HDL AS A TARGET IN THE TREATMENT OF ATHEROSCLEROTIC CARDIOVASCULAR DISEASE

Patrick Linsel-Nitschke and Alan R. Tall

Abstract | Lipid abnormalities are among the key risk factors for cardiovascular disease. Indeed, lipid-modifying drugs — in particular, the statins, which primarily lower plasma levels of low-density lipoprotein (LDL) cholesterol — considerably reduce the risk of cardiovascular events, leading to their widespread use. Nevertheless, it seems that there might be limits to the degree of benefit that can be achieved by lowering LDL-cholesterol levels alone, which has led to increased interest in targeting other lipid-related risk factors for cardiovascular disease, such as low levels of high-density lipoprotein (HDL) cholesterol. In this article, we first consider the mechanisms that underlie the protective effect of HDL cholesterol, and then discuss several strategies that have recently emerged to increase levels of HDL cholesterol to treat cardiovascular disease, including nuclear receptor modulation, inhibition of cholesteryl ester transfer protein and infusion of apolipoprotein/phospholipid complexes.

ATHEROSCLEROSIS
Atherosclerosis is a systemic disease that is characterized by the accumulation of lipid-rich plaques within the walls of large arteries. Major clinical manifestations of atherosclerosis include myocardial infarction (heart attack), stroke and peripheral vascular disease.

Division of Molecular Medicine, Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, New York 10027, USA.
Correspondence to P.L.N. & A.R.T.
e-mails: pl2031@columbia.edu; art1@columbia.edu
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ATHEROSCLEROSIS is the leading cause of mortality in industrialized nations. This is despite substantial therapeutic progress resulting from the widespread use of statins, which inhibit 3-hydroxy-3-methyglutaryl-coenzyme A (HMG Co-A) reductase, a key enzyme in cholesterol biosynthesis. Large-scale clinical trials using statins for both primary and secondary prevention have shown a marked reduction in coronary events, mainly owing to the lowering of plasma concentrations of low-density lipoprotein (LDL) cholesterol[1,2].

So, what can be done to further improve the treatment of atherosclerosis? Recent and ongoing clinical trials with statins are aimed at determining whether more aggressive lowering of LDL cholesterol in primary and secondary prevention beyond the currently recommended guidelines will result in a further reduction in cardiovascular events[3,4]. The recently published PROVE IT–TIMI 22 (Pravastatin or Atorvastatin Evaluation and Infection Therapy–Thrombolysis In Myocardial Infarction 22) study compared the effect of lowering LDL cholesterol in patients with acute coronary syndromes to an average concentration of 95 mg dl^{-1} versus 62 mg dl^{-1} with more aggressive treatment. After a mean follow-up of 24 months, the rate of primary endpoints was 22.4% in the aggressively treated group versus 26.3% in the conventionally treated group, reflecting a 16% reduction in the hazard ratio[4]. This study has been proclaimed as exemplifying the benefits of aggressive lowering of LDL, but nevertheless, the high incidence of residual cardiovascular disease even in the aggressively treated group is remarkable. This and similar studies could herald the limits of statin monotherapy in inhibiting the development of established atherosclerotic disease. The results of similar studies, especially the large-scale secondary prevention Treating to New Targets (TNT) study[3], are pending, but there is a fair possibility that a reduction in coronary endpoints beyond 20–25% is not achievable by LDL lowering alone. There is therefore considerable interest in the therapeutic potential of targeting other lipid-related risk factors.

HDL as a risk factor. HDL-cholesterol levels, which have a strong genetic component (BOX 1), are inversely correlated with the risk of coronary heart disease[5]. A low level

Box 1 | Genetic determination of high-density lipoprotein levels

There is a strong genetic component in the determination of high-density lipoprotein (HDL) levels. In a normolipidaemic US population, this arises from common variation in hepatic lipase and apolipoprotein APOA-I/C-III/A-IV/A-V gene loci[148], but the relevant functional variants have not been identified. Various rare mutations in known genes, and particularly in the ABC transporter A1 (ABCA1), have been found in approximately 10% of patients with very low HDL levels (lowest 5%)[24]. Low HDL levels also occur as a result of mutations in APOA-I and lecithin-cholesterol acyltransferase (LCAT) genes[24]. In the Japanese population, two common low-activity variants in the cholesteryl ester transfer protein (CETP) gene (a missense and a splicing mutation) are present in about 5% of the general population and cause increased HDL levels[149]. The missense variant seems to be widespread in Asia.

of HDL is the most common lipid abnormality observed in men with coronary heart disease; it is the primary lipid abnormality in approximately half these patients[6]. Clinical trials, such as the Veterans Administration HDL Intervention Trial (VA-HIT)[7] and the Helsinki Heart Study[8], have shown a reduced incidence of coronary events in association with an increase in plasma HDL levels in patients treated with fibrate drugs. Epidemiological studies indicate that a 1 mg dl^{-1} increase in the HDL-cholesterol concentration is associated with a 2–3% decrease in cardiovascular risk[9–11]. These studies indicate a potential for HDL-raising therapies to reduce the risk of cardiovascular disease, but this has not yet been directly proven.

Worldwide, there has been a sharp increase in the prevalence of the METABOLIC SYNDROME in both adults and children[12,13] and an associated increase in cardiovascular morbidity. This global epidemic affects not only the industrialized nations, but also the developing world[12,14]. Low HDL levels are one of the hallmarks of the metabolic syndrome, and so the problem of low HDL as a risk factor is likely to increase in the future. Considering the high prevalence of low HDL in the metabolic syndrome and in patients with coronary heart disease (CHD), therapeutic increases in HDL are an obvious approach to decreasing the risk of atherosclerosis. As reviewed below, several new molecular targets and strategies have emerged for increasing HDL levels, which is the next frontier in the prevention of atherosclerotic cardiovascular disease.

Anti-atherogenic mechanisms of HDL

Cholesterol efflux from cells in the arterial wall. The excessive uptake of modified LDL or remnant lipoproteins by arterial wall MACROPHAGES gives rise to cholesterol-loaded cells with foamy cytoplasm due to the presence of cholesteryl ester droplets known as 'foam cells' (BOX 2).

The efflux of cholesterol from foam cells is mediated by HDL or its apolipoproteins and represents a crucial step in the prevention or reversal of atherosclerosis. Although for many years the efflux of cholesterol was thought to occur primarily by passive aqueous diffusion, recently it has become clear that cholesterol efflux is a highly regulated process that is mediated by specific molecules, including ATP-binding cassette (ABC) transporters (FIG. 1).

The ABC transporter A1 (ABCA1) has been identified as the defective molecule in Tangier's disease[15–17]. Patients with Tangier's disease have almost no plasma HDL due to rapid catabolism; they accumulate macrophage foam cells in various tissues and seem to experience moderately accelerated atherosclerosis. ABCA1 promotes the efflux of free cholesterol and phospholipids from cells to lipid-poor apolipoprotein A-I (apoA-I) to generate nascent HDL particles. ABCA1 seems to act as a lipid translocase, increasing the availability of phospholipids and cholesterol at the cell surface. Lipid-poor apoA-I interacts directly with ABCA1, possibly by binding to the two large extracellular domains of this transporter. This binding seems to constitute an essential step in the efflux of phospholipids and cholesterol to apoA-I.

