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STATE OF THE ART REVIEW
Dyslipidaemias

High-density lipoprotein revisited: biological functions and clinical relevance

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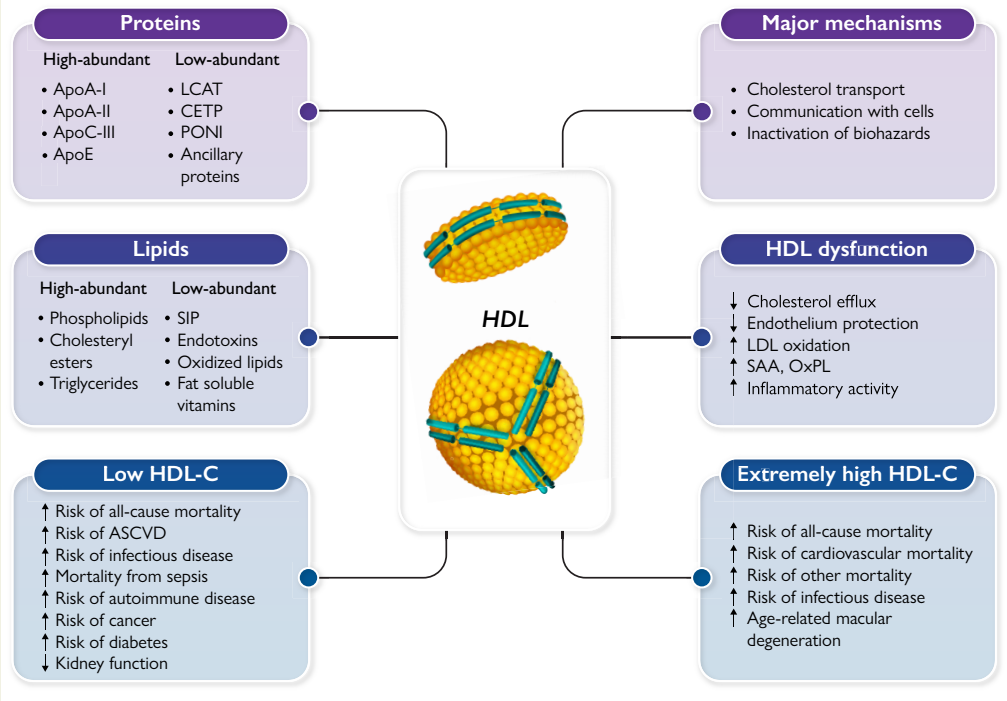
Graphical Abstract



The great complexity of HDL. High-density lipoprotein (HDL) particles carry a large number of proteins and lipids, which contribute to define their compositional and functional complexity. HDLs exert multiple protective activities, essentially by three major mechanisms. HDLs, however, can lose their protective functions and even gain adverse functions in chronic diseases or during infections. U-shaped relationships between HDL-cholesterol (HDL-C) levels and several conditions have been reported, being both low and extremely high HDL-C levels associated with an increased risk of several pathologies and mortality. LCAT, lecithin:cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; PONI, paraoxonase 1; S1P, sphingosine-1-phosphate; ASCVD, atherosclerotic cardiovascular disease; LDL, low-density lipoprotein; SAA, serum amyloid A; OxPL, oxidized phospholipids

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Abstract

Previous interest in high-density lipoproteins (HDLs) focused on their possible protective role in atherosclerotic cardiovascular disease (ASCVD). Evidence from genetic studies and randomized trials, however, questioned that the inverse association of HDL-cholesterol (HDL-C) is causal. This review aims to provide an update on the role of HDL in health and disease, also beyond ASCVD. Through evolution from invertebrates, HDLs are the principal lipoproteins, while apolipoprotein B-containing lipoproteins first developed in vertebrates. HDLs transport cholesterol and other lipids between different cells like a reusable ferry, but serve many other functions including communication with cells and the inactivation of biohazards like bacterial lipopolysaccharides. These functions are exerted by entire HDL particles or distinct proteins or lipids carried by HDL rather than by its cholesterol cargo measured as HDL-C. Neither does HDL-C measurement reflect the efficiency of reverse cholesterol transport. Recent studies indicate that functional measures of HDL, notably cholesterol efflux capacity, numbers of HDL particles, or distinct HDL proteins are better predictors of ASCVD events than HDL-C. Low HDL-C levels are related observationally, but also genetically, to increased risks of infectious diseases, death during sepsis, diabetes mellitus, and chronic kidney disease. Additional, but only observational, data indicate associations of low HDL-C with various autoimmune diseases, and cancers, as well as all-cause mortality. Conversely, extremely high HDL-C levels are associated with an increased risk of age-related macular degeneration (also genetically), infectious disease, and all-cause mortality. HDL encompasses dynamic multimolecular and multifunctional lipoproteins that likely emerged during evolution to serve several physiological roles and prevent or heal pathologies beyond ASCVD. For any clinical exploitation of HDL, the indirect marker HDL-C must be replaced by direct biomarkers reflecting the causal role of HDL in the respective disease.

Keywords Cholesterol efflux • Evolution • Remnants • Triglycerides • Infectious disease • Cancer • Age-related macular degeneration • Autoimmune disease

Historical perspective

The first observation of an inverse relationship between high-density lipoprotein-cholesterol (HDL-C) levels and the risk of developing coronary heart disease (CHD) dates back to the 1950s.[1] Since the 1970s, results from many other studies have reinforced this strong inverse relationship,[2–8] conferring to HDL-C the appellative of 'good cholesterol', as opposed to the low-density lipoprotein-cholesterol (LDL-C) referred to as 'bad cholesterol'. These early observations paved the road for interventional clinical trials testing the hypothesis that increasing HDL-C levels using pharmacological approaches would reduce the cardiovascular (CV) risk.

Drugs that increase circulating HDL-C levels, including niacin, fibrates, and cholesteryl ester transfer protein (CETP) inhibitors except anacetrapib,[9] have essentially failed to demonstrate any CV benefit, at least if added to state-of-the-art treatment with statins.[10–13] However, it is important to reconcile that, with the exception of dalcetrapib, these drugs also alter plasma levels of other lipoproteins so that the futility of these interventions in reducing ASCVD may reflect limitations of the drugs or the study design (e.g. patient selection, combination with statins) rather than the role of HDL in atherosclerotic CV disease (ASCVD). Of note, the benefit shown with anacetrapib was directly proportional to the reduction of non-HDL-C. Genetic studies provided controversial evidence that HDL-C levels are causally associated with CV risk, also because most genetic determinants of HDL-C also affect other lipid traits, notably triglycerides but also LDL-C. Rare variants in genes which cause low HDL-C without altering other lipid traits, namely APOA1, ABCA1, and LCAT, were not associated with any increase of risk of ASCVD in general population studies,[14–16] but associated with a higher prevalence of ASCVD in studies of families with low HDL-C[17] or in a large lipid clinic registry.[18] Variants in genes determining higher HDL-C levels also yielded equivocal results. Some like LIPC[19,20] and SCARB1[21,22] are associated with normal to increased risk of ASCVD. Others like LIPG[23,24] or CETP[25,26] are associated with normal to reduced risk.[23] Data from a genetic score combining 14 variants exclusively related to HDL-C showedno significant association with the risk of CHD.[23] Also, more recently, even larger Mendelian randomization studies failed to show any significant genetic association of HDL-C levels with the risk of ASCVD.[27] Finally, the findings in genetic animal models indicate the importance of specific genes and metabolic pathways as determinants of HDL's role in ASCVD. For example, interferences with apoA-I expression show the expected inverse effects on HDL-C and atherosclerosis, whereas knock-out of Scarb1 increases both HDL-C and atherosclerosis.[28]

Apart from pleiotropic effects of gene variants, an important reason for this controversy is the non-continuous relationship of HDL-C with the risk of ASCVD. Data from six community-based cohorts showed an inverse and linear relationship between HDL-C and CHD risk up to a value of ≈90 mg/dL but for HDL-C values >90 mg/dL, no further reduction in CHD risk was observed.[29] In a meta-analysis of 68 studies, this threshold was the 80th and 60th percentile for unadjusted and adjusted HDL-C levels, respectively.[8] More recently, observational studies have shown a U-shaped relationship, with both low and very high levels of HDL-C being associated with an increased risk of all-cause mortality, CV mortality, infections, and dementia in the general population.[30–32] These new findings have challenged the longstanding premise that raising HDL-C would reduce CV risk, but also suggested that, perhaps, HDL functionality rather than HDL-C levels may be more relevant in terms of drug development and as an ASCVD biomarker.

This new paradigm has reinforced the idea that HDL is not merely a cholesterol transporter, but, rather, possesses several additional functional properties [including cholesterol efflux capacity (CEC), anti-oxidant, anti-inflammatory, and immune-regulating activities].[33] In addition, HDL is a rather complex family of different particles, being composed of sub-species differing in size, density, shape, charge, and composition, undergoing continuous remodelling processes in the circulation.[33] This remarkable heterogeneity of the HDL particle family may explain why HDL cannot always be considered 'anti-atherogenic', but can sometimes become dysfunctional or even 'pro-atherogenic'. Thus, it is not surprising that recent studies have shown that measures of various possible HDL functions, such as CEC and HDL inflammatory index (i.e. the capacity of HDL to inhibit the oxidation of LDL), or the

number of circulating HDL particles are better predictors of CV events than just the cholesterol content of HDL.[34–41]

Is high-density lipoprotein-cholesterol all we need to measure?

High-density lipoprotein structure and composition

HDL is the smallest circulating lipoprotein and is found near all cells. HDL contains both proteins and lipids (*Figure 1*), but the unique arrangement of its two main constituents in different HDL particle subspecies provides insights into understanding its various physiological roles. Lipids in HDL are arranged in a micelle-like configuration, with the abundant amphipathic lipids (phospholipids and free cholesterol) forming a surface monolayer and the more hydrophobic or neutral lipids (cholesteryl esters and triglycerides) in the particle core. Particles with a hydrophobic neutral lipid core form spherical-like structures approximately 8–11 nm in diameter (referred to as α-migrating HDL, based on their migration on agarose gel), whereas particles depleted of neutral lipids form disc-like structures.[42]

Among the two major components of HDL, proteins and lipids, some are abundant whereas others are present in small amounts (*Figure 1*), with concentrations spanning 4–5 orders of magnitude and ranging from sub-micromolar ([ipid transfer proteins, apolipoprotein (apo) L1, sphingosine-1-phosphate, bile acids] to millimolar (cholesterol, phosphatidylcholine).[42] Based on the average plasma concentration of 20 μmol/L, a representative HDL particle carries 50–100 molecules of esterified or unesterified cholesterol or phosphatidylcholine and 2–3 molecules of apoA-I. However, <5% of the particles each carry one molecule of minor constituents.[42]

The most abundant apolipoprotein on HDL is apoA-I, which contributes to the maintenance of HDL structure and to the removal of excess cellular cholesterol through the ATP-binding cassette transporter-1 (ABCA1).[42] The typical large spherical form of HDL has at least three molecules of apoA-I in a trefoil-like configuration,[43] whereas discoidal HDL has two copies of apoA-I wrapped around the side of the disc, shading the hydrophobic acyl chains of its phospholipid bilayer.[44] The low-abundant proteins on HDL can be further divided into lipoprotein-specific proteins and ancillary proteins. Lipoprotein-specific proteins include lecithin:cholesterol acyltransferase (LCAT), CETP, and paraoxonase-1 (less than one copy per particle). Most of the ancillary HDL proteins, which now number over 200,[45,46] are even less abundant and, for the most part, are only loosely associated with HDL. Although low in abundance, these ancillary proteins may, nevertheless, have important biological functions: as an example, haptoglobin or haptoglobin-related protein enables HDL to act as a potent trypanosome-lytic factor,[47] and alpha-1 antitrypsin (an acute-phase protein)[48] may enable a more efficient HDL delivery to sites of tissue damage where it suppresses inflammation.

Phospholipids are abundant, key structural components of HDL and often have been considered just structural, but this is an overly simplistic view. For example, in the absence of HDL due to LCAT deficiency, excess phospholipids generated during lipolysis of apoB-containing lipoproteins reorganize as multi-lamellar vesicles called lipoprotein-X (Lp-X).[49] Lp-X particles get trapped in the glomerulus and can cause end-stage kidney disease, thus one potentially important role of HDL is to prevent this outcome. Moreover, they are substrates for enzymes (e.g. LCAT or endothelial lipase) that generate lysophospholipids and hence bioactive molecules. Although not as abundant as triglycerides on apoB-containing lipoproteins, the transfer of triglycerides to HDL by CETP may extend the time for the delivery of triglycerides for lipolysis in the post-prandial state and, because of the small size of HDL and its ability to enter extracellular fluid, it may enhance the delivery of triglycerides on HDL to peripheral tissues.[50] Low-abundant lipids (or lipid-like substances) on HDL, like sphingosine-1 phosphate (S1P),[51] as will be discussed below, may also have important effects because they are potent biological signalling molecules. HDL can even bind to miRNAs and other types of short nucleic acid fragments but the pathophysiological significance of this is not clear.[52]

High-density lipoprotein sub-fractions

Given the compositional complexity of HDL, it is not surprising that there are numerous sub-fractions or ways to further sub-divide HDL into different structural or functional categories. The main impetus behind this effort was to identify sub-fractions of HDL that may be diagnostically important for predicting CV disease risk, but it also has obvious implications for developing drugs that modulate HDL for the prevention of CV disease or other diseases. Historically, HDL was first sub-fractionated based on the density of lighter (and larger) HDL_2 and heavier (and smaller) HDL_3 subfractions.[53] Another early classification of HDL sub-fractions was based on the presence or absence of apoA-II, the second most abundant protein in HDL. These types of classifications had, however, limited impact on routine diagnostic testing, because of lacking evidence for superiority. Separation of HDL into discrete HDL size fractions can now be readily done by nuclear magnetic resonance (NMR) spectroscopy in clinical laboratories, providing the ratio of large-to-small HDL, which may be useful for assessing not only CV risk but also insulin resistance and other conditions.[53] Recent advances in mass spectrometry allow the comprehensive quantification of the proteome in total HDL as well as distinct subclasses.[45,46,54,55] At this time, there is only limited commercial availability of these advanced HDL sub-fractionation tests and hence they are not widely used but they are being actively investigated for their clinical utility.

