Published in final edited form as:

Curr Opin Lipidol. 2017 February ; 28(1): 52–59. doi:10.1097/MOL.0000000000000382.

Inflammation, Remodeling and Other Factors Affecting HDL Cholesterol Efflux

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Abstract

Purpose of review—The ability of high density lipoprotein (HDL) to promote cholesterol efflux from macrophages is a predictor of cardiovascular risk independent of HDL cholesterol levels. However, the molecular determinants of HDL cholesterol efflux capacity (CEC) are largely unknown.

Recent findings—The term HDL defines a heterogeneous population of particles with distinct size, shape, protein and lipid composition. Cholesterol efflux is mediated by multiple pathways that may be differentially modulated by HDL composition. Furthermore, different subpopulations of HDL particles mediate CEC via specific pathways, but the molecular determinants of CEC, either proteins or lipids, are unclear. Inflammation promotes a profound remodeling of HDL and impairs overall HDL CEC while improving ABCG1-mediated efflux. This review discusses recent findings that connect HDL composition and CEC.

Summary—Data from recent animal and human studies clearly show that multiple factors associate with CEC including individual proteins, lipid composition, as well as specific particle subpopulations. While acute inflammation remodels HDL and impairs CEC, chronic inflammation has more subtle effects. Standardization of assays measuring HDL composition and CEC is a necessary prerequisite for understanding the factors controlling HDL CEC. Unraveling these factors may help the development of new therapeutic interventions improving HDL function.

Keywords

Cholesterol efflux capacity (CEC); HDL; atheroprotection; serum amyloid A; proteomics; lipidomics

Introduction

The inverse relationship between the levels of high density lipoprotein cholesterol (HDL-C) and the risk of cardiovascular disease (CVD) has been well-established for a long time [1].

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Conflicts of Interest:

Dr. Vaisar is named as a co-inventor on patents from the US Patent Office on the use of HDL markers to predict the risk of CVD. Dr Vaisar has served as a consultant for Boston Heart Laboratories.

However, multiple gene association studies [2–4] and recent failures of clinical trials that pharmacologically elevate HDL-C [4–6] provide strong evidence against a causal relationship between HDL-C levels and cardiovascular risk. HDL has been related to multiple potentially anti-atherogenic functions [7], which cannot be fully explained by HDL-C plasma concentration. The HDL plays key role in reverse-cholesterol transport (RCT), a pathway by which excess cholesterol is transported from tissues, including atherosclerotic lesions, to liver for elimination. Three independent clinical trials showed that the ability of HDL to remove cholesterol from macrophages (the first step in the RCT), is a predictor of CVD independent of HDL-C levels [8–10]. Therefore, the HDL function, namely cholesterol efflux capacity (CEC), may be a better measure of HDL cardioprotective effects than HDL-C. However, the molecular determinants of HDL CEC are largely unknown.

This review will summarize the recent advances that have been made in our understanding of the factors that modulate CEC and the effects that inflammation, a hallmark of atherosclerosis, has on CEC.

Pathways for cholesterol efflux from macrophages

Four different pathways for efflux of free cholesterol (FC) from cells have been identified. Two pathways are mediated by ATP binding cassette (ABC) transporters. ATP binding cassette transporter A1 (ABCA1) is thought to export cholesterol mainly to lipid-free apolipoprotein AI (apoA-I) or poorly lipidated apoA-I (pre β -1 HDL) [11], while ATP-binding cassette transporter G1 (ABCG1) mediates cholesterol efflux to mature HDL [12] (Figure 1).

The first evidence implicating ABCA1 pathway as a major route for cholesterol removal from cells came from humans with Tangier's disease. These patients lack ABCA1 activity, have almost no HDL-C, accumulate cholesterol-laden macrophages in many different tissues, and are at increased risk of premature CVD [13]. Because ABCA1 mediates lipidation of apoA-I and formation of nascent HDL [14], apoA-I of Tangier's disease patients is poorly lipidated and is rapidly catabolized. Interestingly, a recent study showed cholesterol efflux through ABCA1 can also be promoted by small, dense HDL [15[■]]. However, the contribution of individual apoA-I derived acceptors to the overall efflux through ABCA1 transport *in vivo* is still unknown. In contrast with ABCA1-mediated efflux, medium and large HDL particles are the preferred substrates for ABCG1 transporter [5, 16]. ABCG1 may mediate efflux alone or in a concerted mechanism with ABCA1. Indeed animals deficient in both ABCA1 and ABCG1 develop more severe atherosclerosis compared to mice deficient in a single transporter or wild type controls [17].