In cholesterol-loaded cells, the oxysterol-activated nuclear receptor liver X receptor (LXR), in partnership with retinoid X receptor (RXR), targets the *ABCA1* promoter, leading to increased *ABCA1* gene expression and augmented cholesterol efflux from foam cells; similarly, *ABCA1* is induced by synthetic LXR agonists[18,19]. Macrophage ABCA1, although not contributing significantly to the maintenance of plasma HDL levels, has an anti-atherogenic function[20–22]. The activity of ABCA1 in hepatocytes has an essential role in the formation of HDL and the maintenance of HDL levels in plasma. However, ABCA1 does not interact with the main forms of HDL that are present in plasma: it shows no interaction with HDL_2 (larger, less dense particles) and minimal interaction with HDL_3 (smaller, more dense particles). Although a wide variety of ABCA1 mutations might cause familial hypoalphalipoproteinaemia (low HDL), common SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) in the *ABCA1* gene probably have only a minor impact on HDL levels in the general population[23–25]. Therefore, although the activity of ABCA1 is essential to initiate HDL formation, it does not readily explain the inverse relationship between HDL levels and atherosclerosis risk.

Wang *et al.*[26] have recently discovered that ABCG1, an ABC transporter of unknown function, can promote cholesterol efflux from cells, including macrophages, to the major forms of plasma HDL — that is, HDL_2 and HDL_3 (FIG. 1). In contrast to ABCA1, ABCG1 is a half-transporter that might act as a homodimer. ABCG1 is an LXR target gene and shows a particularly high expression level in macrophages. In addition to cholesterol efflux via ABCA1 and ABCG1, cholesterol might leave cells via aqueous diffusion or pathways facilitated by the scavenger receptor BI (SR-BI). However, SR-BI efflux pathways seem not to have a major role in macrophage cholesterol efflux, at least in mouse cells[27]. Although the mechanism of action of ABCG1 is poorly understood, it might act to increase the availability of cholesterol at the cell surface, which is followed by efflux of cholesterol through passive diffusion mechanisms.

In contrast to the activity of SR-BI, which promotes bi-directional cholesterol exchange between cells and HDL, ABCG1 and ABCG4 promote net cholesterol efflux to HDL particles. Whereas ABCG4 is primarily

METABOLIC SYNDROME
A common condition associated with increased risk for cardiovascular disease. The diagnosis requires three or more of the following symptoms: central obesity; high blood pressure; low plasma-HDL; high triglycerides; or elevated fasting blood glucose.

MACROPHAGE
A type of white blood cell that is specialized for the uptake of material by phagocytosis.

SINGLE NUCLEOTIDE POLYMORPHISM
(SNP). A specific location in a DNA sequence at which different people can have a different DNA base. Differences in a single base could change the protein sequence, leading to disease, or have no known consequences.

Box 2 | **Cholesterol efflux from the arterial wall and atherogenesis**

Early in the development of atherosclerotic lesions, monocytes transmigrate through the endothelial monolayer into the intima where they can differentiate into macrophages that are capable of taking up lipoproteins. Inflammatory signals are believed to have an important role in this process. These cholesterol-engorged macrophages in the intima have been termed 'foam cells' and are considered a hallmark of early atherosclerotic lesions. Sub-endothelial accumulations of foam cells form the so-called 'fatty streaks' that constitute the earliest morphological change leading to the development of atherosclerotic plaques. At a later stage, the foam cells undergo apoptosis or necrosis and release their cholesterol-rich content, which leads to the development of lesions with a necrotic core. One proposed mechanism that leads to the formation of foam cells is an imbalance between the uptake of lipoproteins and cholesterol efflux from macrophages. Apolipoproteins or high-density lipoprotein (HDL) can serve as acceptor particles for macrophage cholesterol efflux, which explains the anti-atherogenic properties of apolipoprotein A-I and HDL.

expressed in the brain, ABCG1 is expressed in both macrophages and endothelial cells. The ability of ABCG1 to promote cholesterol efflux from cells in the vasculature to HDL might underlie the inverse relationship between HDL levels and atherogenesis.

HDL metabolism and reverse cholesterol transport. The efflux of cholesterol from arterial wall cells onto lipid-poor apoA-I or apoE, or HDL particles, initiates the process of reverse cholesterol transport — that is, the centripetal transport of cholesterol from the periphery to the liver (FIG. 2). Cholesterol that is carried on HDL particles might be esterified in plasma by the lecithin-cholesterol acyltransferase (LCAT) enzyme, thereby generating cholesteryl esters. Cholesteryl esters are subsequently removed from HDL in the liver by a process known as 'selective lipid uptake', which is mediated by SR-BI. SR-BI promotes the selective removal of both free and esterified cholesterol from HDL particles, and facilitates their excretion into bile. The importance of SR-BI in rodent HDL metabolism has been well defined. SR-BI is also expressed in human hepatocytes, which are active in mediating selective uptake[28], but the overall quantitative importance of the selective uptake pathway in humans is unknown.

In humans, a substantial part of the cholesteryl ester that is formed within HDL is transferred to triglyceride-rich lipoproteins (TRLs) as a result of the activity of cholesteryl ester transfer protein (CETP). The TRLs are metabolized into remnants by lipoprotein and hepatic lipase. The remnants of triglyceride-rich lipoproteins are then either directly removed in the liver or converted to LDL and eventually removed by the LDL receptor. The excretion of hepatocyte cholesterol into bile is mediated by ABCG5/ABCG8, which are half-transporters that act as heterodimers. Therefore, at least three of the known mammalian members of the ABCG transporter family (there are six members in total) are LXR targets and have a role in reverse cholesterol transport.

Although there is a widespread belief that upregulating multiple steps in reverse cholesterol transport might be therapeutically desirable as a way to decrease atherosclerosis, it might be sufficient to induce the excretion of cholesterol from arterial wall cells, by inducing ABCA1 and ABCG1 and/or by increasing HDL levels. Although increasing multiple steps in reverse cholesterol transport represents a major therapeutic challenge, a favourable change in the balance of cholesterol between the tiny pool of cholesterol found in atheroma and the plasma lipoproteins might be sufficient to reverse or prevent atherosclerosis.

Other potentially protective mechanisms of HDL. Besides cholesterol efflux from the arterial wall and the 'reverse cholesterol transport' hypothesis, several other theories have emerged to explain the anti-atherogenic properties of HDL. HDL can suppress the induction of cell-adhesion molecules by tumour-necrosis factor (TNF) in endothelial cells[29] and this property has also been shown for reconstituted HDL particles[30], which might be atheroprotective when infused *in vivo*[31]. Recently published studies indicate a regulatory role for HDL in endothelial function. The binding of HDL to SR-BI leads to the activation of endothelial nitric oxide synthase (eNOS) and therefore enhances vasorelaxation[32,33]. The ability of HDL to activate eNOS has been attributed to both oestrogen[34] and lysophospholipids[35] that are contained in HDL particles.