High-density lipoprotein function

Recent proteomic analyses and metabolic turnover studies provided evidence that distinct HDL sub-classes have a pre-defined core-protein composition that remains relatively stable throughout their lifecycle.[56,57] Interestingly, these HDL sub-classes frequently contain specialized proteins that fulfil related or complementary functions, for example, in haemostasis, protease inhibition, or the complement system.[45,46,56] One notable example is the complex consisting of apoA-I, haptoglobin-related protein, and apoL1 by which haptoglobin-related protein provides the binding to *Trypanosoma* and the internalized apoL1 elicits the lysosomal swelling, ultimately killing *Trypanosoma*.[58] By contrast, the lipid composition of HDL particles is highly dynamic. ABCA1 fluxes glycerophospholipids and cholesterol from cell membranes, especially to lipid-free apoA-I as well as to small, lipid-poor HDL. LCAT generates cholesteryl esters and lysophosphatidylcholines by the transfer of sn-2 fatty acids from phosphatidylcholines to the 3-OH group of cholesterol. Endothelial lipase and hepatic lipase hydrolyse phosphatidylcholines and triglycerides of HDL, respectively, generating free fatty acids, lysophosphatidylcholines, and diacylglycerols, which also are bioactive molecules. CETP exchanges cholesteryl esters of HDL for triglycerides from apoB-containing lipoproteins and phospholipid transfer protein (PLTP) transfers phospholipids from

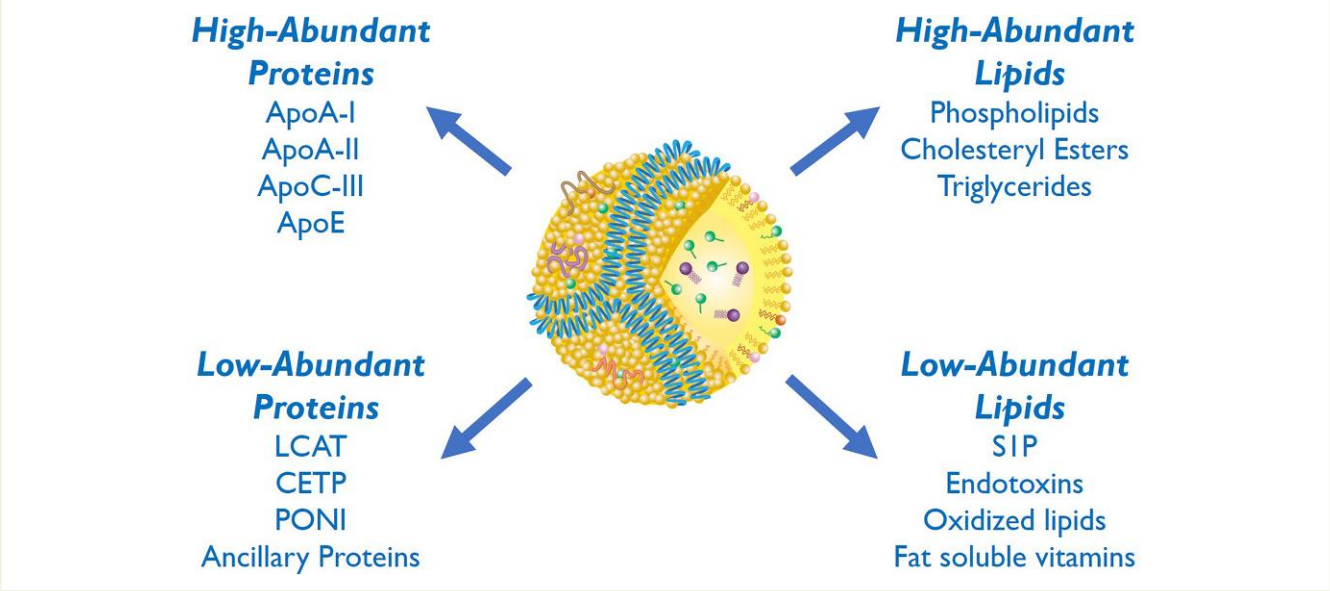

Figure 1 High and low abundant lipids and proteins in high-density lipoprotein particles. Apo, apolipoprotein; LCAT, lecithin:cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; PONI, paraoxonase 1; S1P, sphingosine-1-phosphate.

apoB-containing lipoproteins to HDL as well as between different HDLs.[59] In addition, lipids are fluxed between HDL and cells and between HDL and other lipoproteins following concentration gradients or affinity. For example, unesterified cholesterol is readily accepted by HDL from both lipolysed triglyceride-rich lipoproteins (TGRLs) or cell membranes but also transferred from HDL to LDL or cells[60,61] (*Figure 2*). S1P is readily effluxed from erythrocytes or endothelial cells, especially by HDLs that contain its chaperone apoM.[62,63]

Thus, HDLs are modular scaffolds that combine structural specificity with plasticity. Thereby, HDLs exert multiple functions that protect the organism from chemical or biological harm or help to repair the tissue damage caused by noxious agents. HDLs do so by three principal mechanisms.

Cholesterol transport

The most intensively investigated function of HDL is reverse cholesterol transport (RCT). According to this model, HDLs elicit cholesterol efflux from macrophage foam cells of atherosclerotic plaques either specifically, via sequential interactions with ABCA1 and ABCG1, or by aqueous diffusion, through a process facilitated by scavenger receptor BI (SR-BI) (*Figure 2*).[64] Virtual HDL-deficiency in Tangier disease as well as in mice with systemic or hepatocyte-specific knock-out of ABCA1, illustrates the rate-limiting importance of ABCA1 for the biogenesis of HDL.[59,65] Free cholesterol is then esterified by LCAT and cleared by the liver, either directly, by selective uptake through SR-BI, or indirectly after CETP-mediated transfer to apoB-containing lipoproteins which are then internalized by the LDL receptor.[65] In addition, HDL particles as such are taken up by hepatocytes through a yet poorly understood mechanism (*Figure 2*).[66]

Of note, HDL-C levels reflect neither the capacity nor the intensity of RCT.[67] Neither do increased HDL-C levels upon treatment with CETP inhibitors indicate enhanced RCT.[68] Especially in the context of LDL receptor activation through the concomitant statin therapy, the interference with cholesteryl ester transfer from HDL to LDL blocks RCT by preventing the removal of cholesterol by the LDLR pathway. The doubling of HDL-C levels upon CETP inhibition indicates that the blockage of this indirect pathway is not compensated by the direct removal of HDL and its cholesterol cargo.

RCT is of special relevance for the removal of cholesterol from macrophages: following cholesterol accumulation, macrophages exert several pro-inflammatory activities which are dampened by HDL-mediated cholesterol efflux.[69] Also, adipocytes are enriched with cholesterol to form the biomembranes surrounding lipid droplets. Hydrolysis of triglycerides in white adipocytes upon fasting or in brown adipocytes upon heat production is accompanied by a breakdown of these membranes and the release of considerable amounts of cholesterol for RCT. At least in mouse models, HDL or ABC transporters were shown to play an important role in this process.[70,71]

HDLs also accept unesterified cholesterol from TGRLs, a process enhanced upon hydrolysis of triglycerides by lipoprotein lipase and independent of phospholipids (*Figure 2*).[72]

Communication with cells

HDLs regulate the differentiation, proliferation, migration, survival, and function of many cell types. HDLs modulate the inflammatory action of innate and adaptive immune cells,[69] support the integrity and functionality of endothelial barriers, stimulate angiogenesis,[73] and secure energy homeostasis by stimulating insulin synthesis and secretion by pancreatic beta cells as well as glucose uptake by adipocytes and myocytes.[74] Principally, these cellular responses result from either altered cholesterol homeostasis due to fluxes of cholesterol between cells and HDL, specific molecular interactions between HDL and cells, or combinations thereof.

First, cholesterol efflux alters the cholesterol content of specific plasma membrane domains, so-called rafts, that are enriched with signalling molecules, resulting in different effects depending on the cell type. Examples are the activation of endothelial nitric oxide synthase (eNOS) by HDL/SR-BI interaction in caveolae of endothelial cells[75] as well as the dampening of toll-like receptor-4 response in monocytes[76] or T-cell receptor signalling in lymphocytes;[77] cholesterol efflux also alters the transcription of sterol-regulated genes.[78]

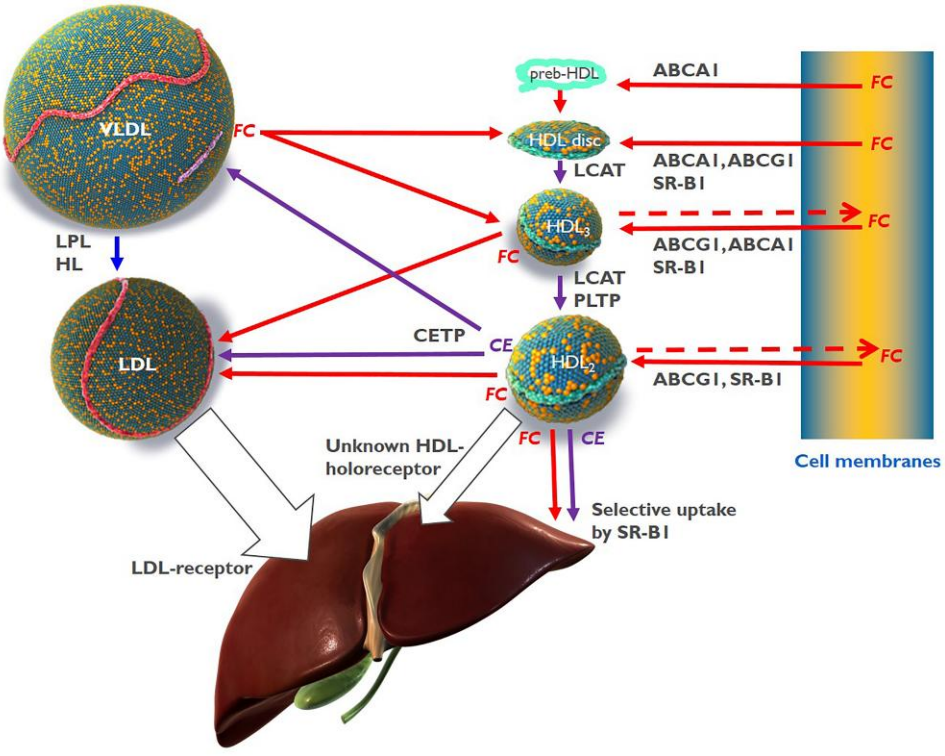

Figure 2 Cholesterol transfers between high-density lipoprotein, very-low-density lipoprotein, low-density lipoprotein, and cells. ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; FC, free cholesterol, unesterified cholesterol; HDL, high-density lipoprotein; HL, hepatic lipase; LCAT, lecithin:cholesterol acyltransferase; LDL, low-density lipoprotein; LPL, lipoprotein lipase; PLTP, phospholipid transfer protein; SR-B1, scavenger receptor B1.

Second, the interactions of HDL with SR-BI or apoA-I with ABCA1 induce signal transduction via the recruitment of intracellular proteins, which in turn activate different cellular responses such as eNOS activation in endothelial cells or glucose uptake into myocytes.[79,80]

Third, HDLs transport agonists of specific signalling receptors; the interaction of S1P with S1P receptors activates diverse signalling cascades and results in many protective effects on endothelial functions, including the induction of nitric oxide (NO) production and the maintenance of the endothelial barrier integrity.[73,81] Furthermore, S1P facilitates the trans-endothelial transport of HDL and thereby entry into extravascular tissues and spaces, where HDL exerts its protective activities.[82] S1P also modulates inflammatory effects on macrophages and lymphocytes and promotes the survival of hearts and kidneys exposed to hypoxia, ischemia-reperfusion injury, or toxic drugs.[81]

Fourth, HDLs deliver cargo into cells either by selective uptake, i.e. independently of the entire particle, or via holoparticle uptake.[66] SR-BI mediates the selective uptake not only of lipids but also microRNAs (miR) carried by HDL.[83] HDL-holoparticle uptake in the liver plays an important role in the metabolism of HDL.[84] In addition, monocyte-derived macrophages and enterocytes also internalize entire HDL particles, but the mechanism or the consequence of this is not understood.[85,86]

In summary, HDLs elicit a plethora of cellular responses by employing several modes of communication. Some mechanisms, for example, S1P receptor activation or cholesterol efflux, lead to many diverse responses in different cell types. *Vice versa*, identical responses can be evoked by several modes of action.

Inactivation of biohazards

By its amphiphilic structure and its cycling between extravascular and intravascular compartments, HDLs bind potentially toxic substances, such as bacterial lipopolysaccharides, oxidized lipids, as well as some lipophilic xenobiotics.[87,88] In plasma, potentially hazardous molecules are either eliminated by reverse transport to the liver or inactivated directly on the surface of HDLs. The best-investigated example for the latter situation is the hydrolysis of oxidized phospholipids by paraoxonase 1, lipoprotein-associated phospholipase A2, and LCAT.[89–91] HDLs also exert direct antimicrobial effects on viruses and even protozoa.[58,88,92] At least *in vitro*, HDL or apoA-I interfere with the entry or fusion of viruses with target cells.[92] Of note, SR-BI is an entry route of several viruses, including SARS-CoV-2, into cells[93] and this process may be competed by HDLs.[94]

Finally, the proteome of HDLs is enriched with proteases and protease inhibitors which modulate platelet aggregation, coagulation, fibrinolysis, complement activation, and tissue degradation. They help to counteract downstream adverse effects of injuries, infections, and inflammation and support wound healing.[48] Of note, functionally related proteins tend to cluster within distinct sub-populations of HDL.[45,46]

High-density lipoprotein dysfunction

HDLs can lose protective functions and even gain adverse functions in chronic diseases, such as rheumatic and autoimmune diseases, CHD, diabetes, chronic kidney disease, or in the course of infectious

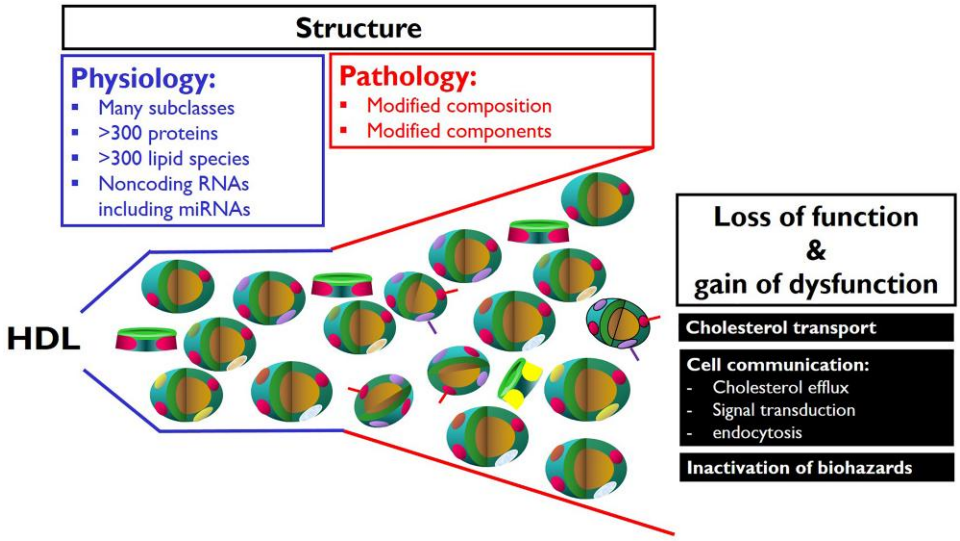

Figure 3 Structure–function relationships of high-density lipoprotein in health and disease. RNA, ribonucleic acid.

diseases.[42,95] HDL dysfunctions include reduced capacities to stimulate cholesterol efflux from macrophages, inhibit LDL oxidation, and regulate apoptosis, NO production, monocyte chemotactic protein-1, or vascular cell adhesion molecule expression in endothelial cells (*Figure 3*). A systematic investigation of HDLs' structure–function relationships in CHD and diabetes showed that the different functionalities of HDL are not correlated with each other and are determined by different features and molecules of HDLs.[96] HDLs of patients with CHD or chronic kidney disease inhibit rather than stimulating NO production because upon interaction with the lectin-like oxidized LDL receptor LOX-1 and the toll-like receptors TLR2 and TLR4 they induce the phosphorylation of inhibitory rather than activating sites in eNOS. The gain of these adverse receptor binding properties results from the accumulation of oxidized phospholipids and apoA-I, serum amyloid protein A (SAA), or symmetric dimethylarginine.[97,98]

Biomarkers of high-density lipoproteins' function or dysfunction

From a functional point of view, HDL-C is not a causal marker because the many functions of HDL are exerted either by entire particles or specific components other than cholesterol. Moreover, low HDL-C is strongly associated with increased levels of TGRL (*Figure 4*). Therefore and because HDL-C levels decrease upon disturbed lipolysis-induced transfer of unesterified cholesterol from TGRL to HDL[100] as well as enhanced CETP-mediated transfer of cholesteryl esters from HDL to TGRL (*Figure 4*), low HDL-C levels are nowadays widely considered as an indirect and non-causal biomarker of elevated ASCVD risk reflecting the atherogenicity of elevated plasma levels of TGRL and their remnants.[101] Any chance for future exploitation of HDL as a therapeutic target will depend on the availability of direct biomarker(s) reflecting a causal contribution of HDL to the pathogenesis of atherosclerosis and other diseases.

A recent meta-analysis reported that the total number of HDL particles, as well as numbers of small and medium, but less so large HDL particles, are associated with incident ASCVD.[102] A recent Mendelian randomization study found genetically causal associations of coronary artery disease with the concentrations of medium and small HDL particles, but not with large HDL.[103] This heterogeneity is potentially important since lipid-modifying drugs cause diverse changes in HDL particle size, number, and composition: treatment with nicotinic acid and CETP inhibitors increases HDL-C levels more profoundly than HDL-P, while treatment with fibrates increases HDL-P more strongly than HDL-C.[104]

The plasma concentration of apoA-I is the most obvious candidate as a direct biomarker of HDL function, because it is an essential structural HDL component, but also exerts several biological activities. In epidemiological and clinical studies, apoA-I levels show inverse associations with ASCVD events, which however are not stronger than those of HDL-C.[8,105] Neither did a Mendelian randomization study, based on a single SNP of *APOA1*, unravel any causal genetic relationship between apoA-I levels and ASCVD.[106] Nevertheless, we believe it is still worthwhile to further validate apoA-I as a biomarker through observational and more comprehensive Mendelian randomization studies, also concerning endpoints other than ASCVD.

Cross-sectional studies identified several proteins and lipid species in HDL, which differ quantitatively between patients with various diseases (including CHD) and healthy control subjects.[42,95,96] However, only a few of them were validated in prospective studies.[42,45] The presence or absence of distinct proteins was found to determine the association of apoA-I levels with incident CV events. For example, apoA-I levels in particles that contain apoE or apoC-I but not their apoE- or apoC-I-free counterparts, or apoA-I levels in apoC-III-free particles but not in apoC-III-containing particles are inversely associated with incident ASCVD events.[54] Enrichment of HDL with SAA was associated with mortality in CHD patients as well as patients with diabetic end-stage nephropathy.[107,108] However, it is not clear whether the enrichment of HDL with apoC-III or SAA are direct measures of HDL dysfunction or indirect reporters of apoC-III's adverse role in the metabolism of TGRL or the presence of inflammation. In support of

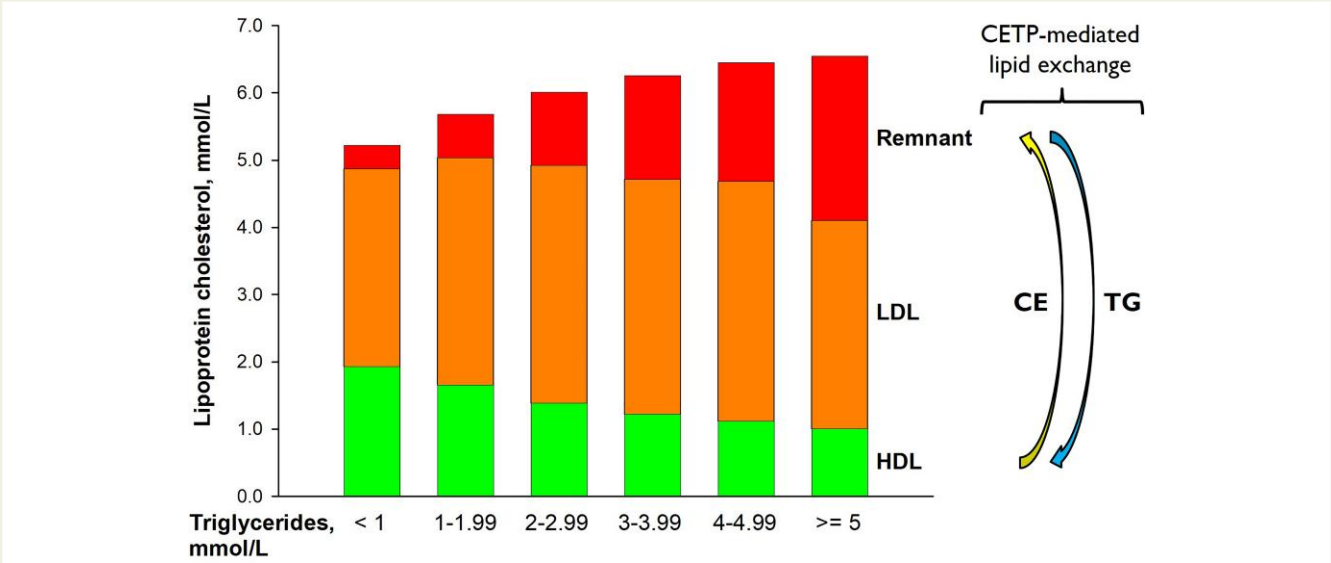

Figure 4 Lipoprotein-cholesterol as a function of increasing levels of non-fasting triglycerides. Based on 60 000 individuals from the Copenhagen General Population Study. CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; TG, triglycerides. Adapted from Chapman *et al.*[99]

the latter, apoC-III interferes with the capacity of HDL to inhibit the apoptosis of endothelial cells and to promote efflux from macrophages and SAA disturbs HDL's ability to activate eNOS.[107,109]

As integrative measures of HDL function, also bioassays were validated in population and clinical studies. CEC was investigated most extensively, using apoB-depleted plasma or serum as a surrogate of HDL. Despite the heterogeneity of results, a recent meta-analysis revealed that CEC is inversely associated with ASCVD events independently of HDL-C.[110] However, the assay is difficult to standardize and is not suitable for clinical routine.[111] Moreover, CEC is no overall proxy of HDL functionality, because other functions of HDL neither correlate nor share molecular determinants with CEC.[96] Although increasing CEC either as monotherapy or as a combination therapy with statins, treatment with evacetrapib did not prevent ASCVD events.[112] Despite these limitations, CEC has been used as a reference to develop molecular biomarkers that can be measured in clinical laboratories. One example is the derivation of an algorithm that integrates the information of differently sized HDL particles as measured by NMR. The estimated NMR-based CEC correlated very well with the *in vitro* measured CEC. However, in contrast to initial encouraging results, the validation of the CEC estimation algorithm failed in large multicentric replication studies.[113] Another example is a proteomic score integrating the information of apolipoproteins A-I, C-I, C-II, C-III, and C-IV, which showed a good correlation with CEC as well as significant association with the presence of coronary artery disease and CV mortality independently of clinical risk factors including conventionally measured concentrations of apoA-I and apoB.[114] Replication studies are needed to validate these surrogate scores of CEC.

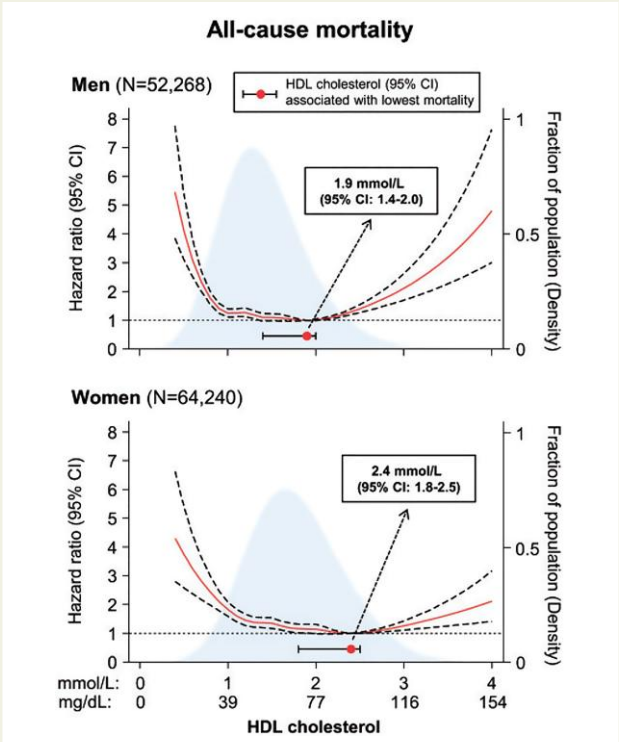

Figure 5 High-density lipoprotein levels on a continuous scale and risk of all-cause mortality in men and women from the Copenhagen General Population Study. Adapted from Madsen *et al.*[30]

High-density lipoprotein in mortality

Both low and extremely high HDL-C levels are associated with an increased risk of all-cause mortality[30] (*Figure 5*). Whereas the association with low HDL-C levels concurs with the repeatedly observed increased risk of ASCVD,[8] the increased mortality at extremely high HDL-C levels is less easily understood. This finding, however, derives from many studies[30,115–117] and raises the question of whether extremely high HDL-C levels have deleterious effects. Demonstrating a causal effect is very difficult, as conventional Mendelian randomization studies assume linear effects (the higher the level, the higher the risk over the entire

concentration range, or *vice versa*). Non-linear Mendelian randomization designs can be used for U-shaped observational associations, but such studies need even more statistical power, and hitherto no such studies have examined whether extremely high HDL-C levels are causally related to increased mortality. Naturally, this limitation is also valid for low HDL-C associated with increased mortality, as well as for the other U-shaped relationships described in the following.

The largest observational studies showed that extremely high HDL-C levels were not associated with a higher risk of cancer mortality, but rather with CV and/or other mortalities.[30,116,117] There are several possible explanations behind these associations. First, 11% of individuals with high HDL-C carry rare genetic variants that not only have a strong effect on HDL-C levels,[118] but may also have concomitant detrimental health effects possibly through dysfunctional HDL, including an altered ability to remove excess cholesterol from cells.[115] Second, the association could be driven by confounding, e.g. extremely high HDL-C levels are found in people with very high alcohol consumption, which could be the actual cause of high mortality.[30,115] Third, high HDL-C levels result from delayed catabolism. On the one hand, this may indicate disturbed delivery of cholesterol to the liver for excretion. On the other hand, like for LDL, the prolonged residence time will promote modifications of the molecular composition and components of HDL, ultimately resulting in HDL dysfunction. At high HDL-C levels, the particle size is larger than normal. Therefore, it is at least theoretically possible that these large, likely dysfunctional HDL particles become trapped in the arterial intima, leading to cholesterol accumulation and eventually to atherosclerosis and ASCVD.[115] Finally, in observational analyses, it is never possible to rule out reverse causation, that is, poor health leading to early death could also lead to extremely high HDL-C levels.

High-density lipoprotein in non-cardiovascular morbidity: from epidemiology to genetic and trial evidence

Both low and high HDL-C levels are associated with an increased risk of infectious disease[31,119] (*Figure 6*, upper panel) as well as mortality from sepsis.[88,92,121,122] There is genetic support for this function of HDL from gain-of-function variants in *CETP* associated with both lower HDL-C levels and higher mortality in sepsis.[123] Possible mechanisms include the inactivation of bacterial lipopolysaccharides,[88,92] but also beneficial effects of HDL on multiple organs and systemic responses, for example, in haemostasis and complement activation which are dysfunctional in hyperinflammation.[48,69,73]

Low HDL-C levels are associated with an increased risk of autoimmune disease[124] (*Figure 6*, middle panel) and cancer[115,120,125,126] (*Figure 6*, lower panel); for cancer, the risk increase was more pronounced for low apoA-I than for low HDL-C,[120] suggesting that HDL particles rather than their cholesterol content drive this association. Currently, there is no convincing genetic evidence linking low HDL-C levels causally to the risk of autoimmune disease or cancer.[115]

Low HDL-C is not only frequently found in individuals with manifest diabetes mellitus Type 2 but is also associated with an increased risk of developing diabetes. There is genetic evidence that low HDL-C levels may be causally related to an increased risk of diabetes in two large studies,[127,128] but not in a third.[129] In randomized trials, CETP inhibition, leading to a 28–132% increase in HDL-C levels, improved glycaemic control and/or reduced the risk of new-onset diabetes.[130,131] The beneficial glycaemic effects could, however, be due to pleiotropic

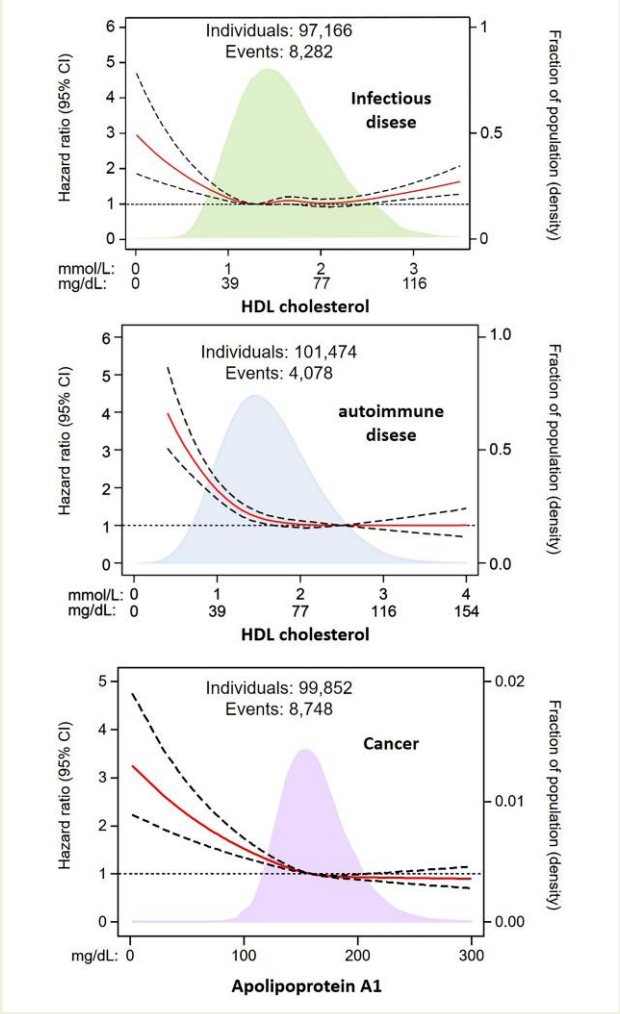

Figure 6 High-density lipoprotein levels on a continuous scale and risk of infectious disease, autoimmune disease, and cancer in individuals from the Copenhagen General Population Study. Adapted from Madsen *et al.*[31] and Pedersen *et al.*[120]

effects of CETP inhibition beyond HDL-C increases. However, infusion of artificial HDL acutely improved glycaemia in patients with diabetes.[132] Moreover, both *in vitro* and in animal experiments, HDL was found to exert potentially anti-diabetic effects on pancreatic beta cells, insulin signalling and glucose metabolism.[74]

Observational[133–135] and genetic[136–138] data show that low HDL-C is associated with decreased kidney function.[115] Finally, smaller studies have linked lower HDL-C levels with asthma; however, there are no genetic studies to support any claim for causality,[115] although overexpression of *ApoA1* or treatment with reconstituted HDL (rHDL) showed beneficial effects in several animal models of lung diseases.[139]

The risk of age-related macular degeneration also increases with elevated HDL-C and apoA1, according to both observational and genetic studies.[140–144] Genetically, well-known genes involved in HDL metabolism (*ABCA1*, *LIPC*, *CETP*, and *APOE*) are drivers of the increased risk of age-related macular degeneration,[140] the most common cause of blindness in the elderly. The use of drugs that specifically increase HDL levels is therefore of concern, and a recent genetic study estimated that the number of individuals who are potentially harmed by developing age-related macular

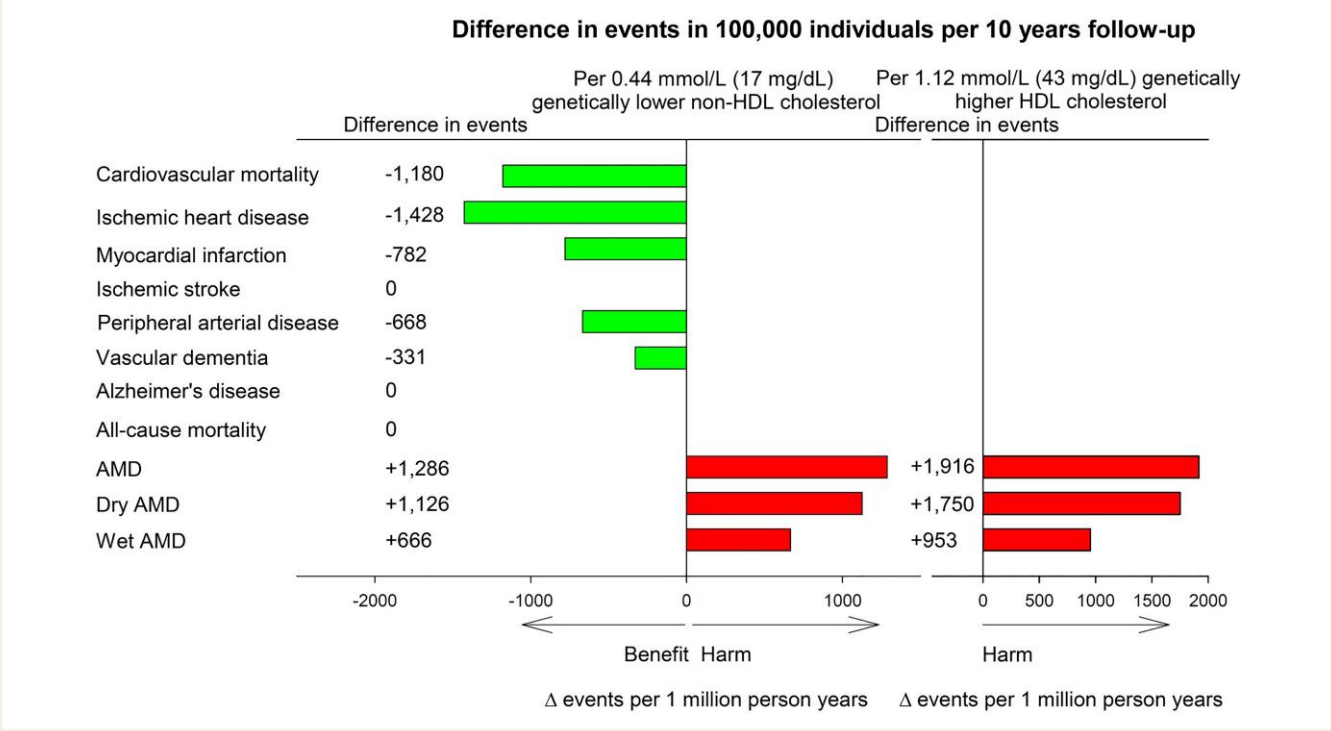

Figure 7 Estimated benefit and harm due to genetically lower non-high-density lipoprotein-cholesterol and genetically higher high-density lipoprotein-cholesterol due to inhibition of cholesteryl ester transfer protein. Based on individuals in the Copenhagen General Population Study. Lower non-high-density lipoprotein-cholesterol by 0.44 mmol/L (17 mg/dL) and higher high-density lipoprotein-cholesterol by 1.12 mmol/L (43 mg/dL) correspond to the changes observed through anacetrapib treatment compared with placebo in the REVEAL trial.[9] AMD, age-related macular degeneration; HDL, high-density lipoprotein; Δ=difference. Adapted from Nordestgaard *et al.*[144]

degeneration via HDL-C increases due to CETP inhibition was of the same order of magnitude as the number of individuals who may have benefited from reduced ASCVD, as a consequence of lowering of non-HDL-C[144] (*Figure 7*). However, in the REVEAL study treatment with anacetrapib did not cause any significant increase in the incidence of age-related macular degeneration (AMD), loss of visual acuity, or blindness.[9] That said, REVEAL participants were followed for 4 years from a median age of 67 years during which time 298 cases of AMD were diagnosed, that is, the study had limited power to exclude an increased risk of AMD if these participants were treated beyond the median age of 72 years where AMD typically develops.[144] Since the retina like the brain is separated from the bloodstream by a tight barrier, one must envisage that HDL-C levels in peripheral blood are only indirectly related to the functions of the HDL genes in the pathogenesis of AMD. For example, the targeted knock-out of *ABCA1* and *ABCG1* in retinal pigment epithelium led to retinal degeneration in mice as seen in human AMD.[139] As interference with LDL metabolism in the liver for example by PCSK9 inhibition does not affect brain function, interference with HDL metabolism in the periphery may have no impact on retina function.

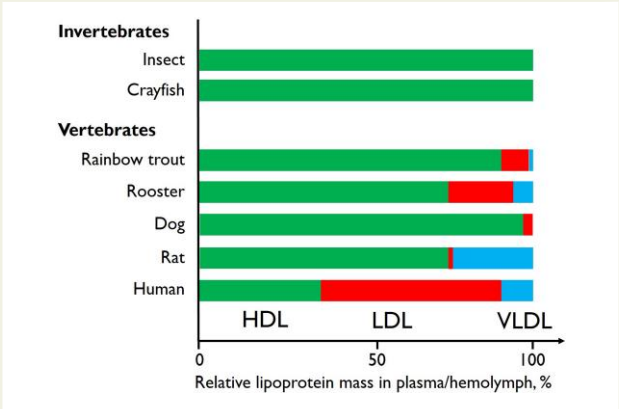

Figure 8 Examples of relative mass distribution of different lipoprotein fractions in different invertebrate and vertebrate species. HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein. Values adapted from Chapman[145] and Van der Horst and Rodenburg.[147]

High-density lipoprotein across species, in search for a role through evolution

HDL is present in essentially all living species: in invertebrates including insects, crabs, and lobsters, different types of HDL-like lipoproteins represent the bulk of lipoproteins in haemolymph (the equivalent of human blood)[145–147] (*Figure 8*). In contrast to vertebrates, invertebrates use only HDL-like lipoproteins for both exogenous and endogenous lipid transport: while circulating in haemolymph between different cells in different tissues, HDL alternately delivers and takes up lipids without being internalized or degraded. Besides lipid transport, haemolymph

HDL is involved in bacterial lipopolysaccharide binding, clot formation, and wound healing, and oocyte maturation in females.[145–147]

The lipoprotein systems of the most primitive vertebrates approach those of mammals and humans, including both HDL and the larger apolipoprotein B-containing lipoproteins like human chylomicrons, very-low-density lipoproteins (VLDLs), and LDL.[145–147] The relative plasma content of HDL, LDL, and VLDL (including chylomicrons) differs between different vertebrates; however, in many species, HDL is the dominant lipoprotein[145–147] (*Figure 8*).

This suggests that, through evolution, HDL particles appeared early, while chylomicrons, VLDL, and LDL first developed in vertebrates. HDL transports lipids to and from different cells like a reusable ferry, while the larger chylomicrons, VLDL, and LDL mediate more targeted delivery including triglyceride hydrolysis via lipases and cholesterol via receptor-mediated lipoprotein uptake. In this regard, it is important to remember that LDL plays an important role in RCT by accepting both unesterified and esterified cholesterol from HDL for LDL-receptor-mediated removal by the liver.[60,148] However, for some organs and functions, for example the steroidogenesis in adrenals, the delivery of cholesterol by HDL rather than by LDL appears to be rate-limiting.[149] As CV disease occurs after reproductive age and mainly in humans, it is unlikely that HDL and RCT, the most intensively investigated function of HDL particles, have developed during evolution to protect from atherosclerosis. It is more likely that HDL evolved as a multimolecular and multifunctional platform and part of the innate host defence to overcome acute crises in early life, such as infection and wounding. In this regard also RCT plays an important role, as tissue degradation, but also the physiological turnover of erythrocytes and platelets as well as lipolysis in the adipose tissue upon prolonged fasting mobilize cholesterol, which is taken up and transported by HDL for biliary excretion.

Therapies modulating high-density lipoprotein

Based on the earlier observations from epidemiological studies, it was assumed that increasing HDL-C levels would produce CV protection. As a consequence, many clinical trials have been conducted with drugs capable of increasing significantly the plasma levels of HDL-C. The results of such clinical trials have been, however, disappointing. Adding niacin to statin therapy did not provide any incremental clinical benefit among patients with ASCVD, despite a 25% increase in HDL-C levels.[10] Several CETP inhibitors have now been tested in clinical trials, most of which failed to show a reduction in CV risk despite significant increases in HDL-C levels,[12,13,150] except for anacetrapib; the latter significantly reduced CV events by 9%,[9] although such reduction was attributed to the observed LDL-C lowering rather than the increase in HDL-C levels.[151] Several studies have also aimed to find further explanations for these results, but neither changes in CEC of HDL[13,112,152] nor variations in HDL sub-fractions,[112,153,154,] could provide clear explanations for the trial results. In addition, different patient populations and different quantification methods were used in these studies, thus further complicating this picture. A recent study showed that the treatment with torcetrapib and evacetrapib increases HDL sub-fractions that are associated with an increased CHD risk, such as those containing apoC-III, suggesting that the pharmacological increase of HDL-C would not be beneficial if an increase in dysfunctional HDL particles is achieved.[155] In contrast with these observations, genetic variations in the CETP gene determining higher HDL-C levels were associated with a reduced risk of 28-day mortality from sepsis, and inhibition of CETP with anacetrapib preserved HDL-C levels decreased the severity of endotoxemia, and improved survival after caecal ligation and puncture in mouse models of sepsis.[123] In *post hoc* analyses of large trials, CETP inhibitors were also found to improve glycaemic control and delay the onset of diabetes.[130,131]

Although pharmacologically increasing HDL-C levels has so far not shown any clinical benefit, several efforts have been launched to develop rHDL that are expected to improve specifically the HDL-mediated RCT rather than increasing its level. These were based on the observation that HDL infusion, as well as the overexpression of apoA-I in experimental animal models, were associated with the prevention or regression of atherosclerosis.[156–158] Over time, three HDL mimetics have been developed and tested in humans.

ApoA-I_{Milano} is a naturally occurring variant of apoA-I determining very low levels of HDL-C and apoA-I and high triglyceride levels but associated with a very low prevalence of CV disease.[159,160] Sera from apoA-I_{Milano} carriers exhibit a higher CEC compared with wild-type apoA-I[161]; this observation has led to the development of a complex (MDCO-216) consisting of purified apoA-I_{Milano} and phospholipids. In one study, MDCO-216 produced significant regression of coronary atherosclerosis, in the absence of any demonstrable change in HDL-C levels, suggesting an improvement in HDL function;[162] this observation, however, was not confirmed in another study.[163] The development of MDCO-216 has now been halted.

Following an acute coronary syndrome (ACS) event, cholesterol efflux is significantly reduced, showing the lowest levels at 2–5 days post-event and returning to baseline approximately after 30 days.[164] Thus, an approach that acutely increases apoA-I and CEC might be beneficial among post-acute myocardial infarction patients. CSL-111 was an early formulation of rHDL consisting of human apoA-I with soybean phosphatidylcholine; although it did not produce significant reductions in coronary atheroma volume in post-ACS patients, improvements in plaque characterization index and coronary score by quantitative coronary angiography were observed.[165] The development of CSL-111 was halted due to adverse hepatic events. CSL-112 is a modified formulation of rHDL. It has threefold less phospholipid than CSL-111, did not show any major organ toxicity or immunogenicity,[166] and is capable of increasing substantially ABCA1-mediated cholesterol efflux from cells.[167] Compared with placebo, CSL-112 was associated with an improvement in measures of CEC (>3-fold).[168] Similar results were reported in another Phase 2a trial.[169] The ongoing Phase 3 AEGIS-II trial is evaluating 4 weekly infusions of CSL-112 can lower the short-term rates of recurrent events among post-AMI patients.[170]

CER-001 is a negatively charged lipoprotein complex, consisting of phosphatidylcholine, sphingomyelin, and recombinant human apoA-I. Although CER-001 promoted the regression of diet-induced atherosclerosis in a mouse model,[171] no changes in coronary atherosclerosis were observed among patients with a recent ACS.[172] It is conceivable that the infusion with HDL mimetics cannot reduce plaque burden beyond the effect induced by intensive statin therapy. Of note, CER-001 was proven to be effective in protecting kidneys in patients with familial LCAT deficiency,[173,174] and intravenously administration of CER-001 in a severe COVID-19 patient increased apoA-I levels while HDL-C levels decreased, accompanied by significant decreases in many inflammatory markers and cytokines,[175] suggesting potential utilization for diseases other than CV disease.

As LCAT plays a key role in HDL metabolism and RCT, ongoing studies are currently evaluating the effect of increasing LCAT activity not only for CV disease but also for other conditions such as familial LCAT deficiency and fish-eye disease. A Phase 2a study in subjects

with stable CHD showed that recombinant human LCAT (MEDI6012) added to statin therapy increased HDL-C and apoA-I levels, increased non-ABCA1-mediated cholesterol efflux while reducing apoB levels and total and small LDL particle number.[176] A Phase 2b trial is currently evaluating the safety and efficacy of MEDI6012 in patients presenting with an acute ST-elevation myocardial infarction (clinicaltrials.gov/ct2/show/NCT03578809).

Final considerations

Taken together, HDL is a dynamic multifaceted lipoprotein that can serve several physiological roles, most of which have been preserved throughout evolution for other reasons than protection from atherosclerosis (*Graphical Abstract*). Clinically, low HDL-C remains a strong and important risk marker for increased risk of ASCVD, likely due to the inverse association with increased levels of triglyceride-rich remnant lipoproteins. For patients with extremely high HDL-C, the documented increased risk of infectious disease and all-cause mortality should inform patients and doctors alike of the possible negative prognostic consequences of high HDL-C. Finally, the observed increased risk of AMD observationally and causal, genetically for any increase in HDL levels is of concern. Future efforts to pharmacologically modulate HDL should likely focus on functional metrics of HDL function rather than HDL-C and other clinical indications besides ASCVD.

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Data availability

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