HDL antioxidant function.

Although plasma levels of native, modified ApoA1, and the ApoB/ApoA1 ratio can be easily measured in clinics by immunoassay methods, more prospective longitudinal studies are needed to validate and establish the clinical superiority of these markers over traditional HDL-C and LDL-C levels in CVD risk assessment before they can be used in clinical practice.

Analysis of Structural Features to Evaluate HDL Functionality

NMR-based Lipidomic Analysis of HDL

The lipid content in HDL particles directly impacts their integrity, stability, and function, and therefore, monitoring the lipid composition of HDL particles reflects HDL functionality. For example, HDL particles enriched with saturated fatty acids are pro-inflammatory; while HDL particles with a greater content of unsaturated fatty acids are anti-inflammatory [92]. HDL particles with an increase in triglycerides and a decrease in cholesterol ester content are pro-inflammatory. A nuclear magnetic resonance (NMR)-based analytical tool is being employed for analyzing the lipid composition of HDL particles in clinical samples. *Kostara* et al. [93] performed lipidomic analysis of HDL isolated from human subjects with low, normal, and high HDL-C by NMR. Total lipids were extracted from HDL particles present in apoB-depleted serum by the standard methanol/chloroform extraction method, dried and redissolved in the same solvent, and then analyzed by NMR. The study revealed that the HDL lipidome in individuals with normal HDL-C levels was significantly different from individuals with high or low HDL-C. The HDL particles from individuals with low HDL-C were composed of high triglycerides and low cholesterol ester content compared to those with normal HDL-C levels. Conversely, HDL particles from subjects with high HDL-C were associated with low triglycerides and a higher percentage of esterified lipids (cholesterol and phospholipid) with unsaturated fatty acids than saturated fatty acids. Further, the surface content of PC, free cholesterol, and sphingomyelin increased with the increase in HDL-C levels. Accumulated evidence from a small number of studies suggests that NMR-based lipidomic analysis of HDL particles is highly promising in characterizing the quality of HDL and could help in assessing the impact of nutritional and pharmacological interventions on HDL quality as well as estimating the extent of dysfunctional HDL particles in plasma.

Analysis of Subpopulation of HDL Particles

Given a growing body of evidence that suggests that not all HDL particles exert athero-protective functions, there is a growing interest in characterizing a subpopulation of HDL particles for CVD risk assessment. HDL particles vary in density, size, and charge based on ApoA1 molecules and lipid content. Various studies have characterized the subpopulation of HDL particles by different analytical techniques such as density gradient ultracentrifugation, non-denaturing polyacrylamide gel electrophoresis, 2-dimensional (2D) gel electrophoresis, and NMR spectroscopy. Native polyacrylamide gel electrophoresis separates HDL particles into six different sizes: HDL2a (α1), HDL2b (α2), HDL3a (α3), HDL3b (α4), and HDL3c (α5), pre-β1 HDL [94]. In a more advanced 2-D gel electrophoresis technique, the HDL particles are first separated in one dimension based on charge (alpha, pre-β, and pre-alpha) on an agarose gel, followed by separation in the second dimension (based on mass) on a non-denaturing gradient polyacrylamide gel. Subsequently, the 2D-gel is transferred to a nitrocellulose membrane, immunoblotted using an anti-ApoA1 antibody to detect HDL, and lastly, imaged to quantify various subpopulations of HDL particles by densitometric analysis. The 2D-gel electrophoresis

revealed 12 HDL subspecies (pre-β1a, pre-β1b, pre-β2a, pre-β2b, pre-β2c, α1, α2, α3, pre-α1, pre-α2, pre-α3, and pre-α4). *Asztalos* et al. reported that patients with coronary heart disease were associated with higher α3 and lower α1 HDL levels compared to control subjects [95]. Patients with acute coronary syndrome showed higher levels of pre-β1 HDL, HDL3b, and HDL3a and lower levels of HDL2a and HDL2b compared to control subjects [96].

More recently, NMR spectroscopy has emerged as an effective tool to measure the total HDL particle (HDL-P) concentration/count, size, and percentage of each subfraction of HDL-P, HDL-C, and ratio of HDL-C to HDL-P [97]. The size and concentration of HDL-P are calculated from the amplitudes of NMR signals obtained from the terminal methyl lipid signals, which are measured spectroscopically. Based on NMR signals, the HDL subpopulation has been classified into 5 subclasses: very large, 10.3–13.5 nm; large, 8.6–10.2 nm; medium, 8.3–8.5 nm; small, 8.0–8.2 nm; and very small, 7.4–7.9 nm HDL particles. However, the cutoff values for the classification of HDL-P size have not been consistent among the studies. The method has been well validated by various clinical studies, and consistently most studies have reported an inverse relationship between small HDL-P and cardiovascular outcomes [97–99]. The major advantage of NMR analysis is that it is rapid, and the serum or plasma samples are directly used for the analysis without the need for HDL isolation.

Challenges and Potential Solutions

The 2D-PAGE analytical tool could be highly useful in monitoring HDL quality; however, performing 2D-PAGE is laborious, time-consuming, and requires specialized expertise. Furthermore, the quantification by densitometric analysis is highly subjective, which limits its utility for clinical application. The use of an NMR-based approach to quantify HDL quality could be extremely useful in generating a comprehensive estimate of HDL function. The NMR-based method for HDL-P size measurement is based on the lipid content only and does not consider the composition of lipid and/or protein molecules in HDL, which are the determinants of its function. There is a lack of consistency and defined criteria related to sample processing, instrument settings, and threshold values for the classification of HDL-P based on size as large, medium, and small. More NMR-based studies are required to characterize specific lipid changes, which distinguish functional versus dysfunctional HDL particles in fresh clinical samples. Establishing standardized protocols for sample processing, instrument settings, and defining threshold values for HDL-P size classification, as well as robust data acquisition and analysis training programs, could significantly help to reduce day-to-day and interlaboratory variations in HDL quality characterization by NMR in hospitals. The requirement for expensive equipment and the cost per analysis are the other key constraints. A higher benefit-to-cost ratio will greatly outweigh the cost limits.

Conclusion and Future Research

Abundant evidence undoubtedly supports that HDL dysfunction contributes to the initiation and progression of atherogenesis, and therefore, HDL function as a biomarker could be highly valuable in primary prevention, risk assessment and prediction, and secondary prevention of CVD in clinics [10]. Because HDL exerts diverse atheroprotective functions, it is challenging to design one single method for capturing HDL functionality (Fig. 3). There are several challenges associated with the currently available assays to assess HDL function, which limits their integration into clinical practice (Table 1). The first major challenge is the lack of standardized and uniform methods for assessing specific HDL function (antioxidant, anti-inflammatory, or CEC) and this has significantly impacted the repeatability and reproducibility of the assays. There is no consensus on which precipitation method for preparation of the HDL fraction (apoB-depleted serum) is best suited for assessing HDL function. There is no uniformity across the various studies related to the concentration of HDL to be used for the assays, the type and concentration of free radical generators (AAPH or CuSO4), and the incubation time of the HDL fraction with LDL or Ox-LDL. The second major challenge is the use of primary cultures of vascular endothelial cells and freshly derived blood monocytes for performing anti-inflammatory assays. The high cost of procurement and limited availability of primary cultures, batch-to-batch variation, risk of contamination with microbes and endotoxins, and high recurring cost for maintenance of primary cultures discourage implementation of these assays in clinical settings. Lastly, there is a need for large prospective longitudinal clinical trials evaluating HDL function and HDL structural changes using fresh plasma or serum samples from healthy control subjects and disease patients with CVD, T2DM, and other metabolic diseases to characterize the values of normal and abnormal HDL function or particles for clinical interpretation. Future research should focus on the above-mentioned challenges and develop a cost-effective and easily adaptable method with improved sensitivity for characterizing HDL function in clinical practice. In recent times, there has been a renewed interest in developing therapeutic strategies to improve the function of HDL rather than HDL-C levels, such as recombinant ApoA1 [100], ApoA1 mimetic D-4F [101] recombinant LCAT [102], and infusions of reconstituted HDL [103]. Therefore, besides CVD risk assessment, HDL function-based assay will greatly help in patient selection for evaluating HDL-targeted therapies in the clinics.

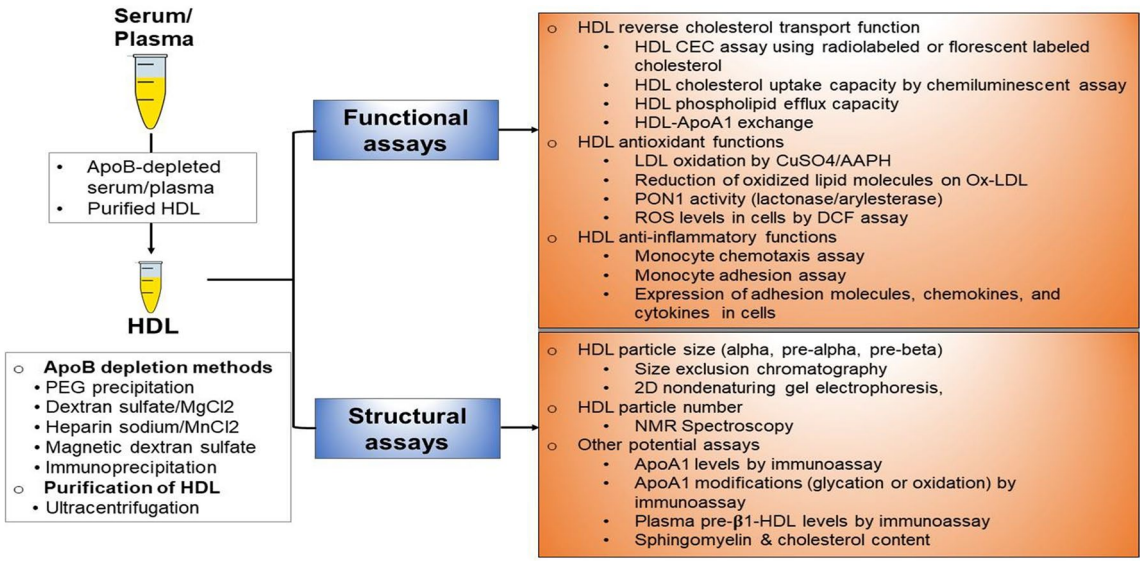

Fig. 3 Various techniques employed for the characterization and quantification of dysfunctional HDL in human serum/plasma

Table 1 Advantages and disadvantages of commonly employed assays for assessing HDL functionality

Assay	HDL sample	Mechanisms	Advantages	Disadvantages
HDL Cholesterol Efflux capacity	• ApoB-depleted serum	• HDL capacity to efflux cholesterol from cholesterol loaded macrophages	• Independently associated with CVD risk and severity • Recapitulates physiologically relevant functionality • Feasibility for automation and high-throughput analysis	• Lack of optimal method for apoB-depleted serum preparation • No consensus on macrophage cell lines • Assay is laborious and time-consuming • Requires a dedicated cell culture facility and expertise in cell culture • Lack of normal reference values • Lack of dynamics in the assay
HDL Antioxidant capacity	• ApoB-depleted serum • Purified HDL	• HDL capacity to inhibit oxidation of native LDL by exogenous oxidant • HDL capacity to reduce oxidized lipid content on preformed oxidized-LDL	• Independently associate with CVD risk and severity • Recapitulates physiologically relevant functionality • Technically easy to perform, implement and cost effective • Feasibility for automation and high-throughput analysis	• Use of non-specific redox probes (DCFDA) for estimating oxidized-LDL • Lack of optimal method for apoB-depleted serum preparation • Purification of HDL by ultracentrifugation is laborious and time consuming • Endogenous antioxidants in apoBdepleted serum may interfere with accurate assessment of HDL-antioxidant capacity • Lack of normal reference values
Paraoxonase 1 activity	• Serum • Purified HDL	• Lactonase activity • Arylesterase activity	• Independently associate with CVD risk and severity • Surrogate estimate of HDL antioxidant capacity • Technically easy to perform and cost effective • Feasibility for automation and high-throughput analysis	• PON1 polymorphism affects activity • Lactonase versus arylesterase activity as an indicator HDL antioxidant function • Lack of normal reference values
HDL Anti-inflammatory function	• Purified HDL	• HDL capacity to inhibit- i) TNF-induced VCAM1 expression in endothelial cells; ii) monocyte chemotaxis and monocyte adhesion to endothelial cells	• Correlates with CVD risk and severity • Recapitulates physiologically relevant functionality	• Requirement of primary cell lines (endothelial cells) • Purification of HDL by ultracentrifugation is laborious and time consuming • Batch-to-batch variations associated with primary cell cultures • Laborious, high cost and Day-to-day variations in the data • Lack of normal reference values

Because of its higher sensitivity, reproducibility, ease of performance, cost-effectiveness, and automation feasibility, the HDL antioxidant capacity assay is easily implementable for patient selection for emerging HDL-targeted interventions, as well as routine monitoring of high-risk populations for CVD development.

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Declarations

Competing Interest The authors report no conflict of interest.

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