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HDL Function and Atherosclerosis: Reactive Dicarbonyls as Promising Targets of Therapy

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Abstract

Epidemiologic studies detected an inverse relationship between HDL-C levels and atherosclerotic cardiovascular disease (ASCVD) identifying HDL-C as a major risk factor for ASCVD and suggesting atheroprotective functions of HDL. However, the role of HDL-C as a mediator of risk for ASCVD has been called into question by the failure of HDL-C raising drugs to reduce cardiovascular events in clinical trials. Progress in understanding the heterogeneous nature of HDL particles in terms of their protein, lipid and small RNA composition has contributed to the realization that HDL-C levels do not necessarily reflect HDL function. The most examined atheroprotective function of HDL is reverse cholesterol transport (RCT), whereby HDL removes cholesterol from plaque macrophage foam cells and delivers it to the liver for processing and excretion into bile. Indeed, in several studies HDL have shown inverse associations between HDL cholesterol efflux capacity (CEC) and ASCVD in humans. Inflammation plays a key role in the pathogenesis of atherosclerosis and vulnerable plaque formation, and a fundamental function of HDL is suppression of inflammatory signaling in macrophages and other cells. Oxidation is also a critical process to ASCVD in promoting atherogenic oxidative modifications of LDL and cellular inflammation. HDL and its proteins including apolipoprotein AI (apoAI) and paraoxonase 1 (PON1) prevent cellular oxidative stress and LDL modifications. Importantly, HDL in humans with ASCVD is oxidatively modified rendering HDL dysfunctional and proinflammatory. Modification of HDL with reactive carbonyl species, such as malondialdehyde (MDA) and isolevuglandins (IsoLG), dramatically impairs the antiatherogenic functions of HDL. Importantly, treatment of murine models of atherosclerosis with scavengers of reactive dicarbonyls improves HDL function and reduces systemic inflammation, atherosclerosis development, and features of plaque instability. Here, we discuss the HDL anti-atherogenic functions in relation to oxidative modifications and the potential of reactive dicarbonyl scavengers as a therapeutic approach for ASCVD.

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Disclosures

Drs. Linton and Davies are inventors on a patent for the use of 2-HOBA and related dicarbonyl scavengers for the treatment of cardiovascular disease (US 11,389,414). All the other authors declare no financial conflicts of interest.

Keywords

Cholesterol efflux; High-density lipoprotein (HDL); Malondialdehyde (MDA); Macrophage; Myeloperoxidase (MPO); 2-hydroxybenzylamine; 5'-O-Pentyl-pyridoxamine (PPM); atherosclerosis

Introduction

In contrast to the direct association of serum LDL-C levels with risk of coronary heart disease, the discovery of the inverse relationship of serum HDL-C levels with the incidence of coronary heart disease in The Framingham Heart Study, resulted in the description of HDL as a protective factor against coronary heart disease.[1] This association was found to be independent of other risk factors, including LDL-C, in several early prospective studies.[2] These observations identified HDL-C levels as a risk factor and a potential target for therapy of atherosclerotic cardiovascular disease and led to decades of research describing multiple atheroprotective functions of HDL. However, the role of HDL-C as a mediator of risk for ASCVD has been called into question by the failure of clinical trials of HDL-C raising drugs, including niacin, fibric acid derivatives, and several CETP inhibitors, to demonstrate a benefit in reducing cardiovascular events.[3] In addition, Mendelian Randomization studies failed to show an association of genetic polymorphisms (SNPs) that impact levels of HDL-C on risk for myocardial infarction and CAD.[4, 5] Furthermore, studies with large cohorts found that the number of HDL particles is a better marker for ASCVD risk than HDL-C levels.[3] Interestingly, mounting evidence shows that very high levels of HDL-C can be associated with increased risk of ASCVD.[6] In this regard, HDL is a collection of subparticles that differ in size, charge, lipid subspecies, and apolipoprotein/protein compositions, all of which can impact HDL functions. Progress in understanding the composition of HDL subpopulations in the areas of proteomics and lipidomics has revealed the complex heterogeneous nature of HDL particles in terms of their protein and lipid composition as well as oxidative modifications.[3] Furthermore, HDL carries microRNAs and small noncoding RNAs that may impact its function.[7, 8] Together these findings suggest that HDL-C levels do not necessarily reflect the HDL particle number or more importantly, HDL function.[3] Consequently, there has been growing interest in HDL's antiatherogenic functions, such as reverse cholesterol transport (RCT), and its anti-inflammatory and antioxidant properties, as markers of risk and potential therapeutic targets.[9] Several HDL based therapies, including recombinant HDL infusions, ApoA-I mimetic peptides, ApoA-I transcriptional supraregulator BET inhibitor RVX-208 (apabetalone), and antagonism of miR-33 are in various stages of development and have been recently reviewed.[3] In this review, we will focus on advances in our understanding of three of HDL's antiatherogenic functions: RCT, anti-inflammatory and anti-oxidant functions of HDL. Furthermore, we discuss recent evidence showing that modification of HDL with reactive dicarbonyl species, such as malondialdehyde (MDA) and isolevuglandins (IsoLG), dramatically impairs all three of these antiatherogenic functions of HDL, and that prevention of these modifications with dicarbonyl scavengers holds promise as a treatment to improve HDL function and reduce the development of atherosclerosis.

The deleterious effects of oxidative modifications of HDL on atherosclerosis have been demonstrated in mouse models showing that administration of oxidized HDL or apoAI promotes inflammatory plaque development, whereas native HDL/apoAI promote lesion regression and anti-inflammatory remodeling.[10, 11] Oxidative modification of HDL occurs upon exposure to reactive oxygen species (ROS) and peroxidized lipids. Enzymes that generate ROS and have been implicated in atherosclerosis include myeloperoxidase (MPO), xanthine oxidase, and NADPH oxidases. Indeed, the majority of apoAI in human atherosclerotic plaques is oxidized and associated with MPO.[12] ROS generated by these enzymes include hypochlorous acid (HOCl), hypobromous acid (HOBr), hypothiocyanous acid (HOSCN), isocyanate (CNO-), nitrogen dioxide radical ($^{\bullet}NO_2$), superoxide ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$) and peroxynitrite ($ONOO^-$).[13] These ROS act on the amino acids of HDL proteins to generate oxidized amino acid species including cysteine (Cys) disulfide bonds (Cys-Cys crosslinks), oxidized Met (oxMet), oxidized tryptophan (oxTrp), modified and tyrosine, including 3-chlorotyrosine (3-Cl-Tyr), nitrotyrosine (NO_2-Tyr), tyrosine dimers (Tyr-Tyr), and *N*-chloroamines. CNO- acts on lysine (Lys) to produce carbamylated-Lys (Cbl-Lys). ROS (especially $^{\bullet}NO_2$ and $ONOO^-$) also act on the polyunsaturated fatty acid (PUFA) tail of a phospholipid (PL) or cholesteryl ester (CE) to extract hydrogen, generating a lipid radical that then reacts with molecular oxygen to form a lipid peroxyl radical. If this lipid peroxyl radical can then extract a hydrogen from an adjacent PUFA, the lipid radical reaction rapidly propagates generating both a lipid hydroperoxide and another lipid radical at each step. Lipid hydroperoxides undergo secondary reactions to form reactive lipid dicarbonyls species, including MDA, IsoLG, 4-oxo-nonenal (ONE), and methylglyoxal (MGO), and also reactive lipid monocarbonyl species such as acrolein (ACR) and 4-hydroxy-nonenal (HNE) (Figure 1). Lipid peroxidation also generates oxidized phospholipids, where the lipid dicarbonyls form esterified to phospholipids, including esterified IsoLGs[14] and structural analogs of ONE such as 9-keto-12-oxo-10-dodecenoic acid (KODA-PC).[15] Various amino acids can then be adducted by these reactive lipid carbonyls to form stable modifications.[13] MDA and IsoLG preferentially modify Lys, but Lys can also be modified by HNE, ONE, KODA-PC, and MGO. Cys are readily modified by ACR, HNE, and ONE. Histidines (His) are modified by HNE, ONE, KODA-PC and ACR and arginines (Arg) are modified by HNE, ONE, and MGO. Detailed evidence for contributions of various reactive lipid carbonyls to reducing individual HDL functions will be given in later sections.

HDL Reverse Cholesterol Transport (RCT)

First described by Glomset,[16] RCT is the process by which HDL removes excess cholesterol from peripheral tissues for transport through the plasma and delivery to the liver for either direct excretion or conversion into bile acids and subsequent secretion into bile (Figure 2). Studies have established the atheroprotective effects of HDL in mediating RCT in atherosclerotic animal models.[17] However, the lack of effects of HDL-C raising treatments on reducing CAD risks in humans have called into question the atheroprotective effects of HDL RCT in humans. In this regard, the heightened oxidative stress in CAD likely impairs every step of RCT by promoting oxidative modifications of HDL thereby negating any

benefit of raising HDL-C. Here, we discuss the steps of HDL RCT and their relevance to atherosclerosis and the role of oxidative stress.

Cholesterol Efflux

The rate-limiting step in HDL RCT for protection against atherosclerosis is the efflux of free cholesterol (FC) to HDL from macrophages. Alleviation of the macrophage cholesterol burden is critical to the inhibition of plaque progression as excess membrane FC promotes inflammatory signaling and cell death (Discussed later). Cholesterol transport from macrophages to HDL/apoAI occurs by four mechanisms (Figure 2). ATP-binding cassette transporter A1 (ABCA1) mediates the efflux of phospholipid and FC to lipid-poor apoAI or pre-β HDL.[17–19] In addition, endogenous apoE is an efficient acceptor for ABCA1-mediated cholesterol efflux, and the absence of macrophage apoE markedly accelerates atherosclerosis.[19, 20] Cholesterol efflux to discoidal and mature spherical HDL occurs via ATP-binding cassette transporter G1 (ABCG1) and scavenger receptor-class BI (SR-BI).[18, 21] In vitro studies have shown that, while a large part of the total cholesterol efflux from mouse macrophages to serum or the apoB-depleted serum fraction occurs via ABCA1 (35%) versus ABCG1 (21%) or SR-BI (9%), a large portion (35%) also occurs by aqueous diffusion and/or unknown mechanisms.[17, 22] In contrast, cholesterol efflux from human macrophage foam cells is largely independent of ABCG1, and SR-BI plays a major role.[17] The finding that apoAI in normal human arteries is mostly lipid-poor, suggests that ABCA1 mediated cholesterol efflux is a major mechanism in preventing atherosclerotic lesion formation.[23] However, studies suggest that the lipid-poor apoAI has a limited capacity to sustain net cholesterol efflux via ABCA1.[24] In this regard, ABCG1, SR-BI, and aqueous diffusion are likely important for driving cholesterol efflux to the discoidal particles formed via ABCA1, and all the mechanisms may act in a synergistic fashion.[18, 21] In mediating cholesterol efflux, ABCA1 interacts with apoAI and recycles back and forth from the plasma membrane to endo/lysosomal compartments to facilitate release of FC derived from hydrolysis of lipid droplet CE by either neutral esterase or acid lipase in the process of autophagy.[25,26] In contrast, ABCG1 does not interact with HDL, but localizes to intracellular vesicles to traffic FC from the golgi and ER to the plasma membrane for release to the HDL.[27] SR-BI acts intracellularly to facilitate CE clearance by operating directly as part of the VPS34/Beclin-1 complex to stimulate autophagosome formation, suggesting that ABCA1 and SR-BI cooperate to enhance lipophagy.[28] The flux of FC to HDL is bi-directional, and the net flux is determined by the direction of the FC gradient, which is influenced by the FC and phospholipid subspecies content of the HDL and plasma membrane.[17, 29] In this regard, HDL with an increased FC/phospholipid ratio, such as HDL from SR-BI deficient mice, promotes the accumulation of cholesterol in tissues.[30] In addition, oxidative modifications of HDL/apoAI, including MDA, carbamylation, and IsoLG, that occur in subjects with FH or CAD, not only can inhibit cholesterol efflux but enhance HDL recognition by scavenger receptors, thereby promoting cholesterol influx via endocytic mechanisms.[31–38] In vivo RCT studies in C57BL/6 mice have shown that cholesterol efflux from macrophage foam cells via ABCA1 and ABCG1 is routed to the liver for excretion.[39] Consistent with a role for ABCA1 in alleviating macrophage cholesterol burden in plaques, deletion of bone marrow ABCA1 accelerates atherosclerosis in mice, and humans heterozygous for loss of cholesterol efflux function in the ABCA1

gene are at increased CVD risk.[18, 40] In contrast, deletion of bone marrow ABCG1 in *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice decreases atherosclerosis, which may stem from compensatory upregulation of macrophage apoE and ABCA1.[41, 42] Indeed, a number of variants of the ABCG1 gene in humans have been implicated in enhancing the risk of CAD.[43] In contrast to ABCA1 and ABCG1, a study using an in vivo RCT assay with cholesterol-normal cells in C57BL/6 mice suggested that macrophage cholesterol released via SR-BI is not preferentially transported to the liver for processing and excretion.[39] However, another study by Zanotti and colleagues showed that cholesterol released via SR-B1 is routed for excretion in mice treated with an LXR agonist.[22] These conflicting results are likely due to differences in assays, as the study by Zanotti et. al. used macrophage foam cells to examine RCT, which would increase the net cholesterol efflux potential via SR-BI, and also treatment of mice with the LXR agonist increased the number of enlarged α-migrating HDL particles, which are a preferred acceptor for SR-BI.[22, 29] It should also be noted that the contribution of ABCA1, ABCG1, and SR-BI mediated cholesterol efflux to RCT in vivo has never been tested in atherosclerotic mouse models (*Ldlr*$^{-/-}$, *Apoe*$^{-/-}$), where oxidative stress clearly plays a role. In this regard, studies have established that cholesterol efflux via both ABCA1 and SR-BI to oxidized forms of HDL can be severely impaired, but some modifications (i.e. carbamylation) impair SR-BI cholesterol efflux without impacting ABCA1 activity.[32, 38] In addition, in vivo MPO-mediated oxidation impairs in vivo RCT from macrophage foam cells in wildtype C57BL/6 mice and reduces HDL CEC.[44] Furthermore, the premise that cholesterol efflux via SR-BI is atheroprotective is substantiated by studies showing that deletion of macrophage SR-BI accelerates plaque development in *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice, and that macrophages in *Scarb1*$^{-/-}$ plaques are severely engorged with lipid inclusions with only 3% of their cytoplasm lipid-free.[45–47] In addition, SR-BI deficient macrophages exhibit lipid engorged lysosomes which are enriched in FC, which is consistent with the role of SR-BI in autophagy.[28, 47] Elucidation of the role of macrophage cholesterol efflux via SR-BI in human atherosclerosis is complicated in that SR-BI is a multifunctional receptor that is expressed in multiple tissues. However, carriers of the P376L variant in SR-BI are at increased risk of CAD, whereas carriers of the P297S variant do not have enhanced CAD risk.[48–50] These differences are likely due to the degree of loss of SR-BI function and HDL dysfunction.[48, 50] Indeed, P376L variant carriers have increased plasma levels of oxidized HDL.[49] Interestingly, macrophage SR-BI internalizes oxidized LDL as well as oxidized forms of HDL for degradation rather than mediating efflux, thereby promoting cholesterol accumulation.[32, 38] At present it is unknown, whether, similar to other scavenger receptors (i.e. CD36, LOX-1, SRA1) that mediate an inflammatory response to oxidized lipoproteins, SR-BI internalization of oxidized HDL is proinflammatory.[31, 51, 52] It is possible that SR-BI provides an anti-inflammatory signaling pathway for removal of toxic HDL/LDL as it does in the efferocytosis of plaque apoptotic cells.[53]

Lecithin:cholesterol acyltransferase (LCAT)

Once in plasma, HDL FC is esterified by LCAT (Figure 2). ApoAI activates LCAT to drive the net movement of cholesterol from the periphery to plasma. The importance of LCAT in RCT was substantiated in studies showing in vivo that there is enhanced macrophage cholesterol efflux and subsequent cholesterol delivery to the liver and biliary excretion in atherosclerotic *Ldlr*$^{-/-}$ mice and cynomolgus monkeys treated with a novel LCAT

activator.[54] In addition, tracer studies in humans suggested that the CE formed by LCAT is preferentially routed for excretion.[55] Nonetheless, the association of LCAT activity with CAD risk in humans is controversial. While there is a strong association between HDL-C levels and LCAT variants in humans, there is no link to increased CAD risk.[56] Similar results were observed in a large Mendelian randomization study.[57] In addition, humans that have complete loss of LCAT activity are not at increased risk for the development of CAD, which is likely due to their extremely low levels of LDL-C.[58] In this regard, subjects that are heterozygous for LCAT loss of function mutations exhibit higher LDL-C and lower HDL-C levels and have increased incidence of CAD.[17] Interestingly, studies have also demonstrated that a number of atherosclerotic mouse models that have increased oxidative stress naturally, and/or from consuming a western diet, have impaired LCAT activity resulting from oxidation of apoAI or LCAT.[59–61] Importantly, overexpression of LCAT or increased activation of LCAT reduces atherosclerosis in a number of mouse models including *Ldlr*$^{-/-}$, *Scarb1*$^{-/-}$, and *Ldlr*$^{-/-}$*;ob/ob* mice.[54, 61, 62] It is also worth noting that the plasma and plaques of humans with CAD contain oxidized forms of apoAI that are known to inhibit LCAT, including nitrated-apoAI, acrolein-apoAI, and carbamylated-apoAI.[32, 63–66] In this regard, studies are consistent with impaired LCAT activity being critical to CAD in humans as LCAT activity is significantly decreased in humans with CAD compared to controls[67]. In a Phase 2a study, MEDI6012, an active recombinant human (rh)LCAT, showed safety and increased HDL-C, apoAI, non-ABCA1 mediated CEC and reduced LDL particles, suggesting enhanced RCT.[68] However, in a recent Phase 2b study, treatment of patients with acute ST-segment–elevation myocardial infarction with MEDI6012 did not result in a significant reduction in infarct size or noncalcified plaque volume at 12 weeks.[69] Limitations of this study included a smaller than anticipated infarct size that may have limited achievement of a significant outcome. Further studies will be required to demonstrate whether treatment with rhLCAT can promote RCT and improve ASCVD outcomes.

Plasma phospholipid transfer protein (PLTP)

PLTP transfers phospholipids among HDL subpopulations and between VLDL and HDL (Figure 2). Remodeling of HDL in interstitial fluid by PLTP results in the formation of lipid-poor apoAI or pre-β HDL to act as acceptors of cellular cholesterol.[70] Despite the increased production of pre-β HDL by interstitial PLTP, increased plasma PLTP activity is associated with enhanced atherosclerosis in humans and mice, effects that are likely due to reduced production of nascent HDL by hepatic ABCA1 and increased VLDL production.[70] Thus far, little is known about the effects of HDL oxidation on PLTP activity. However, in vitro studies found that $HOCl^-$ prevents PLTP mediated dissociation of pre-β HDL from HDL_3, which could impact RCT.[71]

CE Transfer Protein (CETP)

HDL is further remodeled in humans by CE transfer protein (CETP), which is absent in mice. CETP transfers CEs from HDL to VLDL and triglycerides from VLDL to HDL resulting in the conversion of VLDL to IDL and LDL(Figure 2).[9] Importantly, CETP activity improves the ability of HDL to prevent oxidation of LDL by transferring peroxidized lipids from LDL to HDL for inactivation by HDL, as well as by delivery to the liver directly

or via SR-BI.[72, 73] Indeed, humans with homozygous CETP deficiency have increased MDA-LDL[74] and oxidized HDL enriched with modified phospholipids.[75] In addition, CETP expression in mice enhances the hepatic uptake of HDL CE via SR-BI in vivo, which may be due to its transfer activity as well as by CETP activity enhancing SR-BI expression.[76, 77] Furthermore, studies suggested an independent mechanism for direct delivery of CE to the liver by CETP.[78] However, it is worth noting that other studies showed that transgenic expression of CETP in mice increases RCT by promoting clearance of cholesterol via the hepatic LDL receptor.[79] In addition, studies have reported variable results on the effects of transgenic expression of CETP on atherosclerosis in mice, which is likely due to differences in the mouse model tested.[76, 80] In this regard, CETP likely enhances atherosclerosis in mice under conditions where hepatic clearance of LDL/remnant particles is impaired (i.e. *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$). Similarly, the impact of CETP on atherosclerosis in humans has been variable, which may depend upon the populations examined (sex, CETP variants, alcohol consumption) and/or the degree of CETP activity (for an in depth review see,[81]). A number of studies showed that CETP activity was inversely associated with CAD and/or that lower activity was associated with CVD events or mortality including the Framingham Heart Study, the LURIC Study, and KAROLA.[81, 82] In contrast, a recent large Mendelian randomization study in humans with CETP genetic variants found that a decrease in CETP activity was associated with reduced CAD risk, but the effect was suggested to be due more to the decrease in apoB levels (−1.4 mg/dL) than the increase in HDL-C levels (+4.6 mgd/L).[83] However, other studies on CETP genetic variants suggested a higher risk of CAD.[81, 82, 84] In addition, humans with complete loss of function due to mutations in the CETP gene have 3 to 6-fold higher HDL-C levels compared to controls, but they have enlarged HDL that is enriched with apoCIII, apoAII, and apoE and has reduced PON1 activity and CEC.[82, 85] Regardless of the controversy over the atherogenic effects of CETP, the premise that CETP inhibition would raise HDL-C, lower LDL-C, and presumably increase direct delivery of HDL-C to the liver, led to the development of CETP inhibitors such as torcetrapib, dalcetrapib, anacetrapib, and evacetrapib as potential therapeutic treatment for CAD in humans. However, these clinical trials have been largely disappointing. Administration of torcetrapib in humans with enhanced CVD risk, increased CVD events and mortality while raising HDL-C by 72%.[86] Despite raising HDL-C by 31% and 130%, respectively, dalcetrapib and evacetrapib had no effect on CVD events.[87, 88] Although anacetrapib reduced CVD events, the effect was attributed to lower LDL-C levels and not an increase in HDL, and the sponsor decided not to pursue approval.[89] Similar to CETP deficiency, CETP inhibition in humans led to the accumulation of apoC3 and apoE enriched HDL particles, which have impaired CEC and are associated with increased CAD risk.[90] Importantly, CETP inhibition in humans failed to enhance in vivo HDL RCT.[91] These observations suggest that uptake via the LDL receptor is the preferred route for delivery of cholesterol to the liver in humans. However, the findings that CETP can stimulate HDL CE selective uptake via SR-BI and that loss of CETP decreases hepatic SR-BI expression raises the possibility that CETP inhibition may also have impacted cholesterol delivery via the SR-BI pathway.[76, 77] Importantly, CETP inhibition in humans decreases the proportion of HDL particles containing PON1 and in vitro studies have shown that CETP inhibition markedly impairs PON1 activity.[90, 92] Taken together, these studies substantiate that raising HDL-C is not beneficial without improving the number of functional HDL

particles and suggest that CETP inhibition will exacerbate the effects of the heightened oxidative stress in individuals with CAD, thereby worsening HDL dysfunction in promoting RCT and preventing oxidation. Indeed, Hine et al.[73] proposed that the failure of CETP inhibitors to reduce atherosclerosis maybe due to the loss of CETP activity reducing LDL oxidation. In contrast, Schmidt et al. suggested that the failures of CETP inhibitors are likely compound and due to the heterogeneity of effects of the candidate drugs tested.[93] Furthermore, they performed Mendelian randomization analyses that support CETP as an effective target for CHD prevention.[93] A potential limitation of CETP inhibition is that it promotes accumulation of mature HDL; whereas treatment with dicarbonyl scavengers has the potential to block the modification of HDL in vivo and to protect the function of newly formed HDL.