Various other protective properties of HDL in atherosclerosis have been proposed. These include anti-inflammatory, antioxidative, anticoagulant, anti-aggregatory and pro-fibrinolytic properties that are mediated by the different components of HDL (see REFS 36,37 for reviews). The *in vivo* significance of these various proposed mechanisms in the protective properties of HDL has not yet been thoroughly evaluated.

Effect of drugs in clinical usage on HDL

Statins, fibrates and nicotinic acid. Treatment with statins has only moderate effects on HDL concentrations, raising HDL cholesterol by an average of 5–10%[38]. For this reason, fibrates and nicotinic acid (niacin) are the mainstays of therapy to raise HDL. Fibrates exert their indirect effect on HDL levels by activating the nuclear transcription factor peroxisome proliferative activated receptor-α (PPARα). They increase HDL levels and decrease triglyceride concentrations without having a major effect on LDL levels, making the fibrates attractive drugs for use in the metabolic syndrome, which is characterized by high triglyceride and low HDL levels. However, the benefit of fibrate therapy in the secondary prevention of cardiovascular disease is not as clearly established as it is for the statins, as the results from clinical trials, although promising, are not uniformly positive.

Various mechanisms have been proposed by which fibrates increase plasma HDL levels. Induction of PPARα in the liver leads to increased synthesis of apoA-I[39,40] (FIG. 2), thereby enhancing the formation of new HDL particles. Studies in primary hepatocytes from mice have shown a downregulation of hepatic SR-BI protein levels on treatment with fibrates[41]. Downregulation of this receptor in the liver leads to decreased HDL clearance and would provide another mechanism by which fibrates could increase plasma HDL levels.

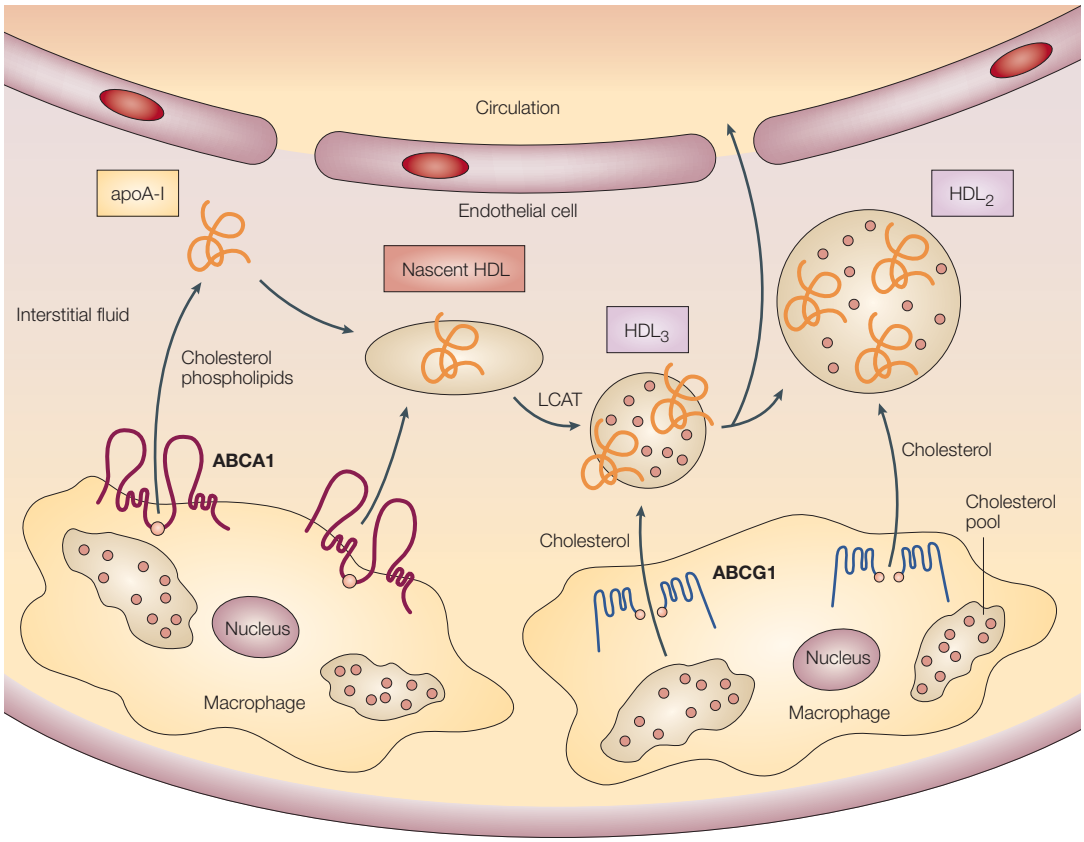

Figure 1 | **Mechanisms of cholesterol efflux from the arterial wall.** On entering the sub-endothelial space, lipid-free or lipid-poor apolipoprotein A-I (apoA-I) can bind to the ABC transporter A1 (ABCA1) on the cell surface of macrophages in the arterial wall and promote efflux of free cholesterol and phospholipids from these cells. This results in the formation of nascent high-density lipoprotein (HDL) particles, which undergo further modification by the lecithin-cholesterol acyltransferase (LCAT) enzyme and develop into spherically shaped HDL_2 (larger, less dense particles) or HDL_3 (smaller, more dense particles), which, in turn, can act as acceptors for ABCG1-mediated cholesterol efflux from macrophages, resulting in further cholesterol enrichment of HDL, before returning to the circulation. Although inter-conversion of HDL subspecies is depicted as occurring in the arterial wall, it probably also occurs in the plasma.

The most beneficial outcome with fibrates so far in both primary and secondary prevention was achieved with the drug gemfibrozil in the Helsinki Heart Study, a primary prevention trial that involved 4,081 men with dyslipidaemia[8], and the VA-HIT Study, a secondary prevention trial that involved 2,531 men with low HDL and LDL (HDL <40 mg%, LDL cholesterol <140 mg%) who were treated with gemfibrozil for a mean follow-up of 5.1 years[7,42]. In the VA-HIT Study, each 5% increase of plasma HDL concentration resulted in an 11% risk reduction in the hazard ratio for CHD events.

The Lopid Coronary Angiography Trial (LOCAT) study compared gemfibrozil with a placebo and looked at the angiographic progression of coronary and vein graft atherosclerosis in men with low HDL who had undergone bypass surgery[43]. However, no significant benefit was observed in the gemfibrozil-treated group in this study. Another secondary prevention trial, the Bezafibrate Infarction Prevention (BIP) study, involved 3,019 subjects[44]. HDL in the bezafibrate-treated group was increased by an average of 18% compared with the placebo group. Subgroup analysis showed a significant endpoint reduction after bezafibrate treatment in those patients who had elevated baseline triglycerides, a typical feature of the metabolic syndrome[44]. At this time, fibrates might be used in patients whose predominant lipid abnormality is an elevation of triglycerides. These patients frequently show other features of the metabolic syndrome, such as low HDL and insulin resistance, which put them at risk for cardiovascular disease.

The HDL-raising effect of nicotinic acid has been known for decades. Inhibition of peripheral lipolysis and hepatic very low-density lipoprotein (VLDL) synthesis, which indirectly lead to increased HDL levels, have been proposed as mechanisms of action for nicotinic acid[45]. *In vitro* studies in adipocytes have linked reduced cyclic AMP concentrations to the antilipolytic effect of nicotinic acid. Accordingly, a G_i-protein-coupled receptor that is highly expressed in adipocytes and to which nicotinic acid acts as a high-affinity ligand[46] has recently been identified. Nicotinic acid has also been suggested to increase the expression of macrophage ABCA1 *in vitro* by a mechanism that is not clearly understood[47].

Although nicotinic acid can effectively increase HDL and lower triglyceride concentrations, its widespread use has been hampered by unfavourable side effects. These side effects include prostaglandin release with intense flushing, impaired glucose tolerance, increased uric acid levels and liver toxicity. Extended-release preparations of nicotinic acid that cause less flushing have become available. However, some of these extended-release forms seem to have an increased risk for liver toxicity[48]. Two clinical trials in the 1970s and 1980s assessed the clinical efficiency of nicotinic acid, and a retrospective analysis suggested decreased mortality in the nicotinic-acid-treated group[49,50]. However, these trials would not meet the stringent criteria that are set for lipid-lowering trials nowadays. Although the clinical efficacy of nicotinic acid is not proven, and the side effects will continue to limit its widespread use, the molecular mechanism of the nicotinic acid receptor might well be an attractive drug target for future drug discovery.

HDL-directed drugs in clinical development

In recent years, a number of promising therapeutic targets to increase HDL levels have emerged from intensive research. This has led to several new drugs that are currently being tested in Phase I–III clinical trials. This section focuses on drugs that have entered advanced clinical testing, which have been reported in the public domain and, in the opinion of the authors, show the greatest promise for introduction into clinical practice in the foreseeable future.

Agonists of PPARα, PPARγ and PPARδ. The PPAR family of nuclear receptor transcription factors consists of the three members PPARα, PPARγ and PPARδ. Each of these nuclear receptors controls a distinct network of target genes. A common feature of many of the PPAR-regulated genes is their involvement in fatty-acid metabolism. The clinical importance of fibrates as agonists of PPARα has already been mentioned. However, unlike the glitazones that act as high-affinity agonists by directly binding to PPARγ, fibrates are comparatively week agonists of PPARα and do not show a high degree of subtype selectivity, necessitating high doses of these drugs (200–1,200 mg per day). Furthermore, direct binding of fibrate drug to the PPARα receptor has never been demonstrated. A number of potent and more selective PPARα agonists have been reported in the literature[51–54], but none of these has entered clinical use so far. More selective PPARα agonists promise to become useful drugs for the metabolic syndrome and atherosclerosis.

PPARγ, another member of the PPAR family of nuclear receptor transcription factors, is most highly expressed in adipose tissue and has been shown to be essential for adipocyte differentiation and normal glucose metabolism[55]. Glitazones as activators of PPARγ have found widespread use as insulin sensitizers in the treatment of type II diabetes. It has been suggested that glitazones could act as potent inducers of ABCA1 and promote macrophage cholesterol efflux[56]; however, these findings have not been uniformly observed[57,58]. The overall effect of glitazones on plasma HDL levels seems to be small, increasing HDL by an average of 3–5%[59]. The insulin-sensitizing effects of PPARγ activators could be important in reversing pro-atherogenic changes in macrophage foam cells reflecting macrophage insulin resistance[60], or in endothelial cells reversing impaired eNOS expression[61]. Recent evidence indicates that *in vivo* PPARγ activators protect against atherosclerosis but do not upregulate ABCA1. By contrast, they induced macrophage ABCG1 and cholesterol efflux to HDL, indicating that this might be a key mechanism of protection by this class of drugs[62].

Another potentially important mechanism by which both PPARα and PPARγ agonists could attenuate the development of atherosclerosis is through an anti-inflammatory effect, which has been suggested for both these nuclear receptors. PPARα and PPARγ are expressed in cell types that are relevant to the regulation of the immune system and the pathogenesis of atherosclerosis, such as macrophages, monocytes, smooth-muscle cells and vascular endothelial cells[63]. Several studies report that activation of both PPARα and PPARγ can inhibit NF-κB signalling and suppress the secretion of various pro-inflammatory cytokines[64]. Other possible anti-inflammatory mechanisms that have been suggested for both receptors include inhibition of the vascular adhesion of monocytes by reduced chemokine-receptor and adhesion-molecule expression and inhibition of monocyte–macrophage migration into lesional areas[64,65]. The *in vivo* significance of the anti-inflammatory effects of these and other nuclear receptor agonists remains to be determined. Indeed, in a recent study, PPARα and PPARγ activators decreased atherosclerosis, whereas PPARδ activators did not. As all three classes of activator had similar anti-inflammatory effects, these results indicated that in this model effects on atherosclerosis were independent of effects on inflammation[62].

Because of potentially multiple benefits on lipid metabolism, insulin sensitivity and vascular inflammation, combined and/or more specific agonists of PPARα and PPARγ hold great promise in the treatment of the metabolic syndrome and associated cardiovascular disease. Several of theses drugs have been developed and are now being tested in Phase III clinical trials[66–68]. Ragaglitazar (DRF272; Novo Nordisk) (FIG. 3) has been shown to increase HDL, lower triglycerides and increase insulin sensitivity in patients with type II diabetes[67,68] and in animal models of diabetes[69–72]. Although ragaglitazar seems to achieve the desired effect on lipid and glucose metabolism, it is still unclear whether an appropriate therapeutic index can be obtained in humans. Observed side effects in both published studies included oedema, weight increase, leukopaenia and anaemia[67,68].

The third member of the PPAR family of nuclear transcription factors, PPARδ (previously also known as PPARβ), has emerged as a powerful regulator of fatty acid catabolism and energy homeostasis and provides another potential drug target in the treatment of the

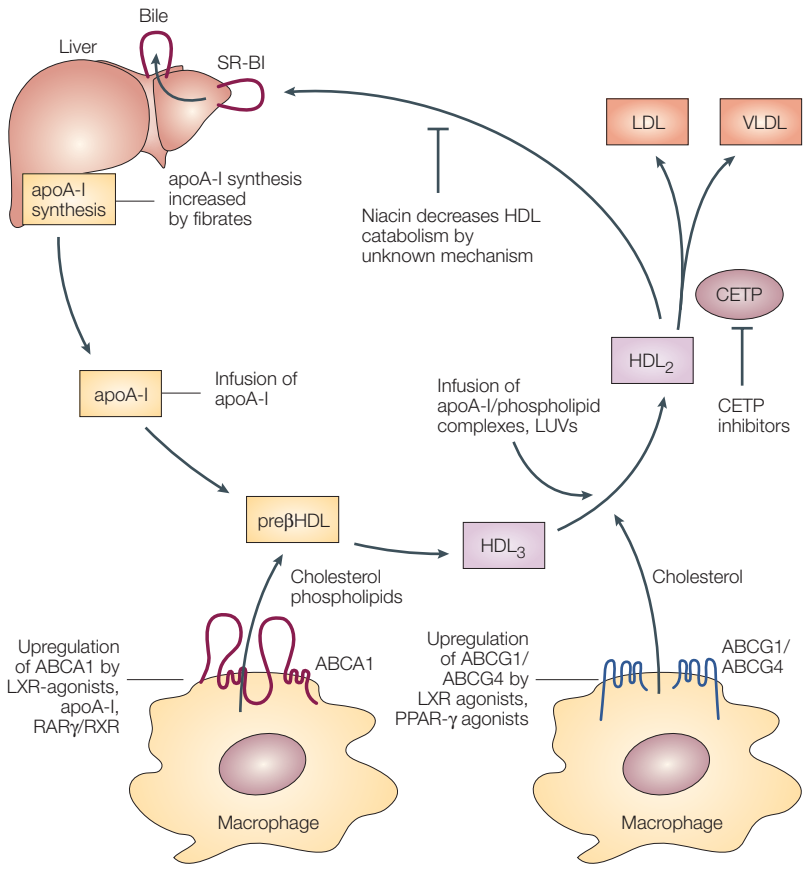

Figure 2 | **Overview of high-density lipoprotein metabolism and potential targets for therapeutic intervention.** Synthesis of new high-density lipoprotein (HDL) particles begins with the secretion of apolipoprotein A-I (apoA-I) from the liver. Fibrates have been shown to increase the expression of apoA-I in human hepatocytes[150,151]. Lipid-free or lipid-poor apoA-I can subsequently serve as an acceptor for ABC transporter A1 (ABCA1)-mediated lipid efflux from hepatocytes or macrophages. Infusion of apoA-I has been shown to attenuate atherosclerosis in animals[101] and possibly in humans[31]. ABCA1-mediated lipid efflux from macrophages can also be enhanced by transcriptional upregulation of this lipid transporter through the nuclear receptors liver X receptor (LXR)/retinoid X receptor (RXR)[18] or retinoic acid receptor-γ (RARγ)[128]. ABCA1-mediated efflux of cholesterol and phospholipids results in the formation of pre-β or nascent HDL particles that are further modified by lecithin-cholesterol acyltransferase (LCAT). The resulting HDL_2 (larger, less dense particles) and HDL_3 (smaller, more dense particles) can serve as acceptors for ABCG1-mediated cholesterol efflux[26]. Expression of this transporter can also be stimulated by LXR activation[152]. Infusion of recombinant phospholipid–apoA-I complexes[31] and large unilamellar phospholipid vesicles (LUVs)[153] have been shown to increase HDL levels, presumably by acting as acceptors for ABCG1-mediated cholesterol efflux. Cholesterol esters from HDL can be transferred to apoB-containing lipoproteins by the action of cholesteryl ester transfer protein (CETP). Inhibition of CETP in humans has recently been shown to increase HDL and lower low-density lipoprotein (LDL) cholesterol[84]. The catabolism of HDL can also be inhibited by nicotinic acid through a mechanism that is largely unknown. Finally, HDL cholesterol can be taken up by the liver and subsequently secreted into the bile in a process that is mediated by scavenger receptor BI (SR-BI).

metabolic syndrome and associated disorders[73,74]. Transgenic mice that overexpress an activated form of PPARδ are resistant to obesity, fat accumulation in tissues and hyperlipidaemia that is induced genetically or by diet manipulation[75]. PPARδ-deficient mice, on the other hand, show increased susceptibility to obesity and have reduced energy uncoupling[75]. Administration of GW501516 (FIG. 3), a PPARδ-selective agonist, to rhesus monkeys resulted in an 80% increase in HDL and a 29% reduction in LDL-cholesterol concentration[76]. PPARδ agonists are currently being tested in clinical trials, and it will be interesting to see whether the favourable effect on plasma lipids can also be observed in human subjects. A recently published study reports that PPARδ activation *in vitro* leads to lipid accumulation in human macrophages[77], which would of course be an undesired effect in the treatment of atherosclerosis. In summary, little is known about the effect of PPARδ agonists in humans, but the results obtained in some animal and cellular models hint at PPARδ as another promising drug target in the nuclear receptor superfamily. However, as noted above, PPARδ activators were recently found to be ineffective in a murine atherosclerosis model[62].

CETP inhibitors. The discovery of human genetic CETP-deficiency states, characterized by markedly increased HDL levels and moderately reduced LDL levels[78,79] (BOX 1), led to the development of drugs that inhibit CETP. CETP is bound to HDL in the circulation and facilitates the exchange of HDL cholesteryl esters with triglycerides in triglyceride-transporting lipoproteins (VLDL, chylomicrons), which results in a net removal of cholesteryl esters from HDL. CETP contains binding sites for cholesteryl ester and triglyceride molecules that might represent targets for some CETP inhibitors[80]. The carboxyl (C) terminus of CETP contains an amphipathic helix that probably acts as a lid on a hydrophobic lipid-binding pocket[80]. The polar face of this helix represents a target of neutralizing monoclonal antibodies that inhibit CETP and increase HDL levels. The binding of antibody to CETP greatly increases the binding of CETP to HDL particles. The C-terminal peptide of CETP has also been used to develop vaccines against CETP that seem to be weakly effective at increasing HDL, which have been evaluated in humans[81]. Small molecules that inhibit CETP have been developed and are in advanced clinical trials (FIG. 3). Some of these have similar properties to the CETP antibodies, increasing the binding of CETP to HDL and thereby probably forming an unproductive intermediate complex in the transfer mechanism[82]. CETP inhibitors that bind to thiol groups, such as JTT-705 (Roche/Japan Tobacco) (FIG. 3), probably interact with a cysteine at the base of the putative amino (N)-terminal lipid-binding pocket.

The first Phase II clinical trial of a CETP inhibitor was published in 2002 by de Grooth and co-workers[83]. In this study, 198 patients with mild hyperlipidaemia were treated with three different doses of the CETP inhibitor JTT-705 for 4 weeks. In the high-dose-treated group, this led to a 37% increase in HDL and a modest decrease of 7% in LDL cholesterol levels. More recently, even more impressive results were reported in a study published by Brousseau and co-workers[84]. In this single-blind, placebo-controlled study, the effect of the CETP-inhibitor torcetrapib (Pfizer) (FIG. 3) on plasma lipoprotein levels was examined in 19 subjects with low HDL cholesterol. Torcetrapib at a dose of 120 mg per day was administered for 4 weeks either alone ($n = 10$) or in combination with 20 mg of atorvastatin (Lipitor; Pfizer) ($n = 9$). A subgroup of six patients that did not receive atorvastatin was given torcetrapib for

an additional 4 weeks. In this last group of patients, the plasma HDL levels were increased by 106%, whereas plasma LDL levels were lowered by 17% after 8 weeks of torcetrapib treatment. In the patients treated for 4 weeks only, the increase in HDL was 46% in the group that received torcetrapib alone and 61% in those patients who were simultaneously given atorvastatin. The results indicate great potential for this CETP inhibitor for the treatment of patients with dyslipidaemia. The fact that an additional effect on HDL elevation and LDL lowering was observed in the group treated with torcetrapib and atorvastatin indicates a synergistic effect between inhibition of CETP and statin therapy. As CETP expression results in downregulation of hepatic LDL receptors[85], the synergy on LDL levels probably relates to increased expression of hepatic LDL receptors.

The key question that now needs to be addressed is whether torcetrapib can reduce surrogate endpoints for atherosclerosis such as carotid intima media thickness (IMT) or coronary atheroma volume as determined by intra-vascular ultrasound (IVUS). Even more important will be the demonstration of reduced morbidity and mortality in clinical trials. It is unclear whether patients with genetic CETP deficiency are protected from atherosclerotic cardiovascular disease or not[86]. This is because there are too few subjects with homozygous deficiency to allow systematic analysis in population-based studies, and subjects with heterozygous deficiency, although common in the Japanese population, have only a modest elevation of HDL levels. Results from animal studies indicate that CETP inhibition can decrease atherosclerosis. In rabbits treated with JTT-705, a chemical inhibitor of CETP, there was a doubling of HDL-levels, a marked decrease in the concentration of apoB-containing lipoproteins and a 70% decrease in lesion size[87]. Moreover, cholesterol-fed rabbits treated with torcetrapib showed a significant reduction in atherosclerosis[88]. Importantly, this benefit was associated only with elevation of HDL, as VLDL and LDL cholesterol levels were not significantly altered over the course of treatment. This suggests a direct benefit from HDL elevation, which is the major effect of this drug in humans.

Inhibition of CETP activity was also achieved by vaccinating rabbits with a synthetic peptide that was homologous to a peptide sequence of the catalytic site in CETP[89]. Vaccination of rabbits with this peptide led to a 39.6% reduction in aortic lesion areas. Studies with a CETP vaccine in humans, however, have so far been less successful, as the effect of the vaccine on CETP activity and HDL has been small[81]. Using NMR spectroscopy, Brousseau *et al.* observed a significant increase in HDL-particle size and a decrease in small, dense LDL particles in the patients treated with torcetrapib[84]. This change in lipoprotein subclass size can be considered to be favourable because earlier studies have found low levels of large HDL and high levels of small, dense LDL associated with coronary heart disease[90–92]. One possible mechanism to explain the protective effect of HDL particles with increased size, as they are observed after inhibition of CETP, might be that these particles act as ideal acceptors for cholesterol efflux to HDL mediated by ABCG1. Recent results from our laboratory indicate that ABCG1-mediated cholesterol efflux to HDL isolated from patients with genetic CETP deficiency is increased when compared with that of control subjects (F. Matsuura *et al.*, unpublished observations). We propose that CETP inhibition and ABCG1-mediated cholesterol efflux to the subsequently formed larger HDL particles provides an effective mechanism that could result in a beneficial clinical outcome in patients treated with torcetrapib.

Infusion of apolipoproteins or synthetic HDL particles that might act as cholesterol acceptors. apoA-I is the major structural apolipoprotein of HDL lipoproteins. Genetic deficiencies of apoA-I in humans have been observed to result in very low levels of plasma HDL and premature atherosclerosis[93,94] (BOX 1). The anti-atherogenic phenotype of transgenic apoA-I overexpression has been well documented in several studies of transgenic mice[95–99] and rabbits[100]. Transgenic overexpression of apoA-I seems to convey one of the strongest and most consistent anti-atherogenic effects observed in animal models so far. On the basis of these promising observations, great efforts have been undertaken in the past two decades to identify pharmacological agents that can upregulate the gene expression of apoA-I or mimic the effect of this apolipoprotein *in vivo*. Several drugs and other compounds, such as phenobarbital, gemfibrozil, fenofibrate, prednisone, oestrogen and alcohol, induce apoA-I synthesis. Despite intensive research, small molecules that could upregulate apoA-I gene expression without the multiple side effects of the above-mentioned substances remain elusive.

For this reason, there has been growing interest in other methods to deliver apoA-I into the circulation — notably the infusion of apoA-I or recombinant phospholipid–apoA-I complexes. Repeated injections of apoA-I into cholesterol-fed rabbits inhibit the progression of atherosclerosis and possibly even lead to a regression in plaque size[101]. Furthermore, intravenous infusion of pro-apoA-I in humans has been shown to result in increased excretion of faecal sterols, a finding that is consistent with enhanced reverse cholesterol transport[102]. One probable mechanism of action is that by providing free apoA-I as an acceptor particle, the infusion of apoA-I enhances the ABCA1-mediated cholesterol efflux. We and others have recently shown that apoA-I binding to ABCA1 protects ABCA1 from calpain-mediated proteolysis[103,104]. After injection of apoA-I into mice, we have observed increased ABCA1 protein levels in macrophages and the liver, providing another mechanism for the atheroprotective effect of apoA-I infusion[104].

A recent study by Nissen and colleagues[31] has drawn considerable attention to apoA-I-infusion as a therapeutic concept. For this trial, patients suffering from acute coronary syndrome received weekly infusions of recombinant apoA-I-Milano–phospholipid complexes over a period of 5 weeks. Atherosclerosis progression

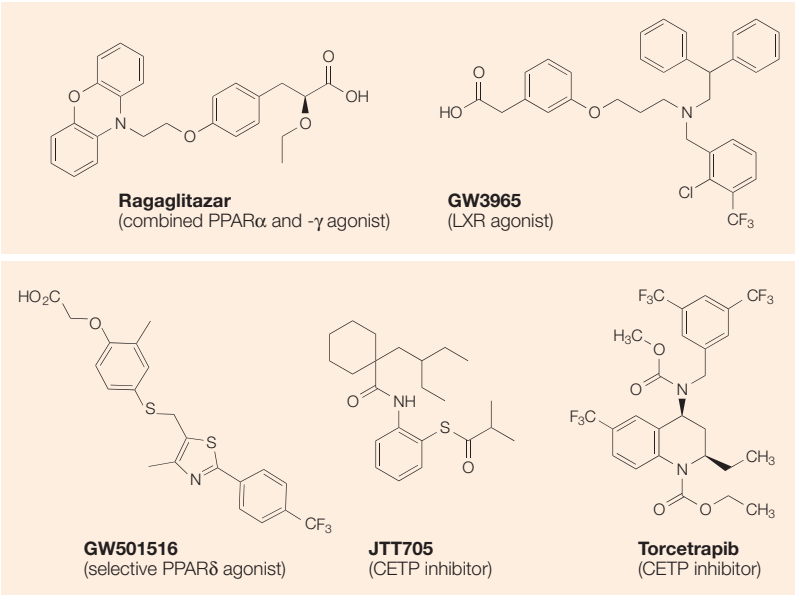

Figure 3 | **Chemical structures of selected investigational agents that target high-density lipoprotein.** CETP, cholesteryl ester transfer protein; LXR, liver X receptor; PPAR, peroxisome proliferative-activated receptor.

was measured at baseline and 2 weeks after treatment using INTRACORONARY VASCULAR ULTRASOUND. Interestingly, in the group of patients that received the weekly apoA-I Milano infusions, there was a significant regression in plaque size compared with baseline, whereas in the placebo group there was no detectable change. This was the pre-declared primary endpoint of the trial. Despite these very encouraging results, there are several limitations associated with this study. The study was designed as a pilot study and compared 45 patients receiving the apoA-I Milano infusions with 12 patients receiving placebo. Because of the small number of patients involved, it lacks the statistical power for direct comparison between the treatment and placebo groups. Moreover, there were side effects in the treated group (stroke and CHOLELITHIASIS) that might have arisen by chance but also could represent complications of therapy that will only become apparent when more extensive studies are performed.

An important issue not addressed by this study is a direct comparison between the wild-type apoA-I and apoA-I Milano. apoA-I Milano is a naturally occurring mutant of apoA-I that has been identified in a number of subjects from a town near Milan in Northern Italy. In apoA-I Milano, there is a cysteine-to-arginine substitution at position 179. This amino-acid substitution results in altered physicochemical properties and a rapid catabolism of apoA-I Milano compared with that of wild-type apoA-I. Whether subjects with apoA-I Milano are truly protected, and whether there is any special property of apoA-I Milano versus wild-type apoA-I, is controversial[105]. The mechanism of protection by phospholipid–apoA-I Milano complexes could involve enhanced cholesterol efflux from macrophage foam cells via ABCA1 and ABCG1 pathways. In animal infusion studies, there seemed to be a rapid disappearance of neutral lipid-staining material from atheroma[101]. Recent studies indicate that macrophages rapidly disappear from lesions during atherosclerosis regression[106]. This could be related to the carriage of platelet-activating factor (PAF) acetylhydrolase on HDL as it enters lesions, leading to the hydrolysis of PAF-like oxidized phospholipids that impair macrophage egress from lesions[106].

Besides native apoA-I and apoA-I Milano, several recombinant peptides with amino-acid sequences that mimic the structure of apoA-I have been developed. Such amphipathic helical peptides that mimic the actions of apoA-I have anti-atherogenic effects in animal models[107–109]. Results of a study with the apoA-I mimetic peptide D-4F, which can be orally administered, indicate that there is also an anti-atherogenic effect of such peptides that is independent of increasing plasma HDL levels[108]. This could be due to antioxidant properties of HDL that are retained in the apoA-I mimetic peptides, such as the ability to modulate the activity of paraoxonase, an apoA-I-associated enzyme with antioxidant activity[110,111]. apoA-I mimetic peptides have also been shown to inhibit inflammation[112,113] and thrombus formation[114], both of which are properties retained from native apoA-I/HDL. apoA-I mimetic peptides can be more easily produced than native apoA-I, and can possibly be orally administered, and so they have become attractive candidates for therapy. However, considerable mystery and uncertainty still surrounds the underlying mechanisms and potential toxicity of apoA-I peptides as therapeutic agents.

HDL-raising drugs in preclinical development

LXR-activators/selective LXR modulators. Intense research has been devoted to developing activators of the LXR as drugs that can raise plasma HDL levels and inhibit the progression of atherosclerosis. LXRs are ligand-activated transcription factors that belong to the nuclear receptor superfamily. LXRs were first named orphan nuclear receptors as their endogenous activators were unknown when they were cloned[115]. Two isoforms, LXRα and LXRβ, have been identified and both have emerged as central regulators of genes that are involved in lipid metabolism[116]. LXRα is primarily expressed in the liver, kidney, macrophages and intestine, whereas LXRβ is ubiquitously expressed[117].

Oxysterols such as 22-OH cholesterol and 24-OH cholesterol have been identified as natural ligands for LXR, and several synthetic ligands have been well characterized[118,119]. LXR target genes include the ABC transporters involved in cholesterol efflux (ABCA1, ABCG1 and ABCG4), HDL-modifying enzymes (CETP and phospholipid transfer protein (PLTP)) and genes involved in cholesterol secretion into the bile (CYP7A, ABCG5 and ABCG8)[120]. Treatment with GW3965 (GlaxoSmithKline) (FIG. 3), a synthetic agonist of LXR, has been shown to inhibit the development of atherosclerosis in mice[121], whereas macrophage-specific knockout of LXR by bone-marrow transplantation aggravates atherosclerosis[122].

INTRACORONARY VASCULAR ULTRASOUND
(IVUS). Procedure in which a miniature ultrasound transducer on the tip of a coronary catheter is used to produce detailed images of the interior wall of coronary arteries.

CHOLELITHIASIS
Presence of gallstones in the gallbladder.

However, the promising results obtained with synthetic LXR agonists are countered by one significant liability: LXR activation leads to increased fatty acid synthesis, accumulation of triglycerides and the development of fatty liver. These effects are caused by LXR-mediated induction of sterol regulatory element binding factor 1c (SREBP1c), a sterol-responsive transcription factor, which, in turn, induces some of the key enzymes involved in fatty acid synthesis, such as fatty acid synthase and acetyl-coenzyme A carboxylase. In addition, LXRs directly target the promoter of fatty acid synthase. For the successful clinical application of LXR agonists, this significant side effect needs to be overcome. The ideal LXR agonist would induce ABCA1 and ABCG1 in macrophages, and possibly ABCG5 and ABCG8 in the liver, without activating genes of fatty acid synthesis in the liver. The development of such selective LXR modulators is therefore an important objective in drug discovery.

One aspect that needs further clarification and might lead to a solution to this problem is the functional difference between the two isoforms LXRα and LXRβ. Knockout mice for both isoforms have been generated[123,124] and a comparison of these animals implies that LXRα is the dominant receptor involved in hepatic lipogenesis. Therefore, a selective LXRβ agonist might be the drug of choice to increase HDL without inducing hypertriglyceridaemia and fatty liver. Several recently published studies point out directions for future research that could lead to the development of a LXRβ-selective agonist. Thomas and co-workers recently published the interesting observation that the relatively simple chemical modification of converting the PPARα agonist fenofibrate from an esterified form into a carboxylic acid converts the PPARα agonist into a high-affinity LXR ligand and activator[125]. This shows that the members of the nuclear receptor superfamily share some degree of conservation in their ligand recognition. Fibrates in the esterified form act as repressors of LXR activation. Interestingly, fenofibrate in its esterified, PPARα-activating form potently represses the LXR-mediated transcription of hepatic lipogenic genes but does not suppress ABCA1, ABCG5 and ABCG8 transcription in the liver[122].

The mechanism for the selective repression of some of the LXR target genes remains to be determined, but could be related to the recruitment of co-activators in a similar fashion, a phenomenon described for the selective oestrogen- and progesterone-receptor agonists[126]. Recently, another interesting study was published examining the effect of the LXR modulator GW3965 on lipid metabolism and hepatic gene expression in mice[127]. At doses that effectively raised plasma HDL levels in the GW3965-treated mice, the authors observed relatively little induction of fatty acid synthase and SREBP1c compared with animals treated with the LXR activator T0901317. GW3965 is a weaker LXR activator than T0901317, which results in less elevation of HDL cholesterol compared with T0901317-treated mice. The authors propose that when activating LXR, there might be a trade-off between inducing genes involved in reverse cholesterol transport and inducing hepatic SREBP1c and fatty acid synthase genes. Using a fluorescence resonance energy transfer (FRET) assay, the authors compared the recruitment of co-activators after treatment with the two LXR activators. Even though the LXR co-activators steroid receptor co-activator 1 (SRC1) and vitamin-D-receptor-interacting proteins were recruited at similar levels with both drugs, GW3965 treatment resulted in less recruitment of the LXR co-activator cAMP-response-element-binding protein (CBP). Although these results need to be confirmed using other methods, selective recruitment of CBP might provide a possible explanation for the lower SREBP1c induction observed with GW3965 (REF. 127). Further research is needed to identify co-activators and co-repressors of LXR that mediate differential gene expression in tissues.

Costet *et al.* have reported upregulation of ABCA1, ABCG1 mRNA and cholesterol efflux in macrophages by activation of the retinoic acid receptor-γ (RARγ)[128]. The induction of ABCA1 by RARγ/RXR is mediated by the same DR4 (direct repeat element spaced by 4 nucleotides) element in the ABCA1 promoter that has been implicated in activation by LXR/RXR[18]. RARγ is not highly expressed in the liver and RARγ activators can stimulate macrophage cholesterol efflux through ABCA1 and ABCG1, so it is conceivable that RARγ-selective activators might have an interesting range of properties that includes increased macrophage cholesterol efflux while tending to spare fatty liver and some of the pro-atherogenic effects of other retinoids.

Endothelial and hepatic lipase inhibitors. Another potential way to decrease the turnover of HDL and increase plasma HDL levels is to inhibit endothelial lipase (EL). Whereas the closely related enzymes lipoprotein lipase (LPL) and hepatic lipase (HL) act mainly as triglyceride hydrolases, EL is believed to preferentially hydrolyse phospholipids from HDL particles[129]. Adenovirus-mediated overexpression of EL in LDL receptor-deficient mice reduced plasma concentrations of VLDL and LDL cholesterol by about 50%, whereas HDL levels were decreased to almost zero[130]. By contrast, inhibition of EL activity by injection of a neutralizing polyclonal antibody resulted in a strong increase in plasma HDL levels in mice[131]. When compared with a control antibody, the infusion of the inhibitory antibody resulted in a 25–60% increase in HDL cholesterol in three different mouse models.

However, the physiological role of EL in lipid metabolism is incompletely understood. A recently published study indicates that EL might not be as HDL-specific as previously suggested, but might have a significant effect on apoB-containing lipoproteins as well[132]. In this study, overexpression of a catalytically inactive form of EL in mice led to inhibition of the endogenous lipase, which in turn led to an increase in LDL and VLDL concentrations[132]. However, despite an increase in apoB-containing lipoproteins, a 70% reduction in aortic lesion area has recently been reported in EL/apoE double-knockout mice[133]. The authors also

observed a decrease in macrophage content in the arterial wall of EL-knockout mice and an inhibition of monocyte adhesion in an *ex vivo* assay, suggesting a further mechanism by which EL could regulate the development of vascular disease. Pharmacological inhibition of EL might therefore have the potential to raise plasma HDL in humans and inhibit atherosclerosis.

Inhibition of EL has been suggested as another means to raise plasma HDL levels[134,135]. However, EL has a role in the removal of remnant lipoproteins, and, therefore, inhibition could result in remnant lipoprotein accumulation and increased atherogenesis.

Other potential drug targets to raise HDL

FXR activators. The farnesoid X receptor/bile acid receptor (FXR) is another member of the nuclear hormone receptor superfamily. One predominant function that has emerged for this nuclear receptor is its involvement in bile-acid homeostasis. Interestingly, FXR-knockout mice show a pro-atherogenic lipid phenotype with increased circulating total cholesterol and triglycerides, decreased hepatic SR-BI and an increase in apoB1 (REF. 136). FXR-knockout mice also show a decreased clearance of HDL lipoproteins, an effect that is probably caused by the downregulation of SR-BI in the liver. High levels of LXR expression have been reported in the vascular wall, and activation of this receptor has been implicated in smooth-muscle-cell apoptosis[137]. Several FXR activators have been described, but so far no reports on their effect on atherosclerosis or plasma lipids have been published[138–140].

SR-BI modulators. SR-BI is believed to have an important role in the catabolism of HDL and the delivery of HDL cholesterol to the liver for excretion into the bile[141]. Moderate overexpression of SR-BI in mice is anti-atherogenic even though HDL is lowered[142], a finding that is consistent with the 'reverse cholesterol transport' hypothesis. Pharmacological upregulation of SR-BI in the liver might therefore be useful to enhance reverse cholesterol transport and inhibit atherosclerosis. An induction of SR-BI levels in the liver has been achieved in animal studies by feeding them polyunsaturated fatty acids[143] and by inhibiting hepatic lipase (HL)[135,144]. However, no drugs that specifically target SR-BI have been reported so far.

Mimetics of lysosphingolipids. Lysosphingolipids, and in particular sphingosine 1 phosphate, are natural components of plasma HDL and have recently attracted considerable attention as possible mediators of vasorelaxation and cytoprotective effects associated with HDL[35,145,146]. Large HDL particles that are formed by pharmacological inhibition of CETP might act as the preferred carriers of lysosphingolipids, which could contribute to their protective function. Pharmacological mimetics of lysosphingolipids, such as the endogenous peptide IGFBP3 (REF. 147), could provide a potential mechanism for HDL-directed therapy to enhance endothelial function. However, it remains unclear whether concentrations of these compounds in HDL are sufficient *in vivo* to exert physiological effects.

Conclusions and outlook

In summary, several promising drug targets have emerged to raise plasma HDL cholesterol and inhibit the progression of atherosclerosis. The next few years will be a watershed as the results of clinical studies with CETP inhibitors, combined PPARα and PPARγ activators and further recombinant HDL-particle-infusion studies become available. If these prove to be negative, it will greatly complicate subsequent HDL-based approaches. However, if they are positive, this will lead to a new approach to treatment and the development of combined therapies — that is, statins plus HDL elevation. A futuristic approach might be to provide additional acceptors for cholesterol efflux (for example, by apoA-I/phospholipid infusion or CETP inhibition) and to upregulate the ABC transporters (ABCA1 and ABCG1) that mediate the efflux of cholesterol from arterial wall cells to HDL or its apolipoproteins (for example, by LXR or PPARγ activation). It is conceivable that in the not-too-distant future a patient with atherosclerotic cardiovascular disease will be treated with a combination of a drug that inhibits cholesterol synthesis and upregulates LDL receptors (such as a statin), a drug that inhibits cholesterol absorption (for example, ezetimibe (Zetia/Ezetrol; Merck/Schering-Plough) and a third drug that raises HDL and enhances efflux of cholesterol from the arterial wall. Combination therapy, which is already commonplace in the treatment of hypertension, is, in the future, likely to become common in the treatment of dyslipidaemia and atherosclerosis and could replace monotherapy with statins.

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Competing interests statement
The authors declare that they have no competing financial interests.

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