High-Density Lipoproteins
– Multifunctional but Vulnerable Protections from Atherosclerosis –
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Low plasma levels of high-density lipoprotein (HDL) cholesterol are associated with increased risks of coronary artery disease (CAD). HDL particles exert many effects in vitro and in vivo that may protect arteries from chemical or biological harm or facilitate repair of injuries. Nevertheless, HDL has not yet been successfully exploited for therapy. One potential reason for this shortfall is the structural and functional complexity of HDL particles, which carry more than 80 different proteins and more than 200 lipid species as well as several microRNAs and other potentially bioactive molecules. This physiological heterogeneity is further increased in several inflammatory conditions that increase cardiovascular risk, including CAD itself but also diabetes mellitus, chronic kidney disease, and rheumatic diseases. The quantitative and qualitative modifications of the proteome and lipidome, as well as the resulting loss of functions or gain of dysfunctions, are not recovered by the biomarker HDL-cholesterol. As yet the relative importance of the many physiological and pathological activities of normal and dysfunctional HDL, respectively, for the pathogenesis of atherosclerosis is unknown. The answer to this question, as well as detailed knowledge of structure-function-relationships of HDL-associated molecules, is a prerequisite to exploit HDL for the development of anti-atherogenic drugs as well as of diagnostic biomarkers for the identification, personalized treatment stratification, and monitoring of patients at increased cardiovascular risk. (Circ J 2013; 77: 2432–2448)

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In both epidemiological and clinical studies, as well as the meta-analyses thereof, low plasma levels of high-density lipoprotein (HDL) cholesterol (HDL-C) identify individuals at increased risk of major coronary events. In line with a causally protective effect, HDLs exert a broad spectrum of potentially anti-atherogenic properties. Moreover, atherosclerosis was decreased or even reverted in several animal models by transgenic overexpression or exogenous application of apolipoprotein (apoA-I), the most abundant protein of HDL. However, to date, drugs increasing HDL-C, such as fibrates, niacin, and inhibitors of cholesteryl ester transfer protein (CETP), have failed to consistently and significantly reduce the risk of major cardiovascular events, especially when combined with statins. Moreover, mutations in several human genes as well as targeting of several murine genes modulate HDL-C levels without changing cardiovascular risk and atherosclerotic plaque load, respectively, in the opposite direction as expected from the increase or decrease in HDL-C levels and cardiovascular risk in epidemiological studies. Because of these controversial data, the pathogenic role and, hence, suitability of HDL as a therapeutic target has been increasingly questioned. Because of the frequent confounding of low HDL-C with hypertriglyceridemia, it has been argued that low HDL-C is an innocent bystander of (postprandial) hypertriglyceridemia or another culprit related to insulin resistance or inflammation.

In this controversy it is important to note that previous interventional and genetic studies targeted HDL-C; that is, the cholesterol measured by clinical laboratories in HDL. By contrast to the pro-atherogenic and, hence, disease-causing cholesterol in LDL (ie, LDL-C), which after internalization turns macrophages of the arterial intima into pro-inflammatory foam cells, the cholesterol in HDL (ie, HDL-C) neither exerts nor reflects any of the potentially anti-atherogenic activities of HDLs. In contrast to LDL-C, HDL-C is only a non-functional surrogate marker for estimating HDL particle number and size without deciphering the heterogeneous composition and, hence, functionality of HDLs. In fact, the MESA study and the EPIC study previously found a stronger and more robust association of intima-media thickness and coronary event rates with HDL particle number than with HDL-C level. Successful exploitation of HDL for therapy and prevention will depend on improved understanding of the structure-function-relationships of HDL in health and disease. This is an essential prerequisite for the development of biomarkers that reflect the functionality of HDLs better than the plasma levels of HDL-C or apoA-I and can guide both the development of anti-atherogenic drugs and the clinical management of patients at increased risk for cardiovascular events.
Multifunctional but Vulnerable HDL

As yet, clinical and epidemiological studies have come to discrepant and inconsistent conclusions on the prognostic performance of HDL subclasses differentiated by size. Sometimes the associations of cardiovascular risk with the various HDL subclass concentrations were not stronger than with HDL-C, and even if so, the associations with size were discrepant: significant associations with risk of cardiovascular events or stroke were found for small HDL in some studies, but for medium-sized HDL or large HDL in others.

Previous proteomic, lipidomic, and transcriptomic studies revealed a much greater structural complexity of HDLs: 80 different proteins, more than 200 lipid species, and several microRNAs have been identified in HDL isolated from plasma by either ultracentrifugation, gel filtration, or immunoaffinity chromatography (Figure 1). Among the proteins, apoA-II is present in HDLs at the highest concentration after that of apoA-I and has been investigated for its cardiovascular risk association by several studies. In contrast to initial findings, large prospective studies did not find any significant differences in the risk association between apoA-II-containing and apoA-II-free HDL.

Despite their much lower concentrations relative to the 2 major proteins apoA-I and apoA-II and relative to the major lipids, many quantitatively minor components of HDLs are not passive cargo but biologically active and thereby contribute to the potentially anti-atherogenic properties of HDLs beyond the molecular composition and vascular functions of HDL in health and disease as well as underlying structure-function relationships.

**Structure and Composition of HDL**

HDLs form a heterogeneous class of lipoproteins that differ by protein and lipid composition, shape, size, and density (Figure 1). A prototypic HDL particle contains 2–5 molecules of apoA-I and approximately 100 molecules of phosphatidylcholine or sphingomyelin. They form an amphipathic shell that contains several molecules of unesterified cholesterol and wraps a core of completely water-insoluble cholesteryl esters and triglycerides. Quantitative variation of apoA-I and the major lipid constituents of HDL (phosphatidylcholine, sphingomyelin, cholesterol, and cholesteryl esters) cause considerable heterogeneity of HDL in shape, density, size, and charge, which can be analyzed by electron microscopy, ultracentrifugation, gel filtration, polyacrylamide gel electrophoresis or nuclear magnetic resonance spectroscopy, and agarose gel electrophoresis, respectively. Although these analytical methods yield differentiations into various HDL subclasses that cannot be simply translated into each other, a consensus group has previously suggested a simplified differentiation into 5 subclasses (very-small-, small-, medium-, large-, and very-large-sized HDL) to facilitate their application to clinical studies as well as the communication of results. As yet, clinical and epidemiological studies have come to discrepant and inconsistent conclusions on the prognostic performance of HDL subclasses differentiated by size. Sometimes the associations of cardiovascular risk with the various HDL subclass concentrations were not stronger than with HDL-C and even if so, the associations with size were discrepant: significant associations with risk of cardiovascular events or stroke were found for small HDL in some studies, but for medium-sized HDL or large HDL in others.

**Figure 1.** Structural and compositional heterogeneity of high-density lipoprotein (HDL). HDL forms macromolecular complexes containing >80 proteins and peptides, >200 lipid species, and several microRNAs. Apo, apolipoprotein; AT, antitrypsin; AP, antiprotease; C3, C4, complement factors 3 and 4, respectively; CETP, cholesteryl ester transfer protein; Coe, ceruloplasmin; Fib, fibrinogen; GPx, glutathione peroxidase; HRP, haptoglobin-related protein; Hb, hemoglobin; hPBP, human phosphatidylethanolamine-binding protein; LCAT, lectin:cholesterol acyltransferase; Lp-PLA2, lipoprotein-associated phospholipase A2; MPO, myeloperoxidase; PI, proteinase inhibitor; PLTP, phospholipid transfer protein; PON, paraoxonase; PSP, parotid secretory protein; SAA, serum amyloid A; TTR, transthyretin. *microRNAs found at very low copy numbers. The structural complexity gives rise to the formation of HDL subclasses that differ by size, shape, density, and charge.
transport and metabolism of lipids (Figure 1). A general theme of these functions is host defense from chemical or biological hazards such as oxidation (eg, paraoxonase-1 [PON1], platelet-activating factor acylhydrolase [PAF-AH], ceruloplasmin) and infection (eg, lipopolysaccharide binding protein, apoL1, serum amyloid A [SAA]), respectively, or the repair of resulting homeostatic disturbances (eg, serpins, antithrombin, antiplasmin) or damages of cells and tissue (eg, sphingosine-1-phosphate [S1P], clusterin). Some minor subcomponents of HDLs appear to form well-defined platforms with distinct functionality; for example, a complex formed by apoA-I, haptoglobin-related protein (HRP), and apoL1 that, despite its very low concentration, protects humans from sleeping sickness caused by the general strain of Trypanosoma brucei. HRP mediates the binding to a cell surface receptor and subsequent internalization, whereas apoL1 causes the lysosomal lysis of these protozoa.

The various lipids and proteins of HDLs cover a broad concentration range from the submicromolar (S1P: 0.5 µmol/L; apoL1: 0.1 µmol/L) to the supramillimolar (cholesterol >1 mmol/L) (Figure 2). Assuming that every HDL particle carries 2–5 molecules of apoA-I, which has an average plasma concentration of 50 µmol/L, the average concentration of HDL in plasma ranges between 10 and 25 µmol/L. The much lower concentration of many HDL-associated proteins, lipids, and microRNAs indicates that they are residing on different HDL particles. This has been confirmed by proteomics and lipidomics of subfractionated HDL particles, which identified the biggest number of microcomponents in the smallest HDL particles. However, these HDL3 particles are also present in plasma at higher concentrations than their microcomponents, which hence appear to be dispersed throughout different HDL3 particles. The great concentration differences between HDL3 particles and particles provide indirect evidence that the established biomarkers HDL-C (1–2 mmol/L) and apoA-I (50 µmol/L), but also the HDL size/density subclasses, lack both sensitivity and specificity to record the complex structure-function relationships of HDLs, which in various inflammatory diseases is further increased by the molecular modification of canonical HDL proteins and the acquisition of atypical constituents.

An intriguing example for the diagnostic potential of minor HDL components is provided by the identification of 2 opposing associations of HDL-C with cardiovascular risk depending on the presence or absence of apoC-III in HDL: In two nested case-control analyses of the Nurses’ Health and the Health Professionals Follow-Up Studies, the cholesterol concentration in both total HDL and apoC-III-free HDL showed the expected inverse association with CAD events, whereas the concentration of cholesterol in apoC-III-containing HDL was positively associated with the risk of cardiovascular events. Interestingly, the enrichment of HDLs with apoC-III was found as one reason for the reduced activity of HDL from CAD patients to protect endothelial cells from apoptosis.

HDL Functions

Traditionally, the anti-atherogenic potential of HDLs is assigned to their capacity to remove cholesterol from peripheral cells and, most importantly, macrophage foam cells in the blood vessel intima. The efflux of cholesterol from macrophages is the first step in the reverse cholesterol transport pathway, a process in which HDLs play a central role by transporting cellular cholesterol back to the liver for final excretion in bile and feces. As HDL research moves increasingly towards HDL functionality, more and more novel functions of HDLs are being uncovered. Some of these functions have been mechanistically linked to the well-known ability of HDLs to induce the activation of cellular cholesterol efflux pathways, whereas many other functions of HDLs are independent of the effects of HDLs on cellular cholesterol homeostasis. Moreover, besides beneficial functions that provide protection against atherosclerotic plaque development, HDLs have been found to positively influence other processes that indirectly affect atherosclerosis such as glucose homeosta-
Cholesterol Efflux and Reverse Cholesterol Transport

A key process in the formation of an atherosclerotic plaque is accumulation of cholesterol in macrophage foam cells. HDLs and their major protein constituent, apoA-I, are efficient acceptors of excess free cholesterol from cells. This ability of HDLs and apoA-I to promote cellular cholesterol efflux might reverse or prevent the formation of macrophage foam cells. Four different pathways have consistently been described by which HDLs and their apolipoproteins remove cholesterol from cells. First of all, cells can release cholesterol toward HDLs by passive aqueous diffusion, which involves spontaneous desorption of free cholesterol molecules from the plasma membranes and diffusion through the intervening aqueous face until collision with and incorporation into acceptor particles.42,43 Such an unmediated bidirectional flux of free cholesterol is rather inefficient and follows a cholesterol concentration gradient between the plasma membrane and HDL.44 The 3 other pathways for cholesterol efflux are more specific and depend on interaction with a cellular receptor. Lipid-free or lipid-poor apoA-I can mediate cellular efflux of both cholesterol and phospholipids via the ATP-binding cassette transporter A1 (ABCA1), resulting in the rapid lipidation of apoA-I to generate nascent HDL particles.45,46 However, the nature of the molecular interaction between apoA-I and ABCA1 is still not entirely understood. The mature HDL particles can then serve as acceptors of cholesterol provided by ABCG1,47 or scavenger receptor class B type I (SR-BI).48 Although it is currently unknown how ABCG1 mediates cholesterol efflux to HDLs, it does not appear to require direct binding of HDLs to the cells.49 Rather, there is considerable evidence that ABCG1 modulates the flux of sterols from intracellular organelles to the plasma membrane for efflux.48 SR-BI facilitates a bidirectional flux of free cholesterol between the cell and HDL, and the net movement of cholesterol is similar to aqueous diffusion determined by the cholesterol concentration gradient.49 In contrast to ABCG1, HDLs have to bind to SR-BI in order to stimulate net cholesterol efflux.50,51

After efflux, cholesterol in HDLs may be esterified by the enzymatic activity of lecithin:cholesterol acyltransferase (LCAT) whereupon HDLs can deliver the excess cholesterol from peripheral cells back to the liver by 3 distinct ways: (1) HDL cholesterol esters, but not the protein component of HDLs, are selectively taken up into the liver via SR-BI.52 (2) HDL particles (lipids together with proteins) can undergo holoparticle endocytosis mediated by the interaction of apoA-I with the ectopic β-chain of Flot-A (Pop) and subsequent activation of the nucleotide receptor P2Y11 and (3) in humans, CETP exchanges cholesterol esters in HDLs for triglycerides in apoB-containing lipoproteins, which in turn are removed from the circulation by the liver via members of the LDL receptor family.54 Ultimately, cholesterol is excreted from the liver into the bile, either directly as free cholesterol or after conversion into bile acids, and eliminated from the body via the feces.55,56 This pathway involving cholesterol efflux from cells toward HDLs, uptake of cholesterol from HDLs into the liver, and finally excretion into bile and feces is termed reverse cholesterol transport. Besides HDL-mediated reverse cholesterol transport through the classical hepatobiliary route, there is also an HDL-independent pathway for body cholesterol removal, the so-called transintestinal cholesterol excretion, which has been described in mice.57-59

The flux of cholesterol from macrophage foam cells represents only a very small fraction of the total cholesterol pool and therefore has no effect on whole body cholesterol homeostasis. However, macrophage-specific reverse cholesterol transport is highly relevant for maintaining normal macrophage cholesterol homeostasis, with profound consequences for atherosclerosis development. Two crucial questions to address regarding the cholesterol efflux and reverse cholesterol transport functionality of HDLs are (1) how HDLs reach macrophage foam cells in the subendothelial space, and (2) how HDLs leave the arterial intima, so that they can transport macrophage-derived cholesterol to the liver for elimination from the body. First, in order to get access to the cholesterol-loaded macrophages within atherosclerotic lesions, HDLs or their major protein constituent, apoA-I, need to cross the endothelium. Our group found that aortic endothelial cells are able to bind, internalize, and transport both HDLs and lipid-free apoA-I in a specific and saturable manner.60,61 Transcytosis of apoA-I through aortic endothelial cells resulted in lipida tion of lipid-free apoA-I and was reduced by knockdown of ABCA1 by small interfering RNA,62,63 providing evidence that ABCA1 is involved in this process. In contrast, binding, uptake, and transendothelial transport of mature HDLs were modulated by ABCG1 and SR-BI, not by ABCA1.64 Further research identified a novel mechanism for transport of both apoA-I and HDLs through endothelial cells, in which binding of apoA-I to the β-chain of cell surface Flot-2 ATPase expressed on endothelial cells stimulates ATP hydrolysis, and the generated ADP selectively activates P2Y12, leading to internalization and transport of initially lipid-free apoA-I and HDLs.53 Once apoA-I and HDLs have been loaded with cholesterol, they need to leave the arterial wall to complete reverse cholesterol transport. It seems conceivable that HDL particles can return from the extravascular space to the systemic circulation either by yet again passing through the endothelium or alternatively via the lymphatic system. Although the first hypothesis has not been disproven, recent data support a role for the lymphatic vessels in reverse cholesterol transport.54,65 Macrophage-specific reverse cholesterol transport was substantially reduced in mice with surgical interruption of lymphatic transport and in Chy mice, which have absent dermal lymphatics because of a mutation in vascular endothelial growth factor (VEGF) receptor 3 (VEGFR3).64,65 Ameliorating the lymphatic function in apoE knockout mice by VEGF-C treatment improved the transport of fluorescent cholesterol from macrophages injected into the footpad to the plasma and liver.64,65 More importantly, the cholesterol label could be detected in lymph nodes regional to the site of injection and in the effluent lymph.65 In vitro observations revealed that lymphatic endothelial cells internalize and transcytose HDLs by a mechanism that involves SR-BI.66 In more complex experiments, aortic arches from apoE−/− donor mice, which are rich in atherosclerotic lesions, were loaded with D6-cholesterol and transplanted into the abdominal cavity of apoE−/− recipient mice.67 Partial inhibition of reformation of lymphatic connections by treating the recipient mice with an antibody blocking VEGFR3 was associated with increased retention of D6-cholesterol in the transplanted aortic segments,67 thereby providing more direct evidence for the specific involvement of lymphatic vessels draining the wall of the aorta in cholesterol clearance.

Antioxidative Effects

Oxidation of LDL yields a more pro-atherogenic particle, and in numerous studies HDLs have been found to be capable of impeding oxidative changes in LDL. An inhibitory effect of HDLs on the oxidation of LDL has been detected both in cocultures of endothelial cells and vascular smooth muscle cells (VSMCs).68-67
and several cell-free systems. A major part of the antioxidative activity of HDLs is attributed to apoA-I. In cell culture experiments, apoA-I removes lipids from LDL and thereby makes LDL resistant to vascular cell-mediated oxidation and prevents oxidized LDL-induced monocyte adherence and chemotaxis. Similarly, LDL isolated from mice and humans injected with apoA-I can no longer be oxidized by coculture with human artery wall cells. There is also evidence indicating that apoA-I binds molecules produced by vascular cells that stimulate the formation of biologically active phospholipids in LDL.

The oxidation of 2 methionine residues in apoA-I to their respective methionine sulfoxide forms is highly important for the ability of HDLs to reduce lipid hydroperoxides.

PON1 is another HDL-associated protein that plays an important role in the antioxidative function of HDLs. Purified PON1 offers protection against oxidation of LDL in vitro by reducing oxidized phospholipids that accumulate in oxidized LDL. Notably, HDL isolated from mice with transgenic overexpression of human PON1 is more potent at inhibiting oxidation of LDL than HDL from wild-type mice, and the lack of a functional PON1 gene in mice renders the HDLs of those mice unable to block accumulation of lipid hydroperoxides in human LDL.

Another HDL enzyme possessing antioxidative activity is PAF-AH, which nowadays is termed lipoprotein-associated phospholipase A2 (Lp-PLA2). Inactivation of HDL-associated Lp-PLA2 abolishes the inhibitory effect of HDLs against LDL modification and production of bioactive oxidized phospholipids. A protective action of Lp-PLA2 against LDL oxidation has been further supported by mouse studies demonstrating that adenoviral-mediated overexpression of human Lp-PLA2 in mice reduced the circulating levels of oxidatively modified LDL.

In contrast to the potentially anti-atherogenic functions of Lp-PLA2, plasma levels of Lp-PLA2 positively correlate with cardiovascular risk. A possible explanation is that the majority of Lp-PLA2 resides in LDL where it generates pro-inflammatory lysosphingolipids. Indeed, the findings of a 3-year prospective follow-up study of patients with stable coronary artery disease (CAD) suggested that a higher total plasma Lp-PLA2 level is associated with increased future cardiovascular mortality, whereas an association in the opposite direction was found with Lp-PLA2 in apoB-depleted plasma.

Finally, LCAT might also contribute to the beneficial effects of HDLs in modulating LDL oxidation. Reports showing that incubation of LDL with LCAT considerably decreased Cu-induced formation of lipid hydroperoxides in LDL and that infection of mice with a recombinant adenosine encoding human LCAT resulted in lower levels of autoantibodies against oxidized LDL support an antioxidative function for LCAT.

**Endothelial Function and Integrity**

HDLs exert several direct protective effects on the vascular endothelium (Figure 3). First, HDLs are presumed endothelium-dependent vasodilators because the addition of HDLs to precontracted aortic segments leads to vasorelaxation. Additional human studies have revealed that heterozygotes for loss-of-function mutations in ABCA1 with low levels of HDL-C display impaired endothelium-dependent vasodilation, which can be restored by a single infusion of apoA-I/phosphatidylcholine disks. Likewise, administration of reconstituted HDLs resulted in normalized endothelial vasodilator function in hypercholesterolemic men. The effect of HDLs on vascular tone is mechanistically explained by the ability of HDLs to induce the phosphorylation of endothelial nitric oxide synthase (eNOS) and thereby stimulate nitric oxide (NO) production. This vasoprotective effect involves the interaction of HDLs with SR-BI and the lysosphingolipid receptor SIP1, which leads to the parallel activation of phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein-kinase signaling. By the same cellular signaling pathways, HDLs also lead to a significant increase in eNOS protein abundance in endothelial cells. Three bioactive lysosphingolipids in HDLs (ie, sphingosylphosphorylcholine, 1P, and lysosulfatide) were attributed to arterial vasodilation by HDLs. Evidence has accumulated that also

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Molecules and receptors mediating endothelial functions of high-density lipoproteins (HDLs). Apo, apolipoprotein; eNOS, endothelial nitric oxide synthase; EPC, endothelial progenitor cell; NO, nitric oxide; S1P, sphingosine-1-phosphate; SR, scavenger receptor; VCAM, vascular cell adhesion molecule.
the cholesterol efflux capacity of HDLs via ABCG1 has some effect in the stimulatory activity of HDLs on eNOS activity. In mice lacking ABCG1 the reduction in endothelium-dependent vasorelaxation is associated with increased aortic levels of 7-oxysterols and decreased levels of active eNOS dimer. Moreover, the inhibitory interaction of eNOS with caveolin-1 in response to cholesterol loading can be reversed by HDLs in an ABCG1-dependent fashion.

HDLs also beneficially affect the vasculature by promoting endothelial cell survival. HDLs suppress apoptosis in endothelial cells induced by several stimuli, including oxidized LDL, tumor necrosis factor-α (TNF-α), and growth factor withdrawal. Studies of the molecular mechanism indicate that HDLs potentially can maintain mitochondrial integrity, thereby preventing the release of cytochrome c and apoptosis-inducing factor from mitochondria and subsequent activation of the caspase cascade. In addition, HDLs are negative regulators of the pro-apoptotic effectors BID and DR5 and positively modulate the expression of the anti-apoptotic protein Bcl-xL. Stimulation of the PI3K/Akt/eNOS signaling pathway again seems to play a role in this endothelial cell protection afforded by HDLs. The anti-apoptotic capacity of HDLs in endothelial cells has been attributed to their protein components apoA-I and clusterin (or apoJ), as well as to lysophospholipids carried by HDLs.

Loss of the structural integrity of blood vessels facilitates the development of atherosclerotic lesions, and electrical cell substrate impedance sensing assays have revealed that HDLs can promote endothelial barrier function. In this context, HDL-associated S1P acts on its receptor S1P1 to stimulate signaling primarily through the Akt/eNOS pathway, an effect that has been shown to be partially dependent on the hydrolysis of HDL-associated phosphatidyls by endothelial lipase and ultimately leads to endothelial barrier enhancement. Recent studies have identified apoM as the binding molecule of S1P in HDLs.

Notably, apoM-deficient mice, which lack S1P in their HDL fraction, exhibit significantly impaired endothelial barrier function in the lungs.

Finally, HDLs may contribute to repair processes after injury. Carotid artery re-endothelialization after perivascular electric injury is blunted in apoA-I knockout mice with very low HDL-C levels, but can be restored through liver-directed gene transfer. In vitro studies indicate that HDLs may in part accelerate re-endothelialization by driving endothelial cell migration through Rac-mediated formation of actin-based lamellipodia in an SR-BI-dependent manner. Moreover, blockage of the SR-BI adaptor protein molecule, PDZK1, prevented HDL-mediated activation of eNOS and endothelial cell migration. In PDZK1 knockout mice, re-endothelialization after perivascular electric injury does not occur. These data suggest that PDZK1 is required for re-endothelialization events activated by HDL/SR-BI. Although somewhat controversial, endothelial progenitor cells (EPCs) are thought to participate in vascular repair. EPCs cultured in the presence of reconstituted HDLs show increased proliferation, differentiation, and cell survival as well as enhanced adhesion to endothelial cells and transendothelial migration. The beneficial effects of HDLs on EPCs can be abolished by either blocking Akt or PI3K in the cells. In a study using mice, the administration of reconstituted HDLs increased the number of EPCs and stimulated the re-endothelialization of injured carotid arteries. In that same study, treatment with reconstituted HDLs improved blood flow and capillary density following hindlimb ischemia, conceivably by enhanced incorporation of bone marrow-derived endothelial cells in the ischemic tissue. The potential relevance for humans is indicated by the positive correlation between HDL-C levels and circulating EPC numbers in CAD patients, as well as by the increase of EPC numbers in the blood of patients with type 2 diabetes mellitus (T2DM) who received infusions of reconstituted HDLs. Furthermore, the administration of HDLs and their constituent, S1P, diminished myocardial damage in a murine model of myocardial ischemia-reperfusion. These protective activities of HDLs require NO as well as the S1P1 receptor and entail the ability of the lipoprotein to suppress the recruitment of inflammatory neutrophils and apoptosis of cardiomyocytes.

**Anti-Inflammatory Effects**

An early event in the pathogenesis of atherosclerosis is upregulated expression of adhesion molecules on the endothelium, leading to the recruitment and infiltration of monocytes. It is generally well established that native and reconstituted HDLs have the capacity to inhibit cytokine-induced expression of adhesion molecules on endothelial cells in vitro. Several lines of evidence confirm that HDL-mediated suppression of adhesion molecule expression also occurs in vivo. Injection of reconstituted HDLs reduced the expression of E-selectin in interleukin 1α-induced skin lesions in a pig model. Moreover, infusions of apoA-I either in the free form or as reconstituted HDL mitigated the vascular inflammation caused by implantation of a parietal collagen in rabbits, characterized among other effects by decreased endothelial expression of various adhesion molecules. Additional human studies have shown that infusions of reconstituted HDLs lower the expression level of vascular cell adhesion molecule 1 (VCAM-1) in atherosclerotic lesions. Lysosphingolipids present in HDLs, such as S1P, have been proposed as mediating the HDL-mediated inhibition of VCAM-1 expression on endothelial cells. However, despite considerable research in this area, the mechanisms underlying this anti-inflammatory effect of HDLs are still not completely understood. TNF-α enhances sphingosine kinase activity and the generation of S1P in endothelial cells, and this can be blocked by pre-incubation with HDLs. Moreover, it has been postulated that HDLs impair VCAM-1 expression on endothelial cells via SR-BI- and S1P1-mediated activation of PI3K and eNOS. Another mechanism may involve repression of TNF-α expression in response to HDLs, thereby preventing nuclear factor-κB (NF-κB) inhibitor degradation, and subsequently reducing nuclear translocation, DNA binding, and the transcriptional activity of NF-κB. More recent studies indicate that the interaction of HDL with SR-BI on endothelial cells increases the expression of heme oxygenase-1 by activation of PI3K/Akt through 3β-hydroxyysteroid-Δ24 and that this may play an important role in the suppression of endothelial inflammation by HDLs.

Although interactions between endothelial cells and monocytes require the expression of adhesion molecules on both cell types, the effect of HDLs on monocyte adhesion molecules has been less extensively investigated. There is one study showing that treatment of human monocytes with native HDL, reconstituted HDL, or apoA-I reduced the expression and activation of the monocyte adhesion molecule CD11b. This inhibition of monocyte activation by HDLs or apoA-I was associated in vitro with decreased monocyte adhesion to endothelial cells, monocyte spreading, and monocyte migration in response to monocyte chemotactrant protein-1 (MCP-1). ApoA-I was found to prevent monocyte activation through efflux of cholesterol via ABCA1, whereas for HDLs this occurred via an ABCA1-independent mechanism.

Besides reducing the number of adhesion molecules, HDLs...
can also affect the infiltration of monocytes in the arterial intima by regulating the expression of chemokines and their receptors.\textsuperscript{125} The beneficial influence of HDLs on the expression of various chemokines is mediated through direct inhibition of the NF-κB-pathway.\textsuperscript{126} On the other hand, the capacity of HDLs to reduce the chemokine receptor, CX-CR1, on monocytes is dependent on peroxisome proliferator-activated protein \( \gamma \).\textsuperscript{125} In addition, diminished production of the chemokine MCP-1 after incubation of VSMCs or isolated rat aortas with HDLs has been attributed to HDL-associated lysosphingolipids and requires signaling through the S1P\( \gamma \) receptor and SR-BI.\textsuperscript{126}

Apart from modulating the functionality of monocytes and macrophages, HDLs were recently found to control adaptive immunity.\textsuperscript{127} A defect in cholesterol efflux after combined knockout of \( Abca1 \) and \( Abcg1 \) in mice was accompanied by excessive expansion of both myelopoietic stem and multipotential progenitor cells in bone marrow as well as by leukocytosis in the peripheral blood.\textsuperscript{127} Moreover, introduction of a human apoA-I transgene into heterozygous LDL receptor knockout mice transplanted with bone marrow of \( Abca1^{-/-} Abcg1^{-/-} \) mice resulted into almost complete normalization of the myelopoietic disorder.\textsuperscript{127} In fact, HDLs were shown to suppress the proliferation of myelopoietic stem cells ex vivo.\textsuperscript{127} Subsequent research by the same group revealed that transplantation of bone marrow from \( Abca1/-\ Abcg1/- \) double knockout mice into wild-type recipients led to interleukin 23-induced secretion of granulocyte colony-stimulating factor, which in turn increased the mobilization of hematopoietic stem and progenitor cells as well as extramedullary hematopoiesis.\textsuperscript{128} Raising the HDL levels in these mice via a human apoA-I transgene could attenuate the number of circulating hematopoietic stem and progenitor cells.\textsuperscript{128}

Findings from other recent work suggest that apart from innate immunity, adaptive immunity is also modulated by apoA-I and HDLs.\textsuperscript{129} LDL-receptor knockout mice lacking apoA-I show signs of autoimmunity in response to a cholesterol-enriched diet, typically reflected by enlarged cholesterol-rich lymph nodes as well as increased T cell activation, T cell proliferation, production of autoantibodies, and skin inflammation.\textsuperscript{129} Administration of human lipid-free apoA-I partly reversed the autoimmunity phenotype of these mice, conceivably by increasing the amount of regulatory T cells in the lymph nodes.\textsuperscript{129}

**Antithrombotic Effects**

Platelet aggregation and thrombus formation directly contribute to the pathogenesis and complications of atherosclerotic cardiovascular disease. There are supporting data from several studies that HDLs are potent inhibitors of platelet activation and aggregation. HDL\( \textsubscript{3} \) exerts positive regulatory effects on the Na\( ^+ \)/H\( ^+ \)-antiport system in human platelets by binding to glycoprotein IIb/IIIa and activation of protein kinase C and phospholipase A\( _2 \).\textsuperscript{130} Infusion of recombinant HDLs into patients with T2DM attenuated platelet aggregation ex vivo.\textsuperscript{130} Moreover, native HDL\( \textsubscript{3} \) interfered with thrombin-induced platelet activation in vitro, as evidenced by decreased platelet aggregation, fibrinogen binding, and P-selectin expression.\textsuperscript{133,134} which was attributed to the inhibitory effect of HDLs on thrombin-induced formation of 1,2-diacylglycerol and inositol 1,4,5-tris phosphate as well as to intracellular calcium mobilization and the ensuing increase in the phosphorylation and activation of protein kinase C.\textsuperscript{133,134}

The capacity of HDLs to inhibit platelet aggregation appears to be dependent on SR-BI,\textsuperscript{134} although this observation could not be confirmed by another group.\textsuperscript{135} Cholesterol depletion from platelet membranes by incubation with either cyclodextrin or HDLs was accompanied by reduced platelet aggregation,\textsuperscript{132} indicating that HDLs may modulate platelet function by cholesterol efflux. In addition, HDL-bound phospholipids blunted platelet activation to a similar extent as native HDL or reconstituted HDL.\textsuperscript{132,134}

Recent investigations have identified a link between HDL-mediated cholesterol efflux via ABCG4 and platelet production.\textsuperscript{135} When Ldlr\( ^{-/-} \) mice were transplanted with bone marrow from \( Abcg4^{-/-} \) mice, the total platelet count and percentage of reticulated platelets were significantly increased,\textsuperscript{135} consistent with enhanced platelet production. The expression of ABCG4 was found to be specific for bone marrow-derived megakaryocyte progenitors, and deficiency of this transporter promoted the proliferation and expansion of these platelet progenitors.\textsuperscript{135} In megakaryocyte progenitors that lack ABCG4, cholesterol efflux to HDLs was defective.\textsuperscript{135} Treatment with reconstituted HDLs reduced platelet numbers and megakaryocyte progenitor proliferation in Ldlr\( ^{-/-} \) mice but not in Abcg4\( ^{-/-} \) Ldlr\( ^{-/-} \) mice.\textsuperscript{135} Taken together, these data suggest that HDLs modulate megakaryocyte progenitor cell proliferation and, hence, platelet production by regulating the cholesterol content of megakaryocyte progenitors via ABCG4. The relevance of this process for human physiology is indicated by the finding of moderately but significantly reduced platelet numbers in patients with peripheral vascular disease who were treated with reconstituted HDLs.\textsuperscript{135}

HDLs might also work in an anticoagulatory fashion by inhibiting steps in the extrinsic coagulation cascade. Already in the 1980s it was documented that HDLs impair the activity of tissue factor and subsequent activation of factor X.\textsuperscript{136} Interestingly, the discovery that HDLs potentiates the anticoagulatory activities of activated protein C and protein S in mediating proteasomal degradation of factor Va was later assigned to contamination of the HDL fraction isolated by ultracentrifugation with anionic phospholipid membranes.\textsuperscript{137,138} Other modes of action that could explain the beneficial effects of HDLs on coagulation include a reduction in platelet-activating factor production by endothelial cells,\textsuperscript{139} inhibition of thrombin-induced endothelial tissue factor expression,\textsuperscript{140} suppression of the procoagulant activity of endothrocytes,\textsuperscript{141} and inactivation of anionic phospholipids that are required to accommodate the prothrombinase complex.\textsuperscript{142}

Consist with these findings proposing a protective function of HDLs against thrombosis, Naqvi et al observed an independent inverse correlation between HDL-C levels and ex vivo thrombus formation in humans.\textsuperscript{143} Furthermore, flow-induced thrombus formation was diminished by reconstituted HDLs as determined by flow-chamber based assays.\textsuperscript{132} Intravenous injection of apoA-I Milano into rats resulted in significantly delayed time to thrombus formation and decreased thrombus weight.\textsuperscript{144}

**Smooth Muscle Cells**

VSMCs play an important role in the stabilization of atherosclerotic plaques. HDLs promote proliferation and cell cycle entry of VSMCs by inducing cyclin D1 expression and retinoic acid-induced protein phosphorylation.\textsuperscript{145} Proliferative effects of HDLs on VSMCs are dependent on the Raf-1/MEK-1/ERK1/2 mitogen-activated protein kinase cascade, but cannot be mimicked by lipid-free apoA-I.\textsuperscript{146} Moreover, HDLs have been shown to inhibit platelet-derived growth factor-induced migration of VSMCs through their S1P1 component.\textsuperscript{146} HDLs also counteract the pro-inflammatory events mediated by oxidized LDL in rabbit VSMCs by reducing the generation of intracellular ROS and thereby activation of NF-κB.\textsuperscript{147} Additional in vitro experimental data indicate that HDLs might regulate the secretory function of VSMCs. On the one hand, HDLs can facilitate the release of the cyclooxygenase-2-derived vasodilator prostacyclin in rat VSMCs via a mitogen-activated protein kinase-dependent mech-
anism.\textsuperscript{148,149} On the other hand, HDLs decrease the production and release of basic fibroblast growth factor from bovine aortic smooth muscle cells.\textsuperscript{150}

**HDL Dysfunction**

Nearly 20 years ago, it was reported that upon an acute-phase response, HDL of both patients and animals loses its inhibitory effect on LDL oxidation and enhances rather than inhibits the expression of MCP-1 by endothelial cells.\textsuperscript{151} Since then, many laboratories worldwide have provided examples of the loss of atheroprotective functions or even gain of paradoxically proatherogenic functions by HDLs (Table S1). Such dysfunctional HDL has been isolated from patients with both acute and stable CAD, T2DM as well as type 1 diabetes mellitus (T1DM), metabolic syndrome (MetS), end-stage (ESRD) as well as mild-to-moderate chronic kidney disease (CKD), rheumatic diseases, or sepsis (Table S1). Dysfunctions include reduced activities or capacities of HDLs to induce cholesterol efflux from macrophages and other cells, to inhibit oxidation of lipids in LDL and cell membranes, and to inhibit cytokine release and expression of cell surface activation markers by macrophages or dendritic cells (Table S1). A broad spectrum of dysfunction has been seen in the interactions of HDLs with endothelial cells: HDL of patients with different disease conditions failed to stimulate NO production and to inhibit superoxide production and thereby modulate downstream activities such as vasorelaxation, expression of VCAM-1 and MCP-1 as well as leukocyte adhesion (Table S1). HDL of CAD, T2DM, or CKD patients was also found to lack the normal activity to promote endothelial integrity by inhibiting apoptosis and stimulating migration of endothelial cells, as well as the repopulation of denuded arteries by EPCs (Table S1).

Pathological structural changes in HDLs were previously found to diminish or eliminate physiological interactions, for example, with ABCA1\textsuperscript{152–154} or SR-BI,\textsuperscript{155,156} resulting in defective cholesterol efflux, or in pathophysiological interactions, for example with the lectin-oxidized lipoprotein receptor LOX-1,\textsuperscript{157} or the toll-like receptor TLR2,\textsuperscript{158} both of which result in inhibition rather than activation of eNOS and, hence, normal suppression of NO production.\textsuperscript{38,39} As yet, the relative importance of the many physiological and pathological activities of normal and dysfunctional HDL, respectively, in the pathogenesis of atherosclerosis or T2DM is unknown. The elucidation of this problem, however, is the major limiting factor in the clinical exploitation of HDL for treatment and prevention of cardiovascular and possibly other diseases. The identification of the most relevant biological activities of HDLs and the mediating molecules within HDLs as well as their cellular interaction partners are pivotal for the development of anti-atherogenic drugs as well as of diagnostic biomarkers for the identification, personalized treatment stratification, and monitoring of patients at increased cardiovascular risk.

Three principle types of molecular changes appear to underlie HDL dysfunction: (1) compositional changes of the proteome, (2) post-translational modifications of proteins, and (3) alterations of the lipid moiety and other cargo molecules.

These phenomena can be interrelated. For example, the displacement of apoA-I, PON1, and PAF-AH by acute-phase proteins reduces the capacity of HDL to protect its own lipids and proteins from oxidation. Oxidized lipids or post-translational modification of proteins by thereof derived advanced lipoxidation endproducts (ALE) can directly interfere with the functionality of HDLs and/or lead to the formation of antigenic neoepitopes; for example in apoA-I, which can bind neutralizing autoantibodies.

**Compositional Changes of the Proteome**

Traditionally, the loss of HDL function has been attributed to replacement of apoA-I by other proteins, notably acute-phase proteins such as SAA, which in inflammation can represent more than 80% of HDL proteins, but also haptoglobin, ceruloplasmin, and fibrinogen.\textsuperscript{153,157} An effect of SAA on HDL’s cholesterol efflux capacity is controversial. The laboratory of van der Westhuysen and de Beer showed in several publications that SAA-enriched HDLs elicit normal cholesterol efflux via ABCG1- and SR-BI-mediated pathways.\textsuperscript{158,159} The SAA-induced remodeling of HDLs liberates apoA-I,\textsuperscript{160} so that ABCA1-mediated cholesterol efflux was also found to be normal in the presence of SAA.\textsuperscript{161} By contrast, Malle’s group found reduced cholesterol efflux capacity by both SAA-enriched HDLs and lipid-free SAA.\textsuperscript{162,163} Interestingly, adenosine-mediated expression of SAA reduced the fecal excretion of macropage-derived cholesterol in mice,\textsuperscript{164} indicating that SAA interferes with macrophage reverse cholesterol transport, possibly by mechanisms other than cholesterol efflux. In addition, SAA was found to reduce the antioxidative capacity of HDLs by displacing PON1 and PAF-AH.\textsuperscript{151} SAA also increases the binding of HDLs to vascular proteoglycans and thereby the retention in the arterial intima as well as exposition to oxidative modifications.\textsuperscript{165} Moreover, SAA mimicked the stimulatory activity of HDL from CKD patients on the production of inflammatory cytokines and chemokines by monocytes and VSMCs.\textsuperscript{166}

Other hypothesis-driven analyses of dysfunctional HDL have identified atypical abundance of myeloperoxidase (MPO) in HDL of patients with T2DM,\textsuperscript{167} which is considered an important causal factor for the oxidative protein modification of apoA-I (see later).\textsuperscript{35} Vice versa, the activity of the antioxidative PON1 was found to be decreased in either sera or HDL of patients with CAD, T2DM, T1DM, CKD, rheumatoid arthritis, or cardiac surgery (Table S1). The decreased PON1 activity showed very plausible associations with reduced antioxidative activities of HDLs in various disease conditions (Table S1). In addition, low PON1 activity was found as a causal factor in the inhibition of NO production in endothelial cells, probably by allowing the formation of malondialdehyde and subsequent lysine residue modifications in HDL-associated proteins.\textsuperscript{168}

Much more compositional changes have been found by recent small but comprehensive proteomic case-control studies of patients with CAD, T2DM, CKD, rheumatoid arthritis, or psoriasis (Table S1). Some findings of previous hypothesis-driven studies were confirmed by this hypothesis-free approach; for example, the enrichment of HDL from patients with rheumatoid arthritis, psoriasis, CAD, or CKD with SAA (Table S1). Enrichment with apoC-III in the HDL of patients with stable or acute CAD, T2DM, or CKD is another very robust observation in proteomic case-control studies from different laboratories.\textsuperscript{33,37,167,168} This finding has been confirmed in 2 nested prospective studies by the opposite associations of apoC-III-containing HDL and apoC-III-free HDL with cardiovascular risk.\textsuperscript{41} However, proteomics studies have also generated controversial findings; for example, the enrichment of HDL from CAD patients with PON1 and apoE in one study,\textsuperscript{167} but deprivation of the same proteins in HDL from CAD and T2DM patients in other studies.\textsuperscript{33,37} Differences in patients and methods of HDL isolation may be the reason, in addition to chance findings in the as yet small sample numbers. Quantitative proteomic studies using single-reaction-monitoring mass spectrometry or arrayed immunossays in larger sample sizes are hence needed to firmly establish proteomic footprints, which differen-
tiate the HDL of healthy and diseased individuals. In addition, the (dy)sfunctional relevance of many differentially expressed proteins in HDLs needs to be shown, especially if this cannot be inferred from previous investigations of candidate proteins such as SAA or PON1. For many newly detected differences in HDL proteomes, the functional relevance needs to be established. For example, enriched apoC-III but decreased clusterin content was found to account for the reduced anti-apoptotic activity of HDL from CAD patients towards endothelial cells.

**Post-Translational Modifications of Proteins**

A broad range of enzymatic and oxidative modifications has been reported for both the proteins and lipids of HDLs. Notably, amino acid residues of apoA-I have been found modified by oxidation through MPO and carbonylation through advanced glycation endproducts (AGE) and ALE. These modifications have been found at increased concentrations in the HDL of patients with inflammatory diseases, including CAD, T2DM, and CKD, and have been associated with dysfunction. However, it must be emphasized that the concentration of these modifications is in the low or even submicromolar range and hence lower than the concentration of apoA-I or HDL particles (Figure 2). It hence appears that defined and functionally highly relevant subfractions of HDL rather than the entire HDL fraction are critically hit by these modifications.

MPO, which is strongly expressed by neutrophil granulocytes and other myeloid cells, plays a pivotal role in the oxidative chlorination and nitration of free hydroxy groups in tyrosine and tryptophan residues, and the sulfoxidation of methionine residues via formation of hypochlorous acid (HOCI), as well as the carbamylation of free amino groups, for example, in lysine residues via the formation of cyanate from thiocyanate.

Patients with CAD were found to have increased concentrations of MPO as well as its products chlorotyrosine and nitrotyrosine in both plasma and HDL. Interestingly, the association of chlorinated or nitrosylated apoA-I with the presence of CAD was found to be stronger than the association of chlorinated or nitrosylated plasma proteins in general, perhaps reflecting the presence of an MPO binding site in apoA-I that allows enrichment of this enzyme and its products in HDLs. Moreover, as compared with plasma apoA-I, apoA-I isolated from atherosclerotic plaques was more strongly modified by MPO. This indicates that either the modification of apoA-I occurs in the extravascular rather than intravascular space or that MPO-modified apoA-I and HDLs are trapped within the vascular wall. Also, carbamylated HDL was found at increased concentrations in advanced atherosclerotic plaques, especially of smokers and CKD patients, probably because cyanate is also released from cigarette smoke and formed from urea independently of MPO. The oxidation of HDLs and apoA-I by MPO or its immediate product, HOCI, severely compromises their ability to induce ABCA1-mediated cholesterol efflux and to activate LCAT, whereas carbamylated HDL has a reduced ability to induce SR-BI-mediated cholesterol efflux. Moreover, treatment of HDLs with MPO or HOCI interferes with their ability to inhibit apoptosis and TNF-α-induced VCAM-1 expression, as well as to stimulate NO production, possibly by reducing the binding of HDL to SR-BI. The observation of decreased macrophage cholesterol transport in mice after injection of MPO brought in vivo evidence for the pathophysiological relevance of the previous in vitro findings. In addition to the loss of protective activities on cholesterol efflux and esterification as well as endothelial function and survival, MPO-modified HDL gains pro-inflammatory activities as it induces NF-κB activation and VCAM-1 expression in endothelial cells by binding to an as yet unknown receptor. Several amino acid residues are oxidatively modified by MPO or HOCI in vitro and have been identified as modified in HDL isolated from patients. However, despite systematic in vitro mutagenesis studies to generate dysfunctional mimetics of MPO-modified apoA-I or MPO-resistant apoA-I mutants, the identity of the pivotal amino acid residues modified by MPO is not unequivocally resolved. As yet, Tyr166, Tyr192, and Met146 have been proposed as being critical.

Another group of protein modifications in HDLs with potential functional relevance is caused by carbonylation of arginine, lysine, and tryptophan residues in their proteins with either AGE including glyoxal, methylglyoxal, and glycoaldehyde or with ALE including malondialdehyde (MDA), 4-hydroxynonenal (HNE), and acrolein. Both AGE (notably methylglyoxal) and ALE (notably MDA) have been found at increased concentrations in HDL of patients with CAD or T2DM. Methylglyoxal interferes with the ability of HDLs to bind phospholipids and to activate LCAT. Exposure of lipid-free apoA-I, native or reconstituted HDL to methylglyoxal as well as to MDA or acrolein, was found to abrogate their capacity to induce cholesterol efflux in some but not all studies. In a recent study, HDL and sera of diabetic subjects were found to display increased rather than decreased cholesterol efflux capacity, which correlated with CETP activity but not with AGE levels. Neither showed AGE levels any association with macrophage reverse cholesterol transport in vivo of db/db mice or uremic mice. HDLs enriched with either AGE in T2DM patients or with MDA in CAD patients failed to inhibit monocyte or neutrophil diapedesis through endothelial cells by suppressing VCAM-1 and intracellular adhesion molecule-1 expression. Both the HDL of CAD patients and that of healthy subjects enriched with MDA bind to the scavenger receptor LOX-1 of endothelial cells. The resulting activation of protein kinase C β II causes the inhibition instead of activation of phosphorylation of eNOS at residues Thr495 and Ser1177, respectively, so that MDA-modified HDL, like the HDL of CAD patients, inhibits rather than stimulates NO production in endothelial cells. The same phenotype and dysfunction were observed in the HDL of PON1 knockout mice and could be corrected by addition of recombinant PON1. Therefore, and because PON1 activity is low in the HDL of CAD patients, PON1 appears to be crucial to protect HDLs from lipidoxidation and hence MDA formation and the resulting dysfunction. In this regard, it is noteworthy that antioxidative activity and paraxonase itself were found to be modified by AGE.

Symmetric dimethylarginine (SDMA) is a natural protein degradation product, which was recently identified as the cause of endothelial dysfunctionality of HDLs in CKD. SDMA is generated intracellularly by the methylation of arginine residues through a protein arginine methyltransferase, secreted by cells, transported by plasma proteins, and mostly excreted by the kidney. Observational studies identified elevated SDMA concentrations as a risk factor for cardiovascular events. As yet, it is not known why the water-soluble SDMA is enriched in the HDL of CKD patients. HDL-associated proteins may serve as SDMA-binding proteins, which are not properly removed from the circulation in CKD. In fact, complementation of HDLs, reconstituted HDLs, or lipid-free apoA-I with SDMA mimics the dysfunction of HDL from CKD patients. Enrichment with SDMA transforms HDLs into agonists of TLR2, which via atypical signaling inhibit eNOS and stimulate the translocation of NF-κB into the nuclei of endothelial cells. In mice, this translates into disturbed vasodilatation and increased blood pres-
sure as well as impaired healing of denuded arteries by EPCs.\textsuperscript{39} In addition, VCAM-1 expression and monocyte diapedesis by cultivated endothelial are enhanced.\textsuperscript{39}

In addition to being direct causes of HDL dysfunction, structural modifications may generate neo-epitopes in apo-A-I that break self-tolerance and allow the appearance of anti-apo-A-I antibodies.\textsuperscript{169} Autoantibodies to apo-A-I are found in the plasma of the normal population at a prevalence of 1–2% but at a prevalence of 10–30% in the plasma of patients with systemic lupus erythematosus (SLE), primary antiphospholipid syndrome (APLS), or rheumatoid arthritis, as well as in the plasma of patients with acute coronary syndrome (ACS) or carotid stenosis.\textsuperscript{196–198} In patients with ACS or rheumatoid arthritis high titers of anti-apo-A-I-autoantibodies increase the risk of cardiovascular events or death.\textsuperscript{199,200} In patients with SLE or APLS the presence of anti-apo-A-I-autoantibodies interferes with the antioxidant properties of HDLs by decreasing PON1 activity.\textsuperscript{196,200} Moreover, the autoantibodies interfere with the negative chronotropic effects of HDLs on cultivated cardiomyocytes.\textsuperscript{206} Finally, there is some evidence that anti-apo-A-I-autoantibodies have anti-idiotypic properties by which they bind to the TLR2/CD14 complex and elicit pro-inflammatory signals.\textsuperscript{207}

**Alterations of the Lipid Moiety and Other Cargo Molecules**

Modern mass spectrometry has enabled differentiation of more than 200 lipid species from 5 major lipid classes (glycerophospholipids, sphingolipids, sterols, acylglycerols, and fatty acids) in the HDL of healthy subjects.\textsuperscript{208}

The content of cholesteryl esters and triglycerides in HDLs is shifted towards enrichment with triglycerides in many clinical conditions characterized by hypertriglyceridemia and increased CETP activity, such as MetS, T2DM, CAD, and the acute-phase response.\textsuperscript{209} By adversely affecting both cholesterol efflux from macrophages and selective uptake of cholesteryl esters by hepatocytes the increased triglyceride content may compromise reverse cholesterol transport.\textsuperscript{28,209–211} However, it is important to note that several laboratories reported positive rather than inverse correlations between plasma concentrations of triglycerides and cholesterol efflux capacity.\textsuperscript{211–213} In addition, the increased triglyceride content of HDL from patients with T2DM or familial hypercholesterolemia was associated with decreased antioxidative,\textsuperscript{214,215} anti-inflammatory,\textsuperscript{216} and vasodilatory activities.\textsuperscript{217} Of note, all these observations reflect associations among disease, altered triglyceride content, and dysfunction. Because of confounding with other changes in the lipidome and proteome of HDLs in those conditions, the causal contribution of triglycerides is as yet not proven.

For the same reason, the causal role of cholesteryl ester enrichment, which is found in CETP deficiency and after CETP inhibition, for cholesterol efflux capacity is unclear.\textsuperscript{218} Unesterified cholesteryl is enriched in the HDL of patients with LCAT deficiency and various inflammatory states, but without any known functional consequence.\textsuperscript{28} In addition to the millimolar concentrations of esterified and unesterified cholesteryl, HDLs carry micromolar or lower concentrations of oxysterols,\textsuperscript{218,219} bile acids,\textsuperscript{220} and steroid hormones,\textsuperscript{221–223} many of which are agonists of nuclear hormone receptors. However, except for some reports on the effect of 7-ketocholesterol, estradiol, and dehydroepiandrosterone on cholesterol efflux capacity,\textsuperscript{221,224} eNOS expression, and endothelium-dependent vasoreactivity,\textsuperscript{222,223} their contribution to the physiological and pathological functions of HDLs in health and disease, respectively, are as yet unknown. By investigating plasma for inborn errors of HDL metabolism, we have previously found that alterations in the activity of ABCA1, LCAT, or CETP affect the concentrations of 27-hydroxycholesterol in HDLs beyond the concentration of cholesterol.\textsuperscript{219}

The phospholipid content and composition have a strong effect on the size, shape, fluidity, and surface charge of HDLs.\textsuperscript{225} Their contribution to the functionality of HDLs has been tested experimentally by reconstituting artificial HDL particles with defined phosphatidylcholine species at different concentrations. Increased concentrations of phosphatidylcholine increase the ability of HDLs to induce SR-BI-mediated cholesterol efflux.\textsuperscript{225} The negatively charged phosphatidylinositol and phosphatidylserine as well as cardiolipin contribute to the inhibitory effects of HDLs on platelet activation and coagulation.\textsuperscript{134,226} A higher content of unsaturated fatty acids in phosphatidylcholines increases the fluidity of HDLs and thereby also their ability to accept cholesterol\textsuperscript{164,226} and lipid hydroperoxides\textsuperscript{227} from cell membranes and the anti-inflammatory activity on VCAM-1 expression.\textsuperscript{228} Surprisingly, however, intervention with a diet enriched with unsaturated fatty acids was not found to alter the cholesterol efflux capacity of HDLs.\textsuperscript{229–231} Phospholipids containing polyunsaturated fatty acids are easily oxidized.\textsuperscript{232} Some of the oxidized phospholipids are bioactive and exert inhibitory effects, for example, on eNOS activity.\textsuperscript{233} Moreover, oxidized phospholipids generate ALE, which modify the proteins of HDLs. It is generally believed that the high antioxidative activity of apo-A-I, PON1, and Lp-PLA\textsubscript{2} (=PAF-AH) as well as LCAT-mediated transacylation prevents the accumulation of oxidized phospholipids in HDLs.\textsuperscript{234} However, as explained earlier, these antioxidative activities are compromised in several clinical conditions. Lipolysis of glycerophospholipids by phospholipases such as endothelial lipase and secretory phospholipase A\textsubscript{2},\textsuperscript{235,236} which are activated by inflammation as well as the LCAT action,\textsuperscript{237} generate lysosphospholipids that in turn are bioactive and can exert pro-inflammatory activities.\textsuperscript{235,237} Changes in the glycerophospholipid composition may hence affect the functionality of HDLs and serve as a biomarker. As yet, however, no data from clinical studies are available that show associations and correlations of phospholipid composition with disease and functionality of HDLs, respectively.

Likewise, the clinical relevance of sphingolipids carried by HDLs needs to be established. By decreasing the fluidity of HDLs but enhancing the affinity for cholesterol, increases in the sphingomyelin content of HDLs have a complex effect on cholesterol efflux capacity.\textsuperscript{28,238} One study reported an inverse association between sphingomyelin in HDL and the presence of CAD in women.\textsuperscript{239} Because of their cytoprotective activities, S1P and other lysosphingolipids are especially interesting candidates to explain HDL dysfunction and to be used as underlying biomarkers. In fact, S1P levels were found by 2 studies to be lowered in HDL or apoB-depleted plasma of CAD patients.\textsuperscript{240,241} Interestingly, in inborn errors of HDL metabolism the concentration of S1P and its binding protein, apoM, do not simply vary with HDL-C or apoA-I levels. Of note, HDL-C increasing mutations in CETP, SCARB1 (coding for SR-BI), LIPC, and LIPG (coding for hepatic lipase and endothelial lipase, respectively) did not increase the S1P levels.\textsuperscript{242} This underlines the potential of S1P to serve as a biomarker beyond HDL-C or apoA-I.

The amphiphilic character and the presence of several specific binding proteins allow HDLs to transport not only lipids but also vitamins and other micronutrients, drugs, xenobiotics, and toxins as well as nucleic acids.\textsuperscript{243} In fact, its broad binding capacity has motivated the generation of reconstituted HDLs for the detoxification of lipopolysaccharides in sepsis as well as for the delivery of drugs for therapy or diagnostic imaging.\textsuperscript{244,245} However, the binding of some exogenous molecules or endog-
enous metabolites may interfere with the normal functionality of HDLs or convey atypical adverse activities. However, this concept has not yet been tested experimentally. Likewise, the role of miRNA transport for physiological and pathological functions of HDLs in health and disease, respectively, is unknown. One study did not find any effect of disease-associated alterations in HDL-associated miRNAs on endothelial functionality.30

Overall, quantitative and qualitative changes in the lipoproteins of HDLs are interesting candidates for explaining interindividual differences in the functionality of HDLs and to be therefore exploited as biomarkers. However, comprehensive lipidomic/metabolomic case-control studies of HDL under different clinical conditions are in their infancy. Ideally, these studies will also record HDL functions to increase the probability of finding functionally relevant lipid species. These candidates then need to be validated by testing reconstituted HDLs with or without this lipid in functional assays to exclude confounding with other alterations in HDLs.

Conclusions

For more than 50 years, a low level of HDL-C has been known as an independent marker of increased cardiovascular risk. Nevertheless and despite the tremendous increase of knowledge about the structure, function, and metabolism of HDL, it has not yet been successfully targeted for the prevention or cure of atherosclerosis.

In part this failure originates from the previous targeting of drug development to the clinical biomarker “HDL-C”, which reflects neither the functionality of HDLs nor the intensity of reverse cholesterol transport. The identification of the most relevant biological activities of HDLs (ie, cholesterol efflux and reverse cholesterol transport or anti-inflammatory or vasoprotective activities) as well as their mediating molecules within HDLs are important bottlenecks in the development of anti-atherogenic drugs as well as of diagnostic biomarkers for the identification, personalized treatment stratification, and monitoring of patients at increased cardiovascular risk. As yet, it is unclear whether such markers will be native proteins or protein modifications or lipids or other cargo molecules. To solve this problem, a systems medicine approach is needed to collect comprehensive data sets on the proteomes, lipoproteins, and functionality of HDLs in different disease conditions for subsequent bioinformatic integration. Proteins, lipids and/or modifications that show the closest correlations with dysfunction and disease status will be the most interesting candidates for subsequent experimental and epidemiological validation in animal and cell culture models as well as prospective population and patient studies.

Another obstacle is the dynamics of HDL function and dysfunction. HDL and apoA-I of healthy humans and animals exert innate host defense against many biological and chemical hazards. However, like temporary and local expansion of acute infections compromise the innate defense of the organism by leukocytes and their humoral mediators and escalate into the damaging and threatening systemic inflammatory response syndrome, chronic inflammatory conditions may compromise the functionality of HDLs and even pervert HDLs into hazardous particles. In this situation it may be harmful rather than helpful to increase the half-life of HDLs, for example by CETP inhibition. The increase in C-reactive protein concentrations observed to increase the half-life of HDLs, for example by CETP inhibition. These candidates may be harmful rather than helpful. Drugs that increase the elimination of existing HDLs and generation of novel HDL particles by increasing de novo synthesis of HDLs or infusion of reconstituted HDLs may be more promising.

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Multifunctional but Vulnerable HDL


Supplementary Files
Table S1. Dysfunction of high-density lipoprotein (HDL) in disease