Delivery of HDL-C to the Liver

In humans, the cholesteryl esters transferred from HDL to LDL by CETP are internalized by the hepatic LDL receptor for processing and/or trafficking of the cholesterol into bile, comprising a major route of delivery of cholesterol from HDL to the liver in humans (Figure 2). The importance of this pathway is exemplified in humans with FH who have markedly elevated levels of LDL-C due to mutations in the *Ldlr* gene[9]. The lack of this route in FH subjects leads to oxidation of LDL and HDL resulting in HDL dysfunction due in part to highly reactive dicarbonyls, including MDA, ONE, and IsoLG.[34–37] In mice, the main route of hepatic delivery is selective uptake of HDL CE and influx of HDL FC via SR-BI (Figure 2).[94, 95] Remodeling of HDL by CETP and hepatic triglyceride lipase enhances HDL CE uptake via SR-BI.[96] Although less substantial than the LDLR route, SR-BI also mediates direct delivery of HDL-C in humans (Figure 2) as substantiated by markedly elevated levels of HDL-C in subjects with loss of CE selective uptake function mutations in the *Scarb1* gene.[48–50] As shown recently, human carriers of the P376L *Scarb1* variant also have elevated oxidized HDL.[49] In addition, human carriers of a number of the *Scarb1* gene variants have markedly elevated levels of lipoprotein (a) Lp(a), an atherogenic LDL particle that is heavily enriched in oxidized lipids, suggesting that SR-BI is the preferential route for selective uptake of Lp(a) cholesterol.[97] In this regard, studies have shown that an in vivo function of SR-BI is to detoxify both HDL and Lp(a) via the selective uptake of oxidized phospholipids and CEs, thereby reducing the atherogenicity of Lp(a) and preserving HDL antioxidant status.[98, 99] Regardless of the pathway, it is likely that the increased oxidative stress in humans with CAD would impair both delivery routes for hepatic processing of cholesterol to the feces as the selective uptake of HDL CE via SR-BI is severely impaired by both HDL and LDL oxidation, and oxidation of LDL/HDL promotes uptake by nonparenchymal cells and macrophages in other tissues.[100, 101]

Measurements of HDL RCT in Humans and Relevance to Atherosclerosis

As the most easily measured step in RCT, emphasis has been placed on measurements of HDL CEC as it relates to CAD and CVD events and mortality in humans. Most studies have used assays geared toward measurement of ABCA1-mediated HDL CEC involving upregulation of ABCA1 with cAMP in cholesterol-normal mouse macrophages. Using this system, Khera and colleagues were the first to report an inverse relationship between CAD and HDL CEC that was independent of HDL-C, suggesting that HDL function is more

relevant to CAD risk.[102] This premise was then bolstered by other studies demonstrating an inverse association between HDL CEC and incidents of CVD events.[103, 104] However, in patients with high C reactive protein (CRP), there was no association of HDL CEC with incident CVD events at baseline, but after statin treatment and lowering of CRP, an inverse association was detected.[105] Similar results were observed in a CKD population.[106] These studies raise the possibility that measurements of ABCA1 CEC do not provide a reliable assessment of CVD event risk in populations with heightened inflammation. However, studies using cholesterol loaded THP-1 cells, which would test the overall ability of HDL to alleviate the macrophage cholesterol burden via multiple mechanisms and are likely more representative of human plaque cells, also found no association between HDL CEC and CAD and/or CVD events in subjects with either Type 2 Diabetes Mellitus or normal glucose metabolism.[107] Similar results were observed in subjects with end stage renal disease.[108] Taken together, the relationship between HDL CEC and CVD events is likely lost in populations with more vulnerable plaques with increased inflammation and oxidative stress. Indeed, other studies using the ABCA1 CEC system have shown positive associations between HDL CEC and major CVD events, including in populations with either CKD or enriched with smokers.[104, 109] Similarly, earlier studies with smaller populations showed a positive association with major CVD events, which is consistent with studies showing that plasma pre-β HDL levels are positively associated with CAD and CVD events.[110–112] Indeed, a number of populations with increased inflammation and oxidative stress (i.e. diabetics, hypertriglyceridemic, renal impaired, postmenopausal women with CAD) have increased pre-β HDL levels and ABCA1 CEC compared to controls.[104, 113–116] Taken together, these observations highlight that in certain populations with systemic inflammation, HDL RCT is impaired in vivo either via decreased expression or function of ABCA1 and/or decreased maturation of HDL (i.e. decreased LCAT). In this regard, a number of these populations have impaired LCAT activity.[67, 116–118] In addition, novel single cell analyses determined that proinflammatory macrophage populations (IL-1β+) in advanced plaques of humans express lower levels of lipid handling genes including ABCA1, ABCG1, and apoE.[119] These observations also stress that measuring whole body HDL RCT may be a better predictor of CAD and CVD events in humans. The novel method recently developed by Cuchel and colleagues using [H^3]-cholesterol-labeled nanoparticles to track the movement of macrophage cholesterol to feces in humans will hopefully make this examination feasible.[120]

HDL Anti-inflammatory Function

The CANTOS trial supports the importance of inflammation in atherosclerotic cardiovascular disease as treatment of humans with an IL-1β monoclonal antibody lowered recurrent cardiovascular events independent of cholesterol lowering.[121] Abundant evidence supports that HDL/ apoAI impairs inflammatory signaling, and the CANTOS trial substantiates the need to preserve HDL anti-inflammatory signaling pathways in order to reduce the residual risk of CVD events. Increased oxidative modifications of HDL/ ApoAI not only impair anti-inflammatory signaling but generate extremely proinflammatory HDL particles.[31, 32, 34, 36, 51, 122, 123] HDL exerts anti-inflammatory effects on all cell types relevant to atherosclerosis, which has been detailed in recent reviews.[124] Here, we

discuss the effects of HDL/ApoAI on endothelial activation, monocytosis, and macrophage inflammation as it relates to atherosclerosis and oxidative stress.

Effects of HDL/ApoAI on Endothelial Cell Activation

One of the early events in atherosclerosis is endothelial cell activation and a compromised endothelial barrier.[9] Triggers such as oxidized LDL and TNF-α activate nuclear factor kappa B (NF-κB) transcriptional activation leading to increased expression of inflammatory monocyte adhesion molecules and cytokines and downregulation of endothelial nitric oxide synthase (eNOS), thereby promoting increased monocyte infiltration (Figure 3). HDL is critical to reducing the inflammatory response by impairing endothelial cell activation and maintaining endothelial barrier integrity via activation of eNOS (see detailed review,[125]). Interaction of endothelial cell SR-BI with HDL activates eNOS by stimulating a signaling cascade involving Src Tyrosine kinase, PI-3K, Akt kinase, and Erk1/2 MAPK and phosphorylation of eNOS.[125] Cholesterol efflux via SR-BI is required for activation of eNOS.[126] ABCG1 also promotes NO production by stimulating efflux of cholesterol and oxidized cholesterol and relocating eNOS away from caveolin-1.[127] In addition, the HDL bioactive lipid, sphingosine-1-phosphate (S1P), which is complexed to apoM on HDL, also mediates activation of eNOS by interaction with $S1P_{1/3}$ receptors to induce PI-3K, Akt signaling.[125] The importance of the HDL/apoM/S1P complex in maintaining vascular integrity has been demonstrated in vivo where deletion and/or transgenic expression of apoM have opposite effects on barrier function.[128, 129] It is also worth noting that both of the HDL SR-BI and $S1P_{1/3}$ receptor signaling pathways stimulate endothelial cell survival and migration.[125] Importantly, the HDL induced production of NO in endothelial cells reduces NF-κB transcriptional activation, thereby decreasing expression of monocyte adhesion molecules (VCAM-1, ICAM-1, and P-selectin) (Figure 3) and proinflammatory receptors (toll-like receptor 2, TLR2) and cytokines (MCP-1 and IL-8).[125] In addition, HDL/ApoAI binding to SR-BI inhibits adhesion molecule expression by activating Akt to upregulate anti-inflammatory heme oxygenase-1 expression and 3-beta-hydroxysteroid-delta reductase.[125, 130] The HDL signaling pathways via $S1P_{1/3}$ receptors and ABCG1 also prevent activation of endothelial cell nucleotide oligomerization domain-like receptor protein with pyrin domain containing 3 (NLRP3) inflammasome and pyroptosis.[131, 132] A number of studies have suggested that HDL induced endothelial cell NO production is relevant to atherosclerosis in humans. HDL from subjects with Type 2 Diabetes have reduced ability to activate eNOS and HDL bound S1P is inversely associated with the severity of CAD.[133, 134] HDL from CAD versus control subjects has reduced capacity to stimulate eNOS and prevent inflammatory/adhesion gene expression in endothelial cells.[51, 135] Importantly, studies have shown that infusion of functional HDL into hypercholesterolemic or diabetic humans and subjects with low HDL due to heterozygous ABCA1 mutations stimulates eNOS activation and vasodilation.[125, 136, 137] In addition, a recent study demonstrated that HDL endothelial cell anti-inflammatory capacity was inversely associated with incident CVD events in a general population, effects that were independent of HDL-C levels and HDL CEC.[135] Interestingly, studies have shown that HDL from CAD subjects activates protein kinase CβII via LOX-1 leading to inhibition of eNOS activity via a mechanism involving reduced HDL PON1 activity and oxidative modification by endothelial cells.[51] In addition, inhibition of PON1 in HDL from control

subjects leads to MDA modification of HDL and activation of the LOX-1/PKCβII pathway, showing that oxidative modification impairs HDL endothelial cell anti-inflammatory capacity.[51] Furthermore, a recent study demonstrated that the plasma levels of HDL with LOX-1 binding capability in a Japanese population were associated with coronary artery calcification independent of HDL-C and particle number.[138] Taken together, these studies highlight that HDL endothelial cell anti-inflammatory capacity and/or HDL oxidative modifications may be a viable marker of incident CVD risk.

Effects of HDL/ApoAI on Monocytosis

The number of blood monocytes are increased in humans with hypercholesterolemia and CAD (Figure 3).[139] Studies in mice have shown that inflammatory Ly6C^{hi} versus anti-inflammatory, patrolling Ly6C^{low} monocytes dominate hypercholesterolemia-associated monocytosis in mice and give rise to inflammatory macrophages in the atheroma (Figure 3).[140] Human monocytes are heterogenous, but have been divided into three major subsets based on the differential expression of the lipopolysaccharide (LPS) receptor (CD14) and the FcγIII receptor (CD16) (for in depth reviews see[139, 141]). The classical CD14^{++}CD16^{-} and non-classical CD14^{+}CD16^{++} cells subsets are the human equivalent of the mouse Ly6C^{hi} and Ly6C^{low} monocytes.[139] However, it was later determined that the CD16+ positive monocytes possess both anti-inflammatory and inflammatory properties, and this subset was further divided into non-classical CD14^{+}CD16^{++} and intermediate CD14^{++}CD16^{+} cells. CD14^{++}CD16^{+} cells share some properties with both classical and non-classical monocytes but are distinguished by CCR5 (RANTES, MIP-1α, and MIP-1β receptor) expression and by the ability to produce inflammatory cytokines (IL-1β and TNF-α) and ROS.[139] Indeed, studies have shown that the numbers of intermediate CD14^{++}CD16^{+} monocytes are independently associated with CVD events in subjects with CKD and CAD and in a randomized population.[139, 142, 143] The increased number of blood monocytes in humans and mouse models with CAD results from enhanced proliferation of bone marrow hematopoietic stem and progenitor cells (HSPCs) due to hypercholesterolemia induced lipid raft signaling and ROS production (Figure 3).[141, 144] LDL and oxidized LDL induce expansion of granulocyte monocyte progenitors (GMPs), whereas functional HDL prevents the expansion via a number of signaling pathways, including ABCA1, ABCG1, and SR-BI.[141, 145, 146] ABCA1 and ABCG1 expressed on HSPCs promote cholesterol efflux to lipid-poor apoAI or HDL, thereby reducing plasma membrane lipid rafts and decreasing cell surface levels of the common β subunit of the IL-3/granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor.[141] In addition, HSPCs express abundant apoE, which promotes phospholipid and cholesterol efflux via ABCA1 and ABCG1 to reduce proliferative IL-3/GM-CSF receptor signaling.[141] SR-BI interaction with HDL limits proliferation of HSPCs by reducing activation of Akt and p38MAPK resulting in less ROS production.[146] The importance of these HDL/apoAI/apoE signaling pathways to HSPCs expansion is substantiated in studies demonstrating that mice with bone marrow deficient in ABCA1, ABCG1, and/or SR-BI have markedly enhanced inflammatory Ly6C^{hi} monocytosis, which exacerbates lesion progression.[141, 146] It is also worth noting that HDL from humans with CAD, diabetes, FH, and CKD and subjects who smoke have oxidative modifications, which are known to impair the ability of HDL to promote cholesterol efflux

and prevent cellular ROS production, which likely contributes to the enhanced monocytosis and accelerated atherosclerosis in these populations.[31–38, 138, 147, 148]

Effects of HDL/ApoAI on Macrophages

Studies have established that macrophage inflammation is a key driver of atherosclerosis progression and that inflammatory resolution is critical to preventing vulnerable plaque formation and promoting lesion remodeling and/or regression.[9] Macrophages can exhibit pro-inflammatory versus anti-inflammatory properties depending upon the environment, and HDL modulates the macrophage response to inflammatory stimuli. In the plaque, TLR ligands such as LPS and oxidized LDL and phospholipids promote inflammatory cytokine production.[9] Intestinally derived HDL_3 binds LPS, thereby preventing entry into the plaque.[149] HDL/apoAI also directly inhibits TLR signaling by promoting cholesterol efflux via ABCA1/ABCG1 to reduce plasma membrane lipid raft content of TLRs, which results in decreased myeloid differentiation primary response 88 (MyD88) and TIR-domain–containing adapter-inducing interferon-β (TRIF) signaling, culminating in suppression of NF-κB and type I interferon (IFN) activity.[150, 151] In addition, HDL reduces inflammatory signaling independently from its CEC function by relocating the TRIF-related adaptor molecule from the plasma membrane as well as by enhancing expression of activating transcription factor 3 to reduce inflammatory gene expression downstream of NF-κB (Figure 3).[151, 152] Furthermore, HDL interacts with SR-BI to activate Akt and suppress p38, Jnk, and NF-κB activation and TLR expression.[94, 153, 154] The suppression of NF-κB activation by HDL/ApoAI reduces expression of the NLRP3 inflammasome components, thereby reducing TLR priming of inflammasome activation and secretion of IL-1β and IL-18 in response to other signals such as excess FC (Figure 3).[155, 156] One of the most important processes to reduce uncontrolled inflammation, necrotic death, and formation of the vulnerable plaque is the efficient efferocytosis of apoptotic cells,[9] and evidence is accumulating that HDL facilitates macrophage efferocytosis. HDL induces an anti-inflammatory macrophage phenotype via JAK1 signaling to activate STAT6 leading to enhanced expression of arginase-1, which metabolizes apoptotic cell arginine, resulting in increased activation of Rac1 to facilitate continual rounds of efferocytosis (Figure 3).[157, 158] In addition, HDL interacts with T regulatory cell-SR-BI to promote survival and IL-13 secretion, which propagates efferocytosis signaling via stimulation of macrophage IL-10 production (Figure 3).[159, 160] As impaired autophagy facilitates plaque inflammasome activation and uncontrolled death, thereby impeding efferocytosis, the roles of ABCA1 and SR-BI in alleviating the efferocyte lysosomal cholesterol burden is also critical to vulnerable lesion formation (Figure 3).[26, 28, 161] The importance of the HDL anti-inflammatory pathways has been demonstrated in atherosclerotic mice, where deletion of bone marrow ABCA1, ABCG1, and/or SR-BI markedly increases systemic inflammation and plaque necrosis.[28, 53, 150, 156] Similar findings have been demonstrated in mice that are deficient in HDL (i.e. *Apoe*$^{-/-}$, *Ldlr*$^{-/-}$*Apoa1*$^{-/-}$) or that have dysfunctional HDL (*Scarb1*$^{-/-}$*Ldlr*$^{-/-}$, *Scarb1*$^{-/-}$*ApoeR61*$^{h/h}$).[162–164] Furthermore, humans that are heterozygous for loss of ABCA1 function have enhanced systemic and plaque inflammation[165]. It is also worth noting that HDL from humans with CAD versus controls have decreased ability to prevent macrophage inflammation in response to TLR ligands, and that oxidative modifications

of the HDL heighten proinflammatory signaling via other pathways (Discussed in detail later).[31, 32, 34, 36, 37]

HDL antioxidant function

Similar to its role in removing cholesterol from peripheral tissues via RCT, HDL also serves to protect other lipoproteins and cells from oxidative damage by removing and inactivating lipid hydroperoxides from other lipoproteins and cells. The mechanisms of HDL's antioxidant activity have been primarily studied using its effects on LDL oxidation as a model.[166] HDL significantly inhibits the peroxidation of LDL lipids by Cu^{2+}, while only undergoing minimal lipid peroxidation itself.[167] Importantly, HDL's antioxidant activity requires enzyme activity rather than its chelation of redox-metals.[168] CETP facilitates the transfer of phospholipid and CE hydroperoxides from oxidized LDL to HDL, where these lipid hydroperoxides are reduced to lipid hydroxides, terminating peroxidation chain reactions.[72, 73] PON1 appears to be the most important antioxidant enzyme associated with HDL. Other key proteins with antioxidant activity associated with HDL include apoAI, apoA2, apoA4, apoE, apoM, serum amyloid A, and potentially others.[166]

PON1 associates with HDL through its cooperative binding to both apoAI and adjacent phospholipids (Figure 4).[169] Purified PON1 effectively inhibits Cu^{2+}-induced peroxidation of LDL.[167] PON1 catalyzes the hydrolysis of lipid peroxides[170] as well as catalyzing lipolactonase, paraoxonase, and arylesterase activities, with all of these activities being strongly correlated.[171] Importantly, the enzymatic activity of PON1 is markedly increased by its association with apoAI.[172] For this reason, apoAI modifications that decrease the ability of PON1 to bind have the net effect of decreasing PON1 activity.[34, 173] Besides its direct antioxidant enzyme activity, PON1 may also exert antioxidant effects indirectly by inhibiting MPO activity (Figure 4). Both PON1 and MPO bind to apoAI, and PON1 binding markedly inhibits MPO's activity (and vice versa).[169] Both animal and clinical studies support an important role for PON1 in protecting against atherosclerosis. Transgenic expression of human PON1 reduces atherosclerosis in *Ldlr*$^{-/-}$*; ob/ob* mice[174], while deleting *Pon1* exacerbates atherosclerosis in Apoe$^{-/-}$ mice[175]. Two common polymorphisms, L55M and Q192R, are found in the human PON1 gene. These polymorphisms alter PON1 activity, as well as the risk for cardiovascular disease.[176–179] Importantly, PON1 activity appears to be markedly reduced in patients with verified cardiovascular disease.[180] One potential mechanism for reduced PON1 activity is oxidative modification of PON1 and apoAI.

ApoA1 directly contributes its own antioxidant activity to HDL, in addition to enhancing PON1 activity (Figure 4). Mature, processed human apoAI includes three methionine (Met) residues, Met86, Met112, and Met148. Incubation of either HDL or isolated apoAI with CE hydroperoxides or phospholipid hydroperoxides reduces these lipid hydroperoxides to lipid hydroxides with concomitant oxidation of Met residues.[181] In canine apoAI, valine and leucine are substituted for Met112 and Met148, respectively, and canine HDL shows far weaker antioxidant activity than human HDL.[181] Interestingly, oxidation of the Met residues of apoAI enhances, rather than diminishes, its capacity to catalyze cholesterol and phospholipid efflux.[182]

ApoA2 can replace one or more apoAI molecules on an individual HDL lipoprotein particle, with apoA2 contributing 12% to 32% of the total apoAI/2 depending on the individual HDL subfraction examined.[183] Recombinant HDL particles generated exclusively with apoA2 show greater capacity to reduce lipid hydroperoxides to hydroxides than those generated with exclusively apoAI (Figue 4).[181] However, transgenic mice overexpressing apoA2 show significantly worsened atherosclerosis.[184] The effect of apoA2 overexpression may be the result of apoA2 association with native HDL displacing PON1, leading to an overall net negative impact on HDL antioxidant activity.[184]

ApoA4 is synthesized in the intestine and initially associates with chylomicrons in lymph. In plasma, about 25% of this apoA4 transfers to HDL, while the remaining circulates in a lipoprotein-free fraction.[185, 186] Purified apoA4 inhibits lipid peroxidation when Cu^{2+} is used to oxidize lymph and LDL.[187] Overexpression of apoA4 reduces oxidative damage and atherosclerosis in vivo,[188] but whether these protective effects depend on its association with HDL remains to be demonstrated.

ApoE associates with chylomicron remnants, VLDL, IDL, and HDL. ApoE has three isoforms (ε2,ε3,and ε4) with ε3 being the most common isoform in human populations. Compared to the ε3, the ε2 and ε4 isoforms increase risk for cardiovascular disease and the ε4 isoform increases risk for Alzheimer's disease.[189] In vitro, all three isoforms provide some measure of protection against against H_2O_2 induced cytotoxicity or Cu^{2+} induced LDL oxidation, with rank order of efficacy being ε2>ε3>ε4,[190, 191] but when antioxidant activity is measured by protection against macrophage-induced LDL oxidation, the ε4 isoform shows greater efficacy.[191] These modest and contradictory differences suggest that the antioxidant activity of apoE is not as relevant to protection against atherogenesis as its effects on lipoprotein clearance and/or the impact of macrophage endogenous apoE effects on cholesterol efflux and inflammation.[9]

ApoM associates with all lipoprotein particles, but is most enriched in HDL, with about 5% of HDL particles carrying apoM.[192] HDL containing apoM more efficiently inhibits Cu^{2+}-mediated LDL oxidation than HDL lacking apoM.[192] One potential mechanism for this antioxidant effect is the ability of apoM to bind and sequester oxidized phospholipids[193]. *Ldlr*$^{-/-}$ mice with transgenic expression of apoM have significantly less atherosclerosis than non-transgenic *Ldlr*$^{-/-}$ mice;[194] however, whether this anti-atherosclerosis effect is the result of HDL antioxidant activity is unclear.

In summary, the antioxidant activities of HDL derive from multiple components associated with these lipoproteins. For some of these proteins, association with HDL may not be essential to exert their protective effects, but it seems likely that HDL acts as an organizing scaffold to coordinate their activities and thereby maximize their effectiveness.

Oxidative modification of HDL proteins in vivo

HDL isolated from patients with cardiovascular disease or directly from atherosclerotic lesions shows strong evidence of enhanced oxidative modification both by amino acid oxidation and adduction of lipid carbonyls, but whether the extent of modification is

sufficient to fully account for HDL dysfunction remains an open question. One challenge to capturing the full extent of HDL modification in vivo is the number of different modifications, especially in terms of those formed by reactive lipid carbonyls. For instance, when IsoLG reacts with Lys, it can form six stable monoadducts (including the IsoLG-lactam-Lys adduct shown in Fig. 1c) and multiple crosslinked Lys adduct species (including the simplest IsoLG-dipyrrole crosslinked adduct shown in Figure 1d). Furthermore, IsoLGs initially form esterified to phospholipids and then undergo hydrolysis by an as-yet-uncharacterized phospholipase, but esterified adducts are difficult to quantitatively convert to their unesterified forms due to their lability to even mild saponification conditions.[195] While MDA primarily forms *N*-propenal-Lys monoadducts (Fig.1a) and Lys-1-amino-3-iminopropene-Lys crosslinks (Figure 1b), it can also form *N*-dihydropyridine-Lys monoadducts, and small amounts of *N*-propenal-His monoadducts and *N*-propenal-Arg monoadducts. ACR can form S-propanal-Cys monoadducts (Figure 1g) and His-propenamine-Cys crosslinks (Fig. 1h), but also *N*-3-formyl-3,4-dehydropiperidino-Lys monoadducts, *N,N*-Propano-Arg monoadducts, *N*-propanal-His monoadducts, and a number other propenamine crosslinks. ONE forms N-4-ketoamide-Lys monoadducts (Fig. 1e) and Lys-pyrrole-Cys crosslinks (Fig. 1f), but also Lys-pyrrole-His crosslinks and Lys-pyrrole-Arg crosslinks. Thus, monitoring all possible post-translational modifications of apoAI or other HDL protein is highly challenging. Given these limitations, typically only levels of the most readily measurable of the oxidative modifications are reported. While this may substantially underestimate the total extent of modification, it does allow for comparison between different patient populations or tissue sites.

Serum HDL isolated from patients with coronary artery disease showed increased levels of 3-Cl-Tyr, NO_2-Tyr, and carbamyl-Lys compared to control individuals.[12, 196] ApoAI isolated from patients with CAD compared to controls also showed increased 3-Cl-Tyr, NO2-Tyr, and carbamyl-Lys modification[196–198]. Levels of Lys modified by IsoLG, ONE, ACR, HNE, and MDA are increased in serum HDL isolated from individuals with CAD and/or FH, who are at increased risk of early onset of atherosclerotic cardiovascular disease.[34–36, 199] In addition, HNE and MDA adducts as well as carbamyl-apoAI are increased in HDL from individuals with CKD, which is associated with increased risk of ASCVD[32, 200]. Urinary apoAI from individuals with CKD shows higher levels of IsoLG modification.[201]

Another challenge to understanding the actual extent of HDL modification in vivo and whether this correlates with disease progression is that modification of HDL proteins such as apoAI appears to primarily occur outside the plasma compartment. This conclusion is based on the findings that levels of apoAI modification are significantly greater for apoAI/HDL isolated from atherosclerotic lesions compared to that isolated from circulation. For example, average levels of apoAI oxTrp72 are 0.07 mmol/mol apoAI in plasma, but are 204 mmol/mol apoAI in aortic plaques.[122] Average oxidized apoAI Tyr levels in apoAI isolated from plasma of CVD patients were 0.63 mmol NO2-Tyr/mol Tyr and 0.5 mmol Cl-Tyr /mol Tyr; while average levels in apoAI isolated from human aortic atherosclerotic lesions were 2.3 mmol NO2-Tyr/mol Tyr and 3.9 mmol Cl-Tyr/mol Tyr[196]. Average levels of carbamyl-Lys in HDL isolated from plasma were approximately 5 mmol/mol apoAI, while in HDL isolated from lesions it is 45 mmol/mol apoAI.[32] Using an ELISA specific for MDA-modification, MDA epitopes were 3.6-fold higher in HDL isolated from

atherosclerotic lesions than HDL isolated from plasma.[33] The ONE-ketoamide-Lys and IsoLG-Lactam-Lys in HDL isolated from plasma of patients with FH are 2.0 mmol/mol apoAI and 0.6 mmol/mol apoAI, respectively, but their levels in atherosclerotic lesions have not been determined. Whether apoAI oxidatively modified in vascular beds enters lymphatics and returns to circulation or is instead trapped by macrophages and dendritic cells in these beds or draining lymph nodes is unknown, but a better understanding of the fate of oxidatively modified HDL would further our understanding of its contribution to disease.

Oxidative modification of HDL causes HDL dysfunction

Effects of HDL oxidiative modifications on RCT

The most direct evidence that oxidative modifications alter HDL function comes from studies where isolated HDL or HDL proteins are exposed to ROS-generating enzymes such as MPO, specific ROS or individual reactive lipid dicarbonyls. The capacity of HDL to facilitate ABCA1-mediated cholesterol efflux from macrophages is markedly reduced after exposure to MPO and cofactors (H_2O_2, Cl^-, and NO_2).[66, 196] Exposure of HDL to MPO leads to oxidation of Met, Tyr, and Trp residues, as well as significantly increasing the levels of IsoLG-, MDA- and ACR-Lys adducts on HDL.[34, 36, 202].

The precise mechanisms whereby oxidative modification of HDL inhibits cholesterol efflux are poorly understood. Although oxidative modification of specific apoAI residues have been implicated in at least some studies, other types of oxidative modification to the same residue have no effect. Specific oxidative modifications may therefore give rise to altered protein structures that in turn alter interactions with key proteins like ABCA1, SR-BI, and CD36, but future studies are needed to assess this hypothesis.. A series of in vitro studies found that mutation of the four apoAI Trp residues to phenylalanine (4WF apoAI) provided significant protection against MPO-induced inhibition of ABCA1-mediated cholesterol efflux and that mutating Trp72 alone was sufficient to provide significant protection.[122, 203] These same investigators found that mutating apoAI Met residues to Val, mutating Tyr residues to phenylalanine (Phe), or chemically methylating Lys to make its less reactive with lipid carbonyls, all failed to protect apoAI from MPO-induced inhibition of cholesterol efflux via ABCA1.[122, 203, 204] Injection of recombinant apoAI engineered to have oxidized Trp only at Trp72 into $Apoa1^{-/-}$ mice showed that this engineered apoAI exhibited reduced lipidation and HDL biogenesis.[205] While these studies suggested that oxidation of apoAI Trp reduces HDL efflux capacity, subsequent in vivo studies with $Ldlr^{-/-}$ mice that overexpressed transgenic 4WF apoAI showed no greater lesion regression than $Ldlr^{-/-}$ mice overexpressing transgenic wild-type human apoAI.[206] Furthermore, 4WF apoAI transgenic mice showed less reverse cholesterol transport to the plasma compartment than mice expressing transgenic WT human apoAI, although they did show similar overall reverse cholesterol transport to liver and feces.[207] Other studies found that in vitro oxidation of HDL3 with either HOCl or MPO reduced HDL LCAT activity in direct correlation to the extent that apoAI Met148 and Trp72 were oxidized, but only Leu mutations of the three apoAI Met residues protected against MPO-induced LCAT inhibition, while deletion of the

four apoAI Trp residues provided no protection.[208] Thus, apoAI Met oxidation may play a role in the overall loss of cholesterol efflux capacity in vivo.

Modification of HDL or apoAI with IsoLG, MDA, ACR, or KODA-PC results in significantly reduced ABCA1-mediated cholesterol efflux, while modification of apoAI with other lipid carbonyls including HNE, ONE, and MGO does not inhibit ABCA1-mediated efflux.[33, 36, 37, 202, 209, 210] MDA modifies a number of apoAI Lys including Lys118, Lys133, Lys 182, Lys195, Lys206, Lys226, and Lys239.[33] ACR modifies Lys12, Lys23, Lys77, Lys88, Lys118, Lys140, Lys182, and Lys226, with ACR modification of Lys226 showing the greatest correlation with loss of efflux activity.[202] The Lys modified by IsoLG have not yet been published. Although KODA-PC and ONE are both γ-keto-alkenals with presumably similar adduction chemistry, ONE modification of apoAI does not alter cholesterol efflux, while KODA-PC inhibits efflux[37, 210] ONE modifies Lys12, Lys23, Lys96, and Lys226[37], while KODA-PC was found to form Lys-pyrrole-His crosslinks between a number of Lys/His pairs (His155/Lys106, His162/Lys94, His162/Lys96, His193,Lys195,His199/Lys195, His135/Lys182, His155/Lys12, His199/Lys133, His199/Lys140, His193/Lys140, and His193/Lys208).[210] Thus, while apoAI Lys modification by specific lipid carbonyls result in reduced ABCA1-medidated efflux, further studies are needed to determine the mechanisms underlying this reduction.

Like ABCA1-mediated efflux, how oxidative modification of HDL alters SR-BI-mediated efflux remains poorly understood. Carbamylation of apoAI Lys inhibits SR-BI mediated efflux and carbamylation of HDL increases its binding affinity to SR-BI, causing carbamylated-HDL to be internalized and promote foam cell formation.[32] Interestingly, carbamylation of HDL does not inhibit ABCA1-mediated cholesterol efflux.[32] In vitro oxidation of lipid poor apoAI using MPO produces carbamylation of Lys40, Lys45, Lys118, and Lys 195, while using CNO- also produces carbamylation on Lys23, Lys40, Lys59, Lys94, Lys96, Lys106,Lys107, Lys118, Lys195, and Lys226.[198] Interestingly, HDL treatment with CNO- that produced less than one carbamyl-Lys per apoAI was still sufficient to inhibit SR-BI cholesterol efflux and CE selective uptake RCT functions, thereby converting SR-BI into a nonproductive and potentially proatherogenic receptor.[32] A similar mechanism may be at play with ACR modification, as ACR modification of HDL inhibits SR-BI-mediated cholesterol efflux and macrophages become foam cells in the presence of ACR-HDL.[209] Similar results have been seen for MPO-mediated modification of other plasma proteins (i.e. BSA) that bind SR-BI with higher affinity than native HDL, thereby impairing SR-BI/HDL RCT functions. In this regard, studies are needed to determine how specific modifications of apoAI residues alter SR-BI interactions.

CD36 is another scavenger receptor that may play a role in modulating cholesterol handling. HDL from humans with CAD interacts with CD36 for uptake and degradation resulting in foam cell formation and studies have shown that both HNE- and MDA-HDL interact with platelet CD36.[31, 200] However, to date, no studies have determined the effects of specific modifications of HDL on interaction with macrophage CD36 or the effects such modifications have on cholesterol flux or inflammation.

In RCT, the esterification of HDL FC by LCAT is critical for maintaining the flux of cholesterol from the periphery to the liver, and humans with CAD and CKD have reduced LCAT activity, and a number of apoAI oxidative modifications impair activation of LCAT. NO_2-Tyr^{166}-apoA-I markedly decreases LCAT activity as a result of decreased LCAT/apoAI interaction.[63] In addition, MGO modification of reconstituted apoAI particles results in glycation of ApoAI Lys, Trp, and Arg residues, which decreases the interaction of ApoAI with LCAT and lipid.[211] Furthermore, studies have shown that ACR and HNE modifications of free Cys and His residues present in the catalytic pocket of LCAT markedly inhibit its activity. In addition, MDA, ACR, and HNE modified HDL particles are poor substrates for LCAT, which is likely due to altered interaction with apoAI.[64, 212]

Effects of HDL oxidative modifications on Inflammation

Oxidative modifications of HDL not only cause it to lose its ability to suppress inflammation, but can convert HDL into proinflammatory HDL. HDL is critical to reducing endothelial cell activation and maintaining endothelial barrier integrity via NO production in response to inflammatory stimuli, and specific MPO-mediated oxidative modifications of HDL/apoAI impair eNOS activation and increase inflammatory activation and death even in the absence of other stimuli (i.e. TNF-α).[125] Indeed, oxTrp72- apoAI isolated from human atheroma enhances expression of proinflammatory VCAM-1 and activates NF-κB without other stimuli.[122] In addition, MDA modification of apoAI-lys causes HDL to activate protein kinase CβII via interaction with LOX-1 leading to decreased activation of eNOS and increased endothelial cell activation.[51] Interestingly, carbamyl-HDL inhibits SR-BI-mediated eNOS activation and promotes apoptosis, which is consistent with carbamyl-apoAI inhibiting SR-B-mediated cholesterol efflux that is required for activation of eNOS.[123, 213] Unoxidized HDL suppresses VCAM-1 surface expression in endothelial cells normally induced by TNFa, but when HDL is oxidized with MPO, it enhances VCAM-1 expression.[214] Similarly, unmodified HDL suppresses expression of Tnfα and Il-1β by macrophages that is induced by LPS, but HDL modified by IsoLG greatly enhances the expression of these cytokines.[36] HDL modified with ONE fails to suppress LPS-induced Tnfa expression, but does not further enhance Tnfa expression, which demonstrates that ONE modification impacts anti-inflammatory function independent of ABCA1-mediated cholesterol efflux signaling pathways that impact TLR signaling.[37] In this regard, ONE modification of HDL could impact its interaction with SR-BI, which also mediates inflammatory signaling in response to LPS.[94, 153, 154] Interestingly, MDA-HDL enhances LPS induced expression of IL-1β and IL-6, but it is not as proinflammatory as IsoLG-HDL.[34, 36] The proinflammatory nature of Iso-LG- and MDA-HDL suggests interaction with receptors that mediate inflammatory signaling pathways such as CD36 and LOX-1. Studies have shown that HDL from CAD subjects interact with both CD36 and LOX-1 to activate NF-κB and enhance proinflammatory gene expression. Studies are needed to determine the mechanisms by which MDA- and IsoLG-HDL are proinflammatory.

Effects of HDL oxidative modifications on antioxidant function.

HDL oxidized by MPO has reduced PON1 activity.[169] Addition of SIN-1 (a peroxynitrite generator) to HDL also reduces PON1 activity.[215] Treatment of HDL with IsoLG, MDA, or ACR reduces PON1 activity,[34, 173] although the mechanisms by which each of these

lipid carbonyls inhibit PON1 may differ. MPO-induced oxidation of HDL leads to IsoLG modification of PON1, and IsoLG added directly to PON1 inhibits its activity.[173] MDA added to HDL modifies apoAI so that it has reduced ability to activate PON1.[34] The mechanism by which ACR modification of HDL inhibits PON1 has not be elucidated. Information as to which specific residues of PON1 may be important for the loss of activity when HDL is oxidized is limited. PON1 Tyr71 is oxidized to 3-Cl-Tyr by MPO and mutation of Tyr71 to alanine, aspartic acid or lysine caused a loss of PON1 activity.[169] The PON1 Lys modified by IsoLG have not been determined.

More definitive evidence that oxidative modifications of HDL proteins such as apoAI and PON1 are a root cause of HDL dysfunction seen in cardiovascular disease is still needed. Studies examining correlation between specific amino acid modifications of HDL proteins and HDL function are limited. One study in individuals with coronary artery disease found no correlation between HDL oxidized Tyr residues and cholesterol efflux.[216] In contrast, a second study reported an inverse correlation between levels of oxidized apoAI Tyr residues and cholesterol efflux.[196] More intriguingly, these investigators immunopurified apoAI from six patients using an antibody that recognized apoAI modified by Trp72 oxidation. This immunopurified apoAI showed poor CEC and when apoAI oxTrp72 levels were examined in 627 individuals, there was a strong correlation between their oxTrp72 levels and coronary artery disease.[122] However, these studies did not attempt to measure individual cholesterol efflux capacities in these samples, so whether there were robust inverse correlations between oxTrp72 levels and efflux capacity is unknown. To the best of our knowledge, there have been no studies that have examined whether there are correlations between specific reactive lipid dicarbonyl modifications and HDL functionality. Clearly, more studies examining the relationship between the extent of oxidative modification and HDL function are needed. If oxidative modification of HDL proteins is an important driver of HDL dysfunction, then blocking or reversing oxidative modification of HDL proteins must be shown to restore HDL function and reduce cardiovascular disease in vivo. A potential approach to test this will be addressed in the next section.

Small molecule scavengers of lipid dicarbonyls as inhibitors of oxidative modification.

A number of small molecules have been identified that are excellent scavengers of reactive lipid monocarbonyls and dicarbonyls and therefore have potential for use to protect lipoproteins from oxidative modification. These include thiol-based scavengers such as 2-mercaptoethanesulfonate (MESNA) and amifostine, imidazole-based scavengers like carnosine and its derivatives, and 2-aminomethylphenol-based scavengers of reactive dicarbonyls (Figure 5).

ACR is efficiently captured and inactivated by thiol-based scavengers including MESNA and the active form (WR-1065) of the prodrug amifostine.[217] Formation of ACR is a byproduct of caner therapies such as cyclophosphamide treatments or radiation therapy and both MESNA and amifostine are widely used as adjuvants during cancer treatment to limit off-target toxicities. In addition to their use in cancer treatments, MESNA and

amifostine have shown efficacy in animal models of other disease conditions with increased oxidative damage including ulcerative colitis, liver injury, Parkinson's disease, and ischemia/reperfusion.[218–222] Given their efficacy at scavenging ACR, it is surprising that to the best of our knowledge there have been no animal or clinical trials examining their effects in cardiovascular disease.

Imidazole-based scavengers effectively capture ACR and other α,β-unsaturated carbonyls like ONE and HNE. In animal models of disease, carnosine or its derivatives, have shown efficacy to protect against complications of diabetes, ischemia/reperfusion injury, and atherosclerosis.[223–229] Very limited clinical studies have looked at the efficacy of carnosine or its derivatives in cardiometabolic diseases, these include a 12-week supplementation study in overweight/obese individuals that showed improved glucose tolerance,[230] and a six month supplementation study that showed improved exercise tolerance in individuals with stable chronic heart failure.[231]

More recently, an entirely new class of scavengers directed against reactive lipid dicarbonyls have been identified and characterized, all of which contain a 2-aminomethylphenol (2-AMP) structural backbone. These novel dicarbonyl scavengers include 2-hydroxybenzylamine (2HOBA) and 5'-O-pentyl-pyridoxamine (PPM), as well as a number of other analogs.[232] The compounds effectively scavenge reactive dicarbonyls including IsoLG, ONE, and MDA, and to a lesser extent some monocarbonyls like ACR. They have shown remarkable efficacy in a number of animal models of oxidative damage including Alzheimer's Disease[233], hypertension[232, 234, 235], proteinuric kidney disease[201] lupus[236], cardiac ischemia/reperfusion injury[237] and gastric cancer.[238]

Dicarbonyl Scavengers Reduce Atherosclerosis

FH is a common autosomal codominant disorder associated with severely elevated levels of LDL-C and increased risk of premature atherosclerotic cardiovascular disease. Pathogenic mutations in the LDLR gene are the most common cause of FH, resulting in delayed clearance of LDL by the liver. Although increased levels of LDL-C and oxidatively modified LDL have long been recognized as critical mediators of the pathogenesis of atherosclerosis in FH, there is substantial evidence that HDL metabolism and function are abnormal in FH.[239, 240] HDL in subjects with FH have reduced capacity to promote RCT, impaired anti-inflammatory and antioxidant activities.[239, 240] Indeed, we have found that HDL from subjects with FH shows increased modification by reactive dicarbonyl species, including MDA and IsoLG, compared to HDL from individuals with normal cholesterol levels.[34–37] Furthermore, HDL from humans with severe FH showed impaired CEC and promote cholesterol loading of macrophages.[35] Therefore, we examined the hypothesis that treatment of *Ldlr*$^{-/-}$ mice, a murine model of FH, with the dicarbonyl scavenger, 2-hydroxybenzylamine (2-HOBA) would reduce modification of HDL and LDL, improve HDL function, and reduce the development of atherosclerosis. Treatment of female *Ldlr*$^{-/-}$ mice fed a western-type diet for 16 weeks with 2-HOBA reduced the extent of atherosclerosis in sections of the proximal aorta by 31% and by 60% in *en face* analysis of the aortas compared to controls treated with water or a geometric isomer 4-HOBA that is an ineffective scavenger.[35] Similar reductions in atherosclerosis were also seen in male *Ldlr*$^{-/-}$

mice treated with 2-HOBA. The reductions in the extent of atherosclerosis were especially impressive given that they occurred in the absence of changes in plasma cholesterol or triglyceride levels or lipid profiles.[35] Importantly, treatment with 2-HOBA dramatically reduced the amount of MDA-protein adducts in the proximal aorta as determined by immunofluorescence and the amount of MDA- and IsoLG-lysyl adducts in the whole aorta as quantitated by LC/MS/MS, compared to controls treated with 4-HOBA. Treatment with 2-HOBA promoted features of plaque stabilization with increased collagen content and fibrous cap thickness and reduced the percentage of necrotic area by 75%. Inadequate efferocytosis of dead cells can promote inflammation and necrotic core formation. Treatment with 2-HOBA was associated with a 72% reduction in the numbers of apoptotic cells and increased efferocytosis in the atherosclerotic lesions compared to treatment with vehicle or 4-HOBA.[35] Furthermore, serum levels of inflammatory cytokines, including IL-1β, IL-6, TNF-α, and serum amyloid A were reduced by 2-HOBA.[35] In vitro studies showed that 2-HOBA treatment reduced the expression of inflammatory cytokines produced by macrophages exposed to OxLDL or H_2O_2. Taken together these results support the ability of dicarbonyl scavenging with 2-HOBA to reduce oxidative stress, inflammation, cell death, destabilization of the plaque, and the extent of atherosclerosis (Figure 6).

Consistent with evidence for increased modification of HDL by reactive dicarbonyls in humans with FH, *Ldlr*$^{-/-}$ mice fed a western diet have increased levels of MDA-apoAI adducts compared to chow fed mice.[35] Most importantly, 2-HOBA treatment of *Ldlr*$^{-/-}$ mice substantially reduced MDA modification of both apoAI and HDL, and HDL isolated from the 2-HOBA treated mice had improved ability to promote macrophage cholesterol efflux compared to HDL from vehicle or 4-HOBA treated controls (Figure 6). Furthermore, 2-HOBA treated *Ldlr*$^{-/-}$ mice had reduced MDA-modification of LDL. Thus, reduced modification of both HDL and LDL by reactive dicarbonyls, including MDA and IsoLG, likely contribute to the antiatherogenic properties of 2-HOBA (Figure 6). In addition, 2-HOBA may prevent other MPO-induced oxidative modifications of HDL (i.e. apoA1 oxTrp72) as in vitro treatment with 2-HOBA negated the MPO-mediated impairment of HDL CEC.and prevented $HOCl^-$ induced crosslinked of lipid-free apoAI.[34, 241] While this possibility needs to examined in vivo, it is worth noting that studies have shown that the scavenger PM prevents in vivo oxidation of Trp192 in renal collagen in a diabetic rat model.[242] Therefore, dicarbonyl scavengers offer a potential new approach to reduce the residual risk of atherosclerotic cardiovascular events that remains in patients treated with statins (Figure 6).[243, 244] Phase 1 studies of treating humans with 2-HOBA have demonstrated its safety,[245] and we are initiating a Phase 2 trial (NCT#04941599) in humans with heterozygous FH to examine the impact of 2-HOBA on modification of HDL and HDL CEC.

Dicarbonyl Scavenger PPM Reduces Insulin Resistance and Hepatic Fat Accumulation

We have recently extended our studies of the therapeutic potential of dicarbonyl scavengers for treating atherosclerosis by examining the impact of another potent dicarbonyl scavenger, 5'-O-pentyl-pyridoxamine (PPM), on the development of atherosclerosis in *Ldlr*$^{-/-}$ mice fed a western diet for 16 weeks.[241] Similar to our results with 2-HOBA, treatment of both male and female *Ldlr*$^{-/-}$ mice resulted in substantial reductions in the extent of atherosclerosis

in the absence of changes in plasma cholesterol and triglyceride levels.[241] In addition, PPM reduced blood Ly6C^{hi} monocytosis, decreased lesion pro-inflammatory macrophages, enhanced efferocytosis, promoting features of plaque stabilization with dramatic reductions in plaque necrotic area (Figure 6).[241] Furthermore, HDL from the PPM treated mice showed improved CEC compared to mice treated with vehicle.[241]

Mounting evidence supports antidiabetic functions of HDL. HDL can increase glucose uptake of by skeletal muscle and HDL can improve blood glucose levels in animal models by increasing the synthesis and secretion of insulin from pancreatic β cells.[246, 247] HDL can prevent oxLDL-induced and ER stress-induced apoptosis of pancreatic β cells.[248, 249] Interestingly, a metanalysis of the CETP inhibitor trials revealed a 12% reduction in the development of T2DM.[250] Furthermore, infusion of reconstituted HDL was found to increase plasma insulin levels and decrease plasma glucose in patients with T2D by activating AMP-activated protein kinase.[247, 251] Therefore, we examined the impact of PPM treatment on insulin sensitivity in male *Ldlr*$^{-/-}$ mice fed a western diet. Interestingly, treatment of *Ldlr*$^{-/-}$ mice with PPM promoted reductions in fasting glucose and insulin levels, reduced the HOMA-IR, and improved glucose and insulin tolerance.[241] Furthermore, PPM protected male *Ldlr*$^{-/-}$ mice fed a western diet from developing hepatic steatosis.[241] Thus, treatment with the dicarbonyl scavenger PPM reduces insulin resistance and hepatic fat accumulation in a model of diet-induced metabolic syndrome. Hence, reactive dicarbonyl scavengers have attractive therapeutic potential for treating multiple features of cardiometabolic disease.

Conclusions and Future Directions

Numerous in vitro and in vivo studies with atherosclerotic animal models support the role of functional HDL/apoAI in limiting atherosclerosis progression and stimulating plaque regression (Figure 6). However, unlike findings in atherosclerotic mouse models, therapeutics which either raise HDL-C or increase apoAI levels in humans have disappointingly produced no benefit to reducing atherosclerotic plaques. The heightened oxidative stress in CAD leads to extensive oxidative modifications of HDL that not only impair its atheroprotective functions, but produce proinflammatory/proatherogenic HDL particles. Indeed, treatment of mouse models with oxidized versus native HDL/apoAI promotes plaque development and inflammation.[10, 11] The findings that human atherosclerotic plaques contain lipid-poor apoAI, which is mostly oxidatively modified and associated with MPO, and that human atherosclerotic lesions have markedly increased MPO levels compared to mouse lesions,[12, 252] likely explains the lack of benefit of HDL/apoAI raising treatments in humans as any HDL particles entering the intima are likely oxidatively modified. However, a limitation to the studies thus far on the role of HDL oxidative modifications in atherosclerosis is that they are largely correlative. In this regard, in vivo studies are needed on the mechanisms by which specific oxidative modifications of HDL proteins and lipids impact atherosclerosis as well as the protein structural changes resulting from specific oxidation modifications that promote HDL dysfunction in atherosclerosis in order to develop more targeted therapeutic strategies. In particular, the impact of HDL modification with specific reactive carbonyls on existing atherosclerotic plaque extent and inflammatory composition needs to be examined, which could lead to the development

of functional ApoAI mutants that are resistant to dicarbonyl modification. It is worth noting that despite the abundant evidence supporting the role oxidative modifications in atherosclerosis, clinical studies assessing the impact of dietary anti-oxidant in humans have shown no impact on lipoprotein modifications and atherosclerosis. However, these dietary anti-oxidants also failed to reduce LOOH levels and thus, prevent toxic reactive carbonyl modifications. The finding that neutralization of reactive lipid carbonyls with 2-AMPs effectively reduces atherosclerosis and improves HDL function in atherosclerotic mouse models offers promise of beneficial treatment of CAD in humans. The observation that 2-AMPs are atheroprotective in the setting of hypercholesterolemia suggests that dicarbonyl scavenging has the potential to alleviate the residual inflammatory risk of CAD that exists even with LDL cholesterol lowering in humans. Furthermore, the atheroprotective effects of 2-AMPs likely go beyond preserving HDL function as AMPs almost certainly prevent carbonyl modification of other plasma and cellular proteins and lipids, which could impact atherosclerosis. In this regard, 2-AMPs offer great potential for targeting CAD in humans.

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List of Abbreviations

ACR	acrolein
ABCA1/G1	ATP-binding cassette proteins A1 and G1
Atf3	activating transcription factor 3
2-AMPs	2-aminomethylphenols
apo	Apolipoprotein
ASCVD	atherosclerotic cardiovascular disease
CAD	coronary artery disease
CE	cholesteryl ester
CEC	cholesterol efflux capacity
CETP	cholesteryl ester transfer protein
CVD	cardiovascular disease
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
FH	familial hypercholesterolemia
FC	free cholesterol
GM-CSF	IL-3/granulocyte-macrophage colony-stimulating factor

HSPC	hematopoietic stem and progenitor cells
HDL-C	high density lipoprotein cholesterol
HNE	4-hydroxy-nonenal
2-HOBA	2-hydroxybenzylamine
IsoLG	Isolevuglandins
IL	interleukin
LCAT	lecithin:cholesterol acyltransferase
LPS	lipopolysaccharide
LDLR	low density lipoprotein receptor
MDA	malondialdehyde
MGO	methylglyoxal
MPO	myeloperoxidase
NLRP3	nucleotide oligomerization domain-like receptor protein with pyrin domain containing 3
OxLDL	oxidized low density lipoprotein
ONE	4-oxo-nonenal
PPM	5’-O-pentyl-pyridoxamine
PL	phospholipid
PON1	paraoxonase 1
ROS	reactive oxygen species
RCT	reverse cholesterol transport
S1P	sphingosine-1-phosphate
SR-B1	scavenger receptor class B type 1
STAT6	signal transducer and activator of transcription 6
TLR	Toll-like receptor

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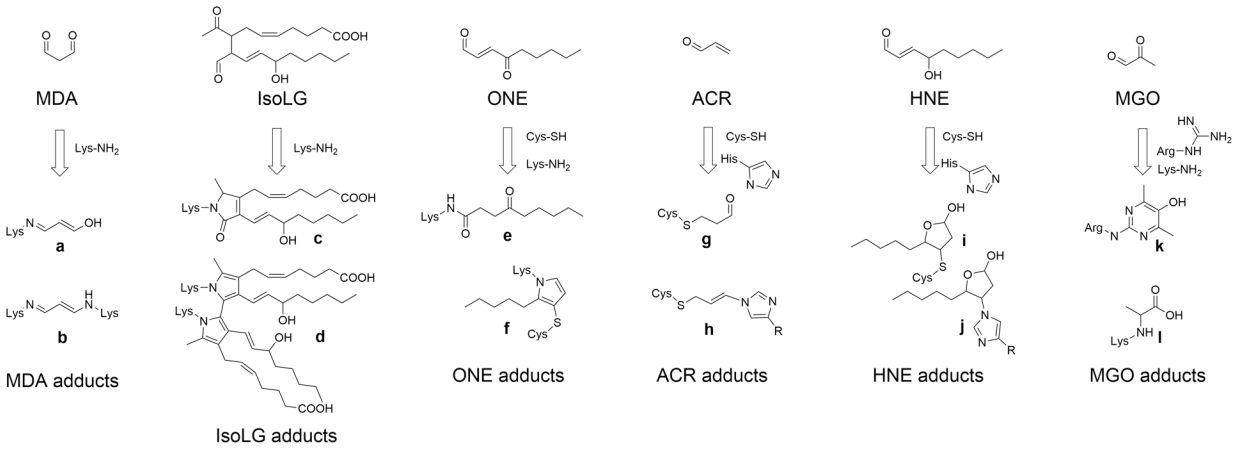

Figure 1. Lipid carbonyls react with amino acid residues to form adducts and crosslinks.

MDA (Malondialdehyde), IsoLG (isolevuglandins), ONE (4-oxo-nonenal), ACR (acrolein), HNE (4-hydroxy-nonenal), and MGO (methylglyoxal) react with their preferred amino acid targets including Lys-NH2 (lysine), Cys-SH (cysteine), His-imidazole (histidine), or Arg-guanidine (arginine) to form various adducts and crosslinks. Crosslinks form when the same lipid carbonyl reacts with two closely adjacent amino acids. In general, multiple species of adducts can form for each lipid carbonyls, two of the most important for each reactive lipid carbonyl are shown here. Adducts shown are: a. MDA N-propenal-Lys monoadduct, b. MDA Lys-1-amino-3-iminopropene-Lys crosslink, c. IsoLG-lactam-Lys monoadduct, d. IsoLG-dipyrrole-Lys crosslink, e. ONE N-4-ketoamide-Lys monoadduct, f. ONE-Lys-pyrrole-Cys crosslink, g. ACR-S-propanol-Cys monoadduct, h. ACR-His-propenamine-Cys crosslink, i. HNE-S-hemiacetal-Cys monoadduct, j. HNE-N-hemiacetal-His monoadduct, k. MGO Argpyrimidine monoadduct, and l. MGO-carboxy-ethyl-Lys.

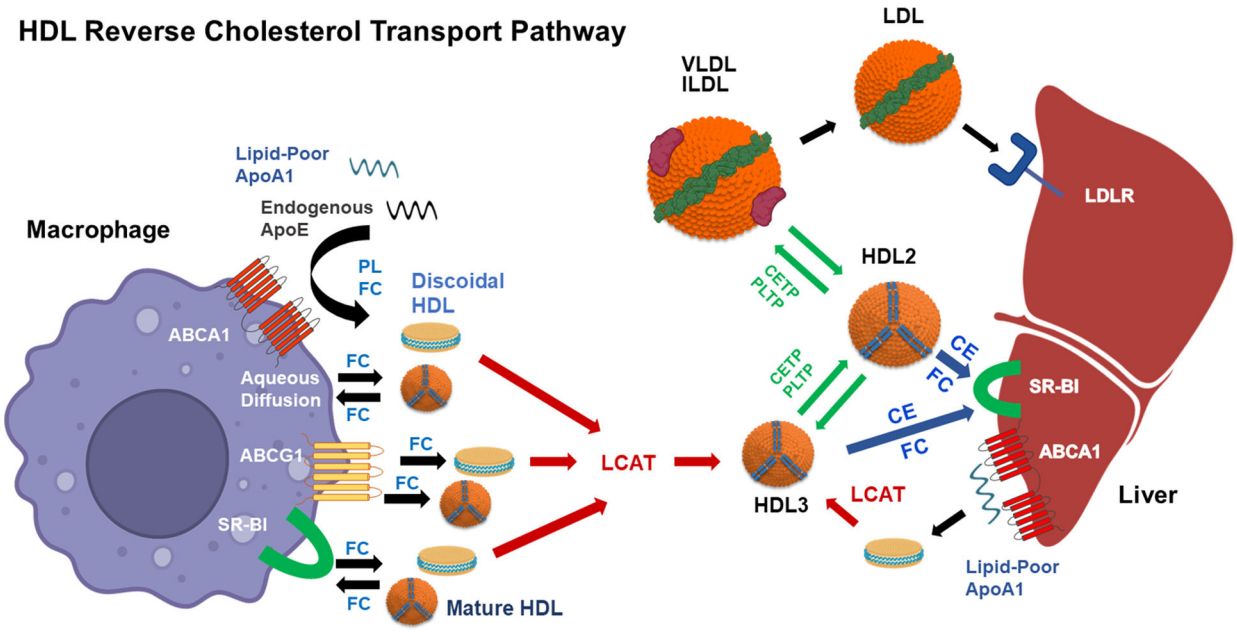

Figure 2. Critical steps of the HDL reverse cholesterol transport (RCT) pathway.

The first step, which is rate limiting, is the removal of free cholesterol (FC) from macrophage foam cells. FC is released from macrophages by four mechanisms. ABCA1 (ATP-binding cassette transporter A1) releases PL (phospholipid) and FC to lipid-poor apoAI or endogenous apoE that is secreted by macrophage foam cells. FC is released to the discoidal HDL formed by ABCA1 and to mature HDL by ABCG1, SR-BI (scavenger receptor-class BI), and aqueous diffusion. The flux of FC between cells and HDL is bidirectional. FC influx occurs by SR-BI and aqueous diffusion. The FC in discoidal HDL particles is esterified by LCAT (lecithin:cholesterol acyltransferase) to form mature HDL3. Plasma HDL is remodeled by CETP (cholesteryl ester transfer protein) and PLTP (phospholipid transfer protein). PLTP transfers PL between VLDL and HDL and among HDL particles. CETP transfers triglyceride from VLDL/IDL to HDL3 to form larger HDL2 particles. CETP also transfers HDL CE to VLDL and IDL to form LDL, which is then internalized by the hepatic LDLR (LDL receptor) for cholesterol routing into bile. An alternative pathway for hepatic delivery of cholesterol and routing to bile is the selective uptake of HDL CE and the influx of HDL FC via SR-BI. CETP also transfers oxidized lipids from LDL to HDL for delivery to the liver by SR-BI. Nascent HDL is synthesized by interaction of hepatocyte or enterocyte (not shown) derived apoAI with hepatic or intestinal ABCA1, and then nascent HDL is routed to peripheral tissue to act as a FC acceptor. Created with BioRender.com.

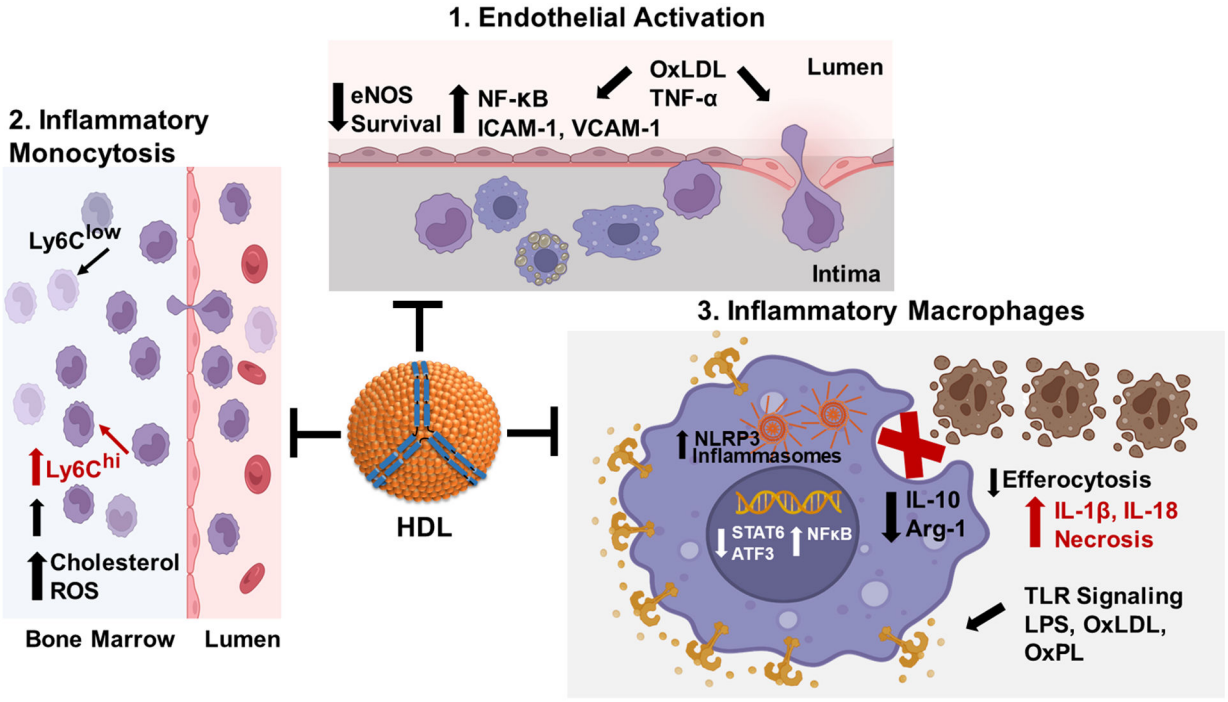

Figure 3. The anti-inflammatory functions of HDL.

1. HDL suppresses endothelial activation in response to proinflammatory stimuli such as TNF-α and oxLDL. Proinflammatory stimuli activate NF-κB (nuclear factor kappa B), which increases expression of monocyte adhesion proteins and chemotactic/inflammatory cytokines, thereby increasing monocyte recruitment and endothelial cell death. HDL signaling pathways stimulate nitric oxide production to decrease NF-κB activation and maintain endothelial barrier integrity. 2. HDL prevents inflammatory monocytosis. Increased LDL and oxLDL, resulting from hypercholesterolemia, induce inflammatory signaling and ROS (reactive oxygen species) leading to expansion of bone marrow granulocyte monocyte progenitors that give rise to inflammatory Ly6C^{high} monocytes. HDL signaling pathways suppress ROS production and inflammatory signaling and expansion of Ly6C^{high} monocytes. 3. HDL prevents the macrophage proinflammatory phenotype. LPS, oxLDL, and other TLR (toll-like receptor) ligands activate NF-κB leading to increased expression of inflammatory cytokines and NLRP3 (nucleotide oligomerization domain-like receptor protein with pyrin domain containing 3) inflammasome components resulting in decreased efferocytosis of apoptotic cells and necrotic death. HDL suppresses TLR signaling and NF-κB activation via cholesterol efflux dependent and independent pathways to promote activation of Atf3 (activating transcription factor 3) and STAT6 (signal transducer and activator of transcription 6), thereby reducing activation of inflammasomes and enhancing efferocytosis of apoptotic cells by promoting expression of Arg-1 (arginase-1) and IL-10. Created with BioRender.com.

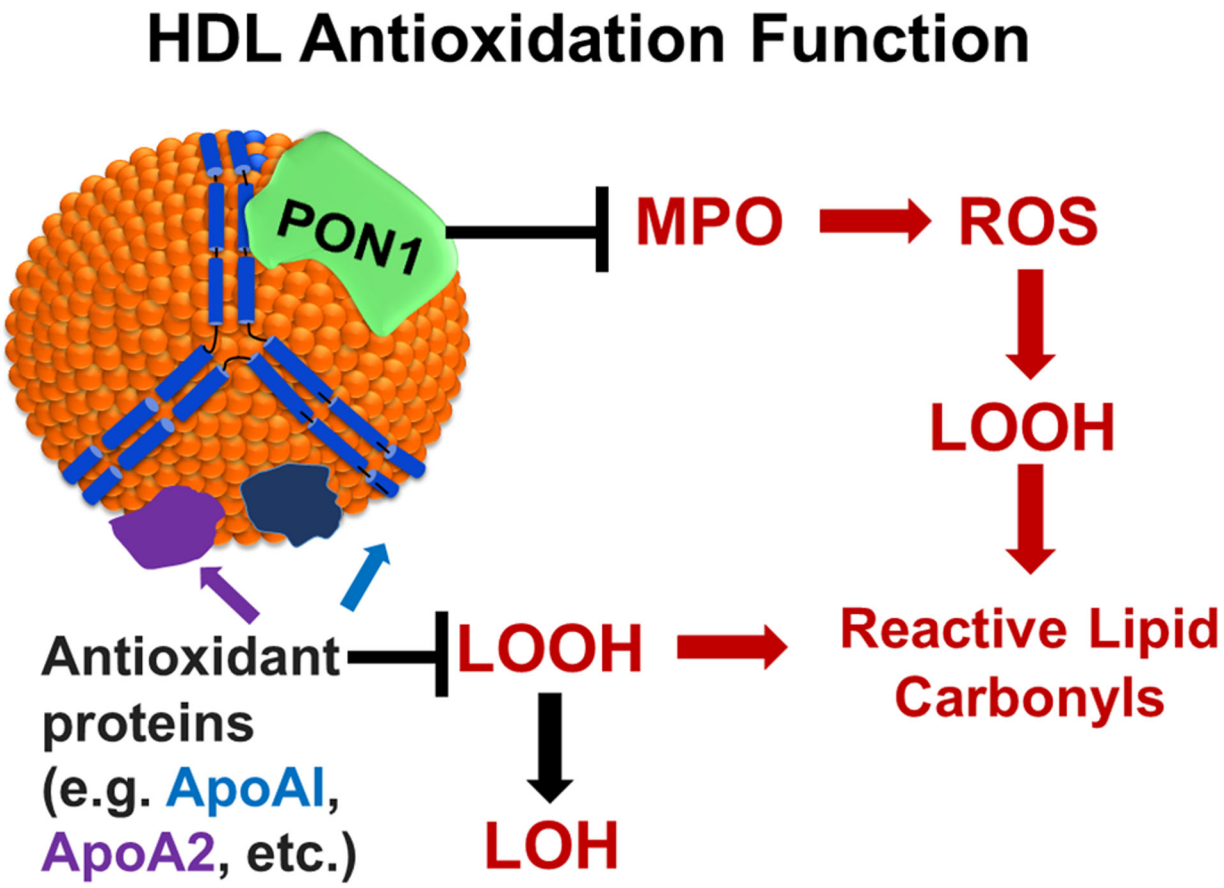

Figure 4. The antioxidant functions of HDL.

A number of proteins associated with HDL including apoAI and apoA2 prevent the formation of reactive lipid carbonyls by reducing LOOH (lipid hydroperoxides) to LOH (lipid hydroxides). MPO (myeloperoxidase) generates ROS (reactive oxygen species) leading to sequential formation of LOOH and reactive lipid carbonyls. The association of PON1 (paraoxonase 1) with apoAI both inhibits MPO activity and also directly inhibits formation of lipid hydroperoxides and reactive lipid carbonyls.

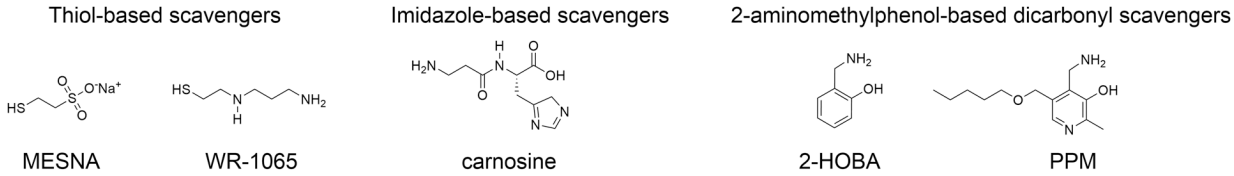

Figure 5. Current classes of small molecule scavengers available to target reactive lipid carbonyls.

Thiol-based scavengers capture ACR (acrolein), ONE (4-oxo-nonenal), and HNE (4-hydroxy-nonenal). Imidazole-based scavengers capture ACR, ONE, HNE, and MGO (methylglyoxal). 2-aminomethylphenol-based dicarbonyl scavengers capture IsoLG (isolevuglandins), ONE, MDA (malondialdehyde), and MGO.

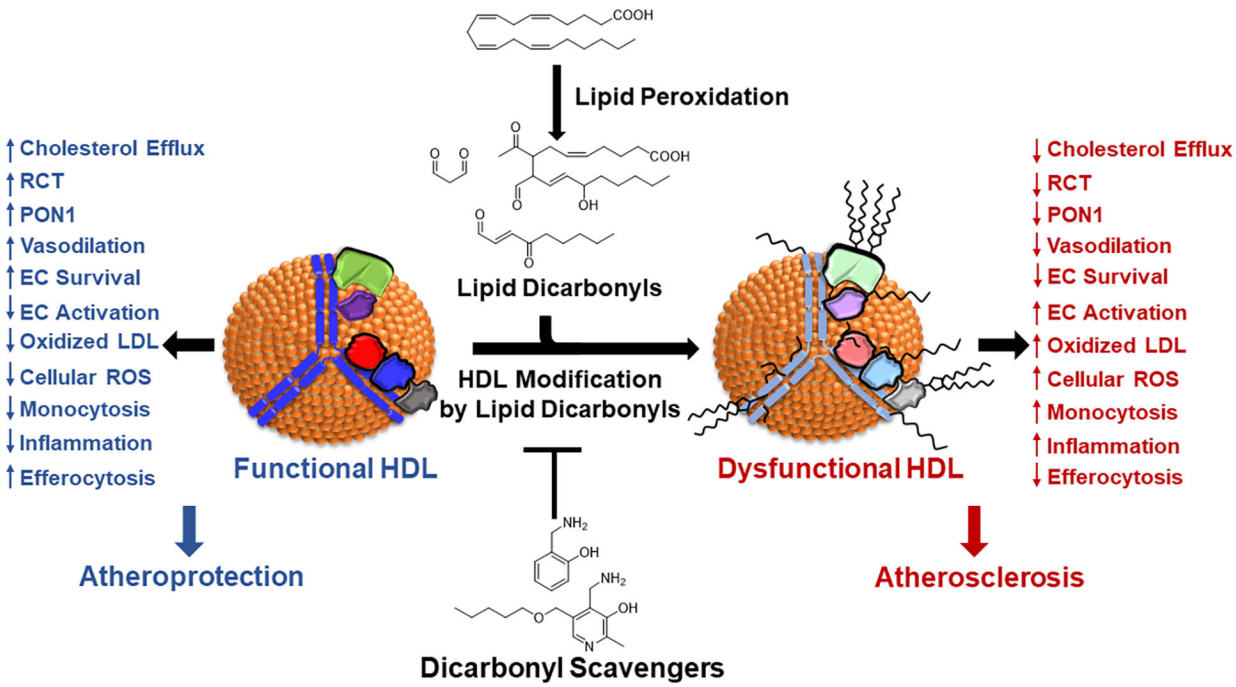

Figure 6. Dicarbonyl scavengers protect HDL functionality.

Under oxidative conditions, lipid peroxidation generates lipid dicarbonyls that react with HDL proteins to render HDL dysfunctional. Dicarbonyl scavengers can intercept these lipid dicarbonyls because the primary amines of these scavengers is even more reactive with dicarbonyls than the lysyl residues of proteins are reactive with the dicarbonyls. Thus, these scavengers protect HDL proteins from modification and thereby prevent HDL dysfunction. Shown are the atheroprotective functions of HDL that dicarbonyl scavengers can protect against dysfunction including EC (endothelial cell) activation and survival.