Review

MOLECULAR METABOLISM

High density lipoprotein and metabolic disease: Potential benefits of restoring its functional properties

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ABSTRACT

Background: High density lipoproteins (HDLs) are thought to be atheroprotective and to reduce the risk of cardiovascular disease (CVD). Besides their antioxidant, antithrombotic, anti-inflammatory, anti-apoptotic properties in the vasculature, HDLs also improve glucose metabolism in skeletal muscle.

Scope of the review: Herein, we review the functional role of HDLs to improve metabolic disorders, especially those involving insulin resistance and to induce regression of CVD with a particular focus on current pharmacological treatment options as well as lifestyle interventions, particularly exercise.

Major conclusions: Functional properties of HDLs continue to be considered important mediators to reverse metabolic dysfunction and to regress atherosclerotic cardiovascular disease. Lifestyle changes are often recommended to reduce the risk of CVD, with exercise being one of the most important of these. Understanding how exercise improves HDL function will likely lead to new approaches to battle the expanding burden of obesity and the metabolic syndrome.

Keywords High density lipoprotein (HDL); Metabolic disease; Glucose homeostasis; Physical activity; Atherosclerotic plaque regression; HDL function

1. INTRODUCTION

Plasma High Density Lipoproteins (HDLs) are small, dense, lipoprotein particles, composed of proteins, phospholipids, unesterified (free) sterols (mostly cholesterol), cholesteryl esters and triglycerides [1]. Mass spectrometry studies have revealed that HDL particles collectively contain more than 80 different proteins and more than 200 lipid species [2]. Although HDL is primarily known to facilitate cholesterol uptake from tissues and transport it back to the liver, it has been recently discovered that HDL has alternative functions that are independent of its ability to promote cholesterol flux and mediated by other molecules it contains, including specific proteins, small RNAs, hormones, carotenoids, vitamins, and bioactive lipids [3,4]. Most known HDL functions are atheroprotective and therefore reduce the risk of cardiovascular disease (CVD). The classical HDL particle function is defined by its efflux capacity, that is, the ability of HDL to accept free cholesterol from cells and transport cholesteryl esters (CEs) to cells and tissues, especially the liver, where cholesterol is processed for excretion through the bile [5]. Peripheral cells, such as macrophages, return excess cholesterol to the liver via this reverse cholesterol transport (RCT) pathway, via unmediated aqueous diffusion, and/or specific receptor-mediated processes [1]. In addition to its classical function many recently discovered functions of HDL include antioxidant, antithrombotic and anti-inflammatory properties [6–8], improvement of endothelial function, promotion of endothelial repair and angiogenesis [9], inhibition of cell apoptosis [10], enhancement of glucose uptake by skeletal muscle [11] and stimulation of secretion and synthesis of insulin from pancreatic β cells [12]. With regard to the growing world-wide diabetes epidemic, this recently discovered insulin-secretagogue effect of HDL on beta cells as well as HDL-induced stimulation of glucose uptake and turnover in skeletal muscle [11,13,14], liver and adipose tissue [15], underscore the importance to maintain HDL function in humans.

Herein, we will review the functional role of HDL to improve metabolic disease and to induce regression of CVD with a particular focus on

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current pharmacological treatment options as well as lifestyle interventions, particularly exercise.

2. HDL FUNCTION AND GLUCOSE HOMEOSTASIS

Several independent studies have determined that HDL and its main protein component apolipoprotein AI (apoA-I) directly increase glucose uptake and glycogen synthesis in murine and human skeletal muscle cells as well as in adipocytes through an insulin-independent manner [9,16–18]. HDL also enhances glycolysis and mitochondrial oxidative phosphorylation rates of glucose in skeletal muscle [11]. That this cell autonomous effect of HDL on intracellular glucose metabolism may have clinical implications has been determined by in vivo experiments showing that overexpression of human apoAI resulted in protection against age-induced fat mass gain, age-induced decline of endurance capacity and diet-induced hyperglycemia [11]. Although HDL and apoAI may modulate glucose turnover directly, there is recent evidence that HDL in parallel affects insulin-mediated glucose uptake in skeletal muscle indirectly by stimulating secretion of circulating factors in immune cells [13]. Since these latter actions were not linked to changes in common pro- or anti-inflammatory mediators or insulin signaling via Akt additional studies are required to identify the secreted factors responsible for mediating this novel function of HDL.

Besides the frequently observed low HDL levels in type 2 diabetes patients, reduced HDL functionality has been described in these patients, together with the presence of smaller HDL particles that exhibit abnormal metabolism and chemical composition [16,19–21]. One of the reasons for the presence of small, dense HDL particles (HDL-P) in type 2 diabetic patients might be elevated cholesteryl ester transfer protein (CETP) activity, leading to lower HDL cholesterol (HDL-C) concentrations [20]. HDL functionality may also play a role in the development of diabetic complications since it has been shown that changes in cholesterol efflux, mediated by cholesterol transporters, contribute to lipid accumulation in kidneys promoting the development of diabetic nephropathy [22]. The transporters in question include adenosine triphosphate binding cassette transporter A1 (ABCA1), which mediates the efflux to apoA-I and pre-β-HDL, ABCG1 and scavenger receptor class B type I (SR-BI), which manage cholesterol efflux to mature HDL particles [23] since their expression was markedly decreased in renal cells under hyperglycemic conditions and in diabetic mice with nephropathy [22].