In addition to the active cholesterol efflux pathways, aqueous diffusion and diffusion facilitated by scavenger receptor class B, type 1 (SRB1) are two pathways primarily driven by cholesterol concentration gradient, with medium and large HDL particles as the major acceptors [16]. SRB1 receptor is responsible for the selective uptake of HDL cholesteryl esters into cells. It is expressed mainly in the liver and non-placental steroidogenic tissues [18], but also in endothelial cells, vascular smooth cells and macrophages, and can promote

free cholesterol efflux to HDL [19]. In cholesterol-fed LDL receptor-deficient mice, SRB1 hepatic overexpression reduced atherosclerosis [20]. In humans, a rare loss-of-function variant in the gene encoding the SRB1 receptor raises HDL-C, but also increases the risk of CVD [21[■]]. Extensive review of cholesterol efflux pathways is provided elsewhere [16].

Assays of cholesterol efflux are generally based on measurement of the amount of cholesterol released from cells to the acceptor in the media, however the details of the assay can vary widely between different laboratories. The first clinically relevant HDL CEC assay measured CEC from murine J774 macrophages stimulated with cAMP and labeled with ³H-cholesterol to serum HDL (apoB depleted serum) [8]. In this arrangement the measured CEC represents efflux by ABCA1 (34 %), SRB1 (20 %), ABCG1 and aqueous diffusion (46 %) pathways, respectively [22]. In contrast, the first evidence of association of cholesterol efflux capacity and incident CVD used a modified assay [9], in which J774 macrophages were labeled with a fluorescent probe - BODIPY-labeled cholesterol. It was implied by the authors that this assay primarily measures ABCA1-mediated cholesterol efflux [23]. Although radiolabeled and fluorescent assays agreed on the clinical outcome (serum HDL CEC was decreased in prevalent and incident CVD independent of HDL-C or apoA-I), the general agreement between the two assays was only modest ($r = 0.54$, 30% variance between assay explained) [9]. Moreover, cholesterol efflux from RAW264.7 cells labeled with ³H-cholesterol to apoB-depleted serum was paradoxically associated with increased prospective risk for myocardial infarction, stroke, and death [24]. Although the cell type was different, the authors showed in a subset of the samples that HDL CEC from J774 and RAW264.7 are in excellent agreement ($r = 0.92$).

Since first efflux studies with fibroblasts and Fu5AH hepatoma cells, many different cellular models have been used to measure specific aspects of cholesterol efflux (Figure 1). Key examples focusing on specific efflux pathways include baby kidney hamster (BHK) cells expressing inducible human ABCA1 or ABCG1 transporter under mifepristone control [25, 26]. Thus, ABCA1-BHK cells were used to show that atorvastatin specifically reduces ABCA1 mediated CEC to serum HDL [27] (Figure 2). Specific ABCA1-mediated efflux can also be estimated as the difference in CEC from J774 macrophages with and without cAMP stimulation, providing good agreement with ABCA1-specific CEC measured in ABCA1-BHK cells [22]. Alternative approach uses inhibition of ABCA1-mediated efflux in cAMP stimulated J774 macrophages by probucol (an ABCA1 inhibitor) [22]. Despite the general agreement between different ways of measuring ABCA1-specific efflux, there are important differences. For instance, the effects of cAMP and probucol on J774 macrophages are only partly specific for ABCA1, and there is a low level of ABCA1-mediated efflux present even in unstimulated J774 cells [22]. Thus, the CEC assay methodology may have significant effect on the measurement results (Figure 1).

The acceptor of cholesterol (the “HDL”) used in the assay is another source of measurement variation. While the initial, mechanistically driven studies employed mostly ultracentrifuge isolated-HDL (uc-HDL), the clinical studies have been using apolipoprotein B-depleted serum (referred to as “serum HDL”), as the cholesterol acceptor. The CEC of the two types of acceptors showed only partial agreement ($r = 0.49$) [28]. While absence of pre β -1 HDL in uc-HDL or damage of HDL upon ultracentrifugation may explain the lack of agreement,

these results also suggest there might be other unknown efflux effectors in plasma distinct from HDL particles.

Collectively, the complexity of the CEC measurement (Figure 1) significantly confounds the ability to determine molecular mediators of HDL CEC and their association with clinical outcomes. Therefore, assay standardization is highly desired.

Determinants of HDL cholesterol efflux capacity

Cholesterol efflux capacity of serum HDL can vary substantially, despite similar levels of HDL-C and apoA-I [22]. This observation suggests that there are other factors that determine HDL CEC that are largely independent of the HDL-C. It is well-established that HDL is highly heterogeneous, consisting of a wide range of subspecies with different sizes, gravitational densities, concentration and composition. This structural and compositional heterogeneity is also likely to confer functional diversity. For instance, the small lipid poor pre β -1 HDL particles, which are thought to efficiently mediate ABCA1-specific CEC, are found at low, yet quite variable concentration compared to larger HDL particles, which are considered less efficient cholesterol acceptors through ABCA1 pathway (Figure 2). The heterogeneity of HDL is also reflected by its proteomic diversity, with as many as 95 proteins reported, depending on the isolation method utilized [29]. Robust isolation methods based on gradient ultracentrifugation, where contamination by plasma proteins is minimal, typically result in about 50 proteins consistently identified over large population of subjects. Alternative methods based on combination of size and lipid-binding properties identify even more proteins [30].

The proteins found in HDL are not limited to lipid metabolism; researchers have consistently found proteins in HDL particles linked to inflammation, complement activation and immunity. So far, studies linking proteins in HDL to CEC in humans are lacking. In mice, a recent study using a panel of mouse strains, together with transgenic and knockout animals [31] found a negative association between the relative abundance of apolipoprotein E in HDL and ABCA1-specific HDL CEC ($r = -0.72$), but not total HDL CEC ($r = -0.32$) [31]. To improve studies relating protein composition to function, methods for relative quantification of up to 45 HDL proteins have been developed [32–34]. However, different diseases predisposing to CVD, i.e. kidney disease, may be associated with proteins not normally found in HDL [35] and the panel of HDL proteins quantitatively monitored must be expanded.

Significant attention has been devoted to HDL protein composition, but HDL lipidome is also very rich and may be associated with HDL function [36]. Most studies so far have only investigated the overall content of major lipid classes (free cholesterol, cholesteryl esters, phospholipids, and triglycerides) as possible modulators of HDL function, suggesting that free cholesterol to phospholipid ratio or phospholipid content of HDL associate with HDL CEC [37]. Whether individual subclasses (i.e. long chain or short chain fatty acid containing phospholipids, saturated vs. unsaturated phospholipids, etc.) have specific effects on CEC is currently unknown.

HDL composition is dynamic with a number of HDL proteins exchangeable with other lipoproteins. The concept of HDL dynamics was exploited by HDL-apoA-I exchange assay, in which stability of apoA-I on HDL is measured by displacement of native apoA-I from the HDL particle (either isolated or directly in plasma) by apoA-I labeled with an EPR probe. The rate and extent of apoA-I exchange is then reflected by EPR signal of HDL after the exchange, with the EPR signal intensity dependent on the apoA-I conformation and its environment (lipid-free vs. lipid associated) [38]. The apoA-I exchange rate measured by this assay was shown to associate with both total and ABCA1-mediated HDL CEC suggesting that changes in HDL dynamics may also contribute to the overall HDL CEC [39].

The current accepted model of HDL metabolism and cellular efflux involves secretion of lipid-poor apoA-I and nascent phospholipid-rich, cholesterol-poor HDL particles by liver and intestine. These nascent particles undergo maturation and remodeling in the circulation by acquiring additional phospholipids and cholesterol via cellular efflux as well as by transfer with other lipoproteins [40]. Recent studies on HDL dynamics challenged this established concept. A study using endogenous stable isotopic labeling and kinetic modeling of apoA-I in 4 major HDL sizes suggested that a given HDL particle population appears in the circulation in a specific size range and remains mainly in that range throughout its lifetime [41[■]]. Another recent study also concluded that 6 HDL apolipoproteins originate in specific HDL sizes, mainly directly from liver and intestine [42]. Therefore, HDL metabolism would occur mainly within its secreted size rather than progressively mature from nascent particles. How these new models of HDL metabolism would fit in the current knowledge of RCT remains to be determined.

Although great advances have been made in characterization of HDL heterogeneity, our understanding of how this heterogeneity relates to HDL function is still limited. Inflammation, a hallmark of CVD, is one of the most studied modulators of HDL composition, and recent findings related to its effects on HDL remodeling and CEC will be discussed in detail.

Inflammation, HDL remodeling and efflux capacity

Inflammation gives rise to pronounced metabolic and structural changes in HDL, which may affect its ability to mediate RCT. In mice, acute inflammation induced by either LPS or silver nitrate (“sterile inflammation”) results in decrease in HDL-C, and increase of circulating triglycerides [43]. Moreover, mouse studies demonstrated that acute inflammation impairs RCT [44, 45]. In humans, inflammation leads to changes in lipid content, marked by increase in free cholesterol and triglycerides and decrease of phospholipids [46]. Despite extensive remodeling of HDL, the effects exerted by inflammation on the HDL function, primarily CEC, are controversial. A hallmark of HDL remodeling during inflammation is major increase in the content of the acute phase protein serum amyloid A (SAA) (~500–1000x in mouse acute inflammation). Studies using purified SAA showed its ability to mediate efflux [47] while other studies noted impaired efflux capacity of HDL in acute phase (review in [46]). Several recent studies in mice and humans attempted to resolve this controversy. An *in vivo* RCT study in SAA1.1 and SAA2.1 deficient mice concluded that while inflammation compromises RCT, SAA played only a

minor role in this impairment [48], although the study may have measured the RCT at the onset rather than the peak of SAA plasma concentration. SAA as well as myeloperoxidase were linked to reduced RCT in mice [49], although decreased biliary sterol excretion may have contributed to RCT suppression [49]. A combined human and mouse study of acute inflammation showed that in addition to overall protein and lipid content changes, nearly 1/3 of HDL proteome was altered by inflammation. The CEC to both uc isolated and serum HDL from inflamed mice was markedly reduced in WT but not in *Saa1/2^{-/-}* mice. Moreover, the CEC was strongly inversely correlated with HDL content of SAA in both humans and mice, but not with its free cholesterol and phospholipid content [50[■]] (Figure 2). Inflammatory HDL also exhibited impaired CEC from adipocytes in obese mice and in humans with systemic lupus erythematosus, and lost its anti-inflammatory properties [51]. SAA was shown to promote early lesion formation in a study using *Saa1/2^{-/-}/Ldlr^{-/-}* mice [52], however, in the same SAA-deficient model on *ApoE^{-/-}* background the SAA had no effect on atherosclerosis [53]. Mice subjected to a high fat diet enriched with saturated fatty acids also showed increase in SAA levels in their small HDL particles when compared with mice on a low fat diet or in a high fat diet enriched in monounsaturated fatty acids [54]. Moreover, these mice fed with saturated fatty acids had an increased movement of radiolabeled cholesterol from macrophages to the plasma in an *in vivo* RCT assay, but their hepatic secretion of cholesterol for fecal excretion was impaired [54].

In addition to remodeling of HDL protein composition, inflammation also has profound effects on its lipidome. Therefore, several studies investigated the relationship of HDL's lipidome with its function. Thus, on a phospholipid basis, small, dense HDL-3, is more able to promote cholesterol efflux from THP-1 cells than larger HDL particles [55]. This efflux capacity was positively associated with the negatively charged phosphatidylserine content of the HDL-3 particles. Moreover, inflammation-driven HDL remodeling led to increase in ABCG1-dependent efflux in mice likely due to phospholipid enrichment rather than the presence of SAA on inflammatory HDL [56].

In summary, the inflammatory remodeling of HDL primarily through SAA enrichment impairs HDL CEC, however, it appears that the SAA levels associated with chronic inflammation may not be sufficient to have significant effect on CEC and atherosclerosis.

HDL modulation, efflux capacity, and clinical outcomes

Many unresolved issues remain regarding the “functional, atheroprotective, HDL particle”. For instance, while lipid-free and poorly lipidated apoA-I are considered to be the most efficient mediators of CEC via ABCA1 pathway [11], in a large cohort levels of pre- β_1 HDL were associated with coronary heart disease independently of other risk factors [57].

These observations raise questions about what property of HDL should be targeted to increase cardioprotection. Therapies that increased HDL-C (primarily in large HDL particles) yielded disappointing results. Niacin therapy increased HDL-C, large HDL particles and overall CEC from macrophages [27, 58], but failed to improve ABCA1-specific efflux capacity of serum HDL [27] and failed to reduce cardiovascular risk [59]. Inhibition of cholesterol ester transfer protein (CETP) efficiently elevates HDL-C levels

through elevation of large HDL particles; however three clinical trials with CETP inhibitors, torcetrapib, dalcetrapib and evacetrapib were stopped due to increased mortality (torcetrapib) or futility [60, 61]. Overall CEC of either uc isolated HDL (torcetrapib, HDL increase 47%) or serum HDL (dalcetrapib, HDL increase 34%) was increased in treated subjects, but the ABCA1-mediated CEC was unchanged [62, 63]. Evacetrapib, which increased HDL by as much as 130%, increased both total and ABCA1-mediated CEC to serum HDL along with plasma levels of pre- β_1 HDL concentrations in dyslipidemic patients [64] (Figure 2), however the drug showed no clinical benefit.

Therapies aimed at increasing HDL levels with injection of reconstituted discoidal HDL particles (rHDL) increased plasma apoA-I and HDL-C levels and increased cholesterol efflux to serum HDL [65]. The injection of the rHDL resulted in extensive particle remodeling via initial particle fusion followed by fission and formation of 3 distinct particle populations - lipid poor apoA-I, small HDL3-like particles and large HDL particles with the former two primarily mediating ABCA1-specific and latter ABCG1 and SRB1 sterol efflux [66[■]]. Collectively, these studies indicate the direction for the future therapeutics; rather than increasing indiscriminately HDL-C the focus should be on increasing specific “functional” populations of HDL particles.

Conclusions

HDL cholesterol efflux capacity has proven to be a better metric to predict CVD risk than HDL-C levels. However, the molecular determinants associated with HDL CEC are only beginning to emerge (i.e. impaired CEC of inflammatory HDL, mediated by HDL enrichment with SAA). Linking HDL composition and metabolism with its CEC will be critical to understand how HDL's composition contributes to CVD risk. This may also facilitate the development of therapeutics aimed at improvement of HDL function. To achieve these goals, a careful standardization of functional and compositional assays must be undertaken. For HDL's proteome and lipidome, a targeted quantification, instead of the semi-quantitative, label free quantification, need to be undertaken on large clinical populations. For CEC assays, standardization of the efflux acceptor, isolated HDL or serum HDL, and the assay methodology (i.e. cell type; efflux substrate - ^3H -cholesterol or fluorescently labeled cholesterol; method of ABCA1-induction) has to be implemented across multiple laboratories to achieve reproducible results across multiple clinical trials.

Furthermore, the heterogeneity of HDL particles is tightly linked to the dynamic nature of lipoprotein metabolism. Static measurements of HDL may not be sufficient to provide information about its atheroprotective effects. Indeed, recent studies on HDL dynamics challenged the established concept of HDL maturation. Further studies and perhaps development of novel approaches capturing the dynamic nature of HDL are necessary to advance our understanding of how HDL composition and dynamics affect CEC, RCT and atheroprotection.

Acknowledgments

Funding Sources:

The work was supported by funding from National Institutes of Health grant HL092969.

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Key points

- HDL cholesterol efflux capacity is a predictor of prevalent and incident cardiovascular disease, independent of HDL-C or ApoA-I levels.
- HDL is highly heterogeneous and specific molecular determinants that control HDL cholesterol efflux capacity are unknown.
- Inflammatory conditions remodel HDL and impair its cholesterol efflux capacity.
- A careful standardization of functional and compositional assays is critical to improve our understanding of factors controlling HDL cholesterol efflux capacity.

Figure 1. Diversity of measurements of HDL cholesterol efflux capacity

HDL CEC has been assessed using wide variety of assays in number of cell types, cell-labeling strategies (${}^3\text{H}$ -cholesterol vs. BODIPY-cholesterol), cholesterol acceptors (HDL isolated by ultracentrifugation or serum depleted of apoB particles - serum HDL), and strategies to specifically assess individual efflux pathways. This diversity makes direct comparisons of results and generalizations from published studies very difficult (M Φ - primary macrophages - elicited peritoneal macrophages or bone marrow derived macrophages).

Figure 2. HDL remodeling and functional changes with inflammation and therapeutic interventions

Heterogeneity of HDL particles is associated with heterogeneity of CEC via different efflux pathways. Inflammation results in enrichment of HDL with SAA accompanied by decreased content of PL and impairment of HDL CEC. Therapeutic interventions (lipid lowering or HDL-C elevation) have diverse effects on both distribution of HDL particle populations and on HDL CEC (white and grey wedges illustrate changes in CEC via ABCA1 and ABCA1-independent pathways, respectively, with changing size of HDL particles).