3. HDL AND PHYSICAL EXERCISE

Based on the emerging evidence that HDL may contribute to the pathophysiology of type 2 diabetes mellitus (T2DM) through direct effects on plasma glucose [24], it is important to understand whether physical exercise is beneficial for HDL-mediated effects on skeletal muscle function especially because skeletal muscle accounts for the majority of plasma glucose disposal [24]. In humans, high levels of aerobic activity and low levels of muscle inactivity have been associated with increased levels of HDL-C when compared to participants having longer muscle inactivity times [25–27]. Even as little as 30 min of moderate intensity (50–70% of maximum heart rate) on cycle ergometers has been shown to effectively increase HDL-C levels in T2D patients [28].

Classically, HDL particles have been separated into two main subfractions, high-density lipoprotein 2 (HDL_2) and 3 (HDL_3), with HDL_3 being more dense and larger when compared to HDL_2 particles, which contain more effluxed cholesterol. Interestingly, enhancing physical activity in people with low levels of HDL-C results in the acute increases of the HDL_3 subfraction in untrained people and of the HDL_2 subfraction in trained individuals [29]. A very different situation has been described for individuals with insulin resistance and T2D, which are characterized by reduced levels of both the large (HDL_2) and small (HDL_3) subfractions and a shift towards the HDL_3 subfraction associated with a reduction in anti-atherogenic (efflux-related) function of HDL once MetS progresses [30,31]. In this patient population, a 4-month aerobic training program did not modify plasma HDL levels per se, but, it did improve the protective effect of the HDL_3 subfraction against LDL oxidation by 15% [32]. This finding underscores the benefits aerobic training can have for T2D patients, even when plasma HDL levels do not change.

A recent meta-analysis aimed to answer how much exercise is required to raise HDL-C levels evaluated 25 randomized, controlled studies, which were designed to investigate the impact of exercise training on HDL-C levels [33]. The conclusion was that 120 min of exercise (or equivalent of 900 kcal of energy expenditure) per week is sufficient for increasing HDL-C levels. Every prolongation of exercise for 10 min per session was associated with an approximately 1.4 mg/dl (0.036 mmol/l) increase in HDL-C. Interestingly, participants with a body mass index (BMI) less than 28 with total cholesterol level of 220 mg/dl or more experienced larger HDL-C increases post-aerobic exercise intervention than people with BMI of 28 or more, with total cholesterol levels less than 220 mg/dl [33]. Similarly, a systematic review of 2808 T2D patients revealed that supervised exercise is effective in raising HDL-C levels of 0.04 mmol/l (95% CI, 0.02–0.07 mmol/l), and heterogeneity was partially explained by duration/intensity of exercise as well as age and dietary co-intervention [34]. Comparing the effectiveness of high intensity interval training versus moderate-intensity, continuous aerobic exercise interval training appeared to be more effective than continuous aerobic activity for improving the aerobic capacity of patients with coronary artery disease [35]. Furthermore, there is mounting evidence that the whole exercise regimen does not have to be completed in a single daily 30-min session to have a positive effect on glycemic control and cardiorespiratory fitness, but moderate-to high-intensity training can be done in 3 daily, 10-min sessions [36].

Although there are a number of studies reporting a positive effect of exercise in raising HDL-C levels [28–31,33–35,37–39], no change in HDL-C levels has also been detected after exercise intervention [40,41]. A limitation in these studies, however, is that they did not measure VO_{2max}, an indicator of cardio-respiratory capacity [42], meaning that evidence about the efficiency of exercise intervention or whether the participants exercised as instructed was not provided. The only 2 parameters measured were heart rate (HR) and Rating of Perceived Exertion (RPE), and it is questionable whether those are valid indicators of exercise intensity [43]. In contrast to a study in humans where an acute bout of exercise did indeed raise HDL-C levels [29], an experimental study in rats running at 3 different intensities (18, 24 and 30 m/min for 30 min) could not detect an increase in HDL-C levels at any time point measured after exercise (before running, immediately after running, 30 min and 5 h post-running) [44]. Although even in the few human studies that did not detect an effect of exercise on HDL-C levels, the researchers still found evidence that exercise improved HDL function [32,45], e.g. by increasing antioxidant activity (paraoxonase) to inhibit LDL and cellular oxidation in vitro [45]; by enhancing HDL particle maturation through loading of cholesterol and proteins [46]; by increasing RCT and increasing lecithin cholesterol acyl-transferase (LCAT) activity [47,48]; or by raising nitric oxide (NO) bioavailability, which, in turn, reduces oxidative modification of HDL [49–51]. Besides

these classic HDL functions, a novel mechanism by which HDL may reduce endothelial dysfunction in chronic diseases is emerging. Expression of pro-angiogenic micro-RNAs [miRNA-126, miRNA-21 and miRNA-222] that are significantly reduced in endothelial cells of chronic heart failure (CHF) patients are restored to levels of healthy controls after a 15-week of exercise intervention on bicycle ergometers [49]. The observed HDL-induced improvement of endothelial function in CHF patients potentially shifts the therapeutic approach from raising HDL-C concentration towards improving HDL function.

Although lifestyle interventions and in particular exercise are known to promote cardiovascular health, the underlying mechanisms leading to the improvements of atherosclerosis are not fully known. Running exercise reduces neo-intimal growth and stabilizes vascular lesions by reducing the number of Mac-3-positive, oxidized LDL-containing macrophages in the vessel wall and by increasing the content in collagen fibers in rodent models for atherosclerosis [51]. Contributing factors may be enhanced traffic of cholesterol from macrophages to the liver and modification of inflammatory regulators [52,53]. More importantly, combined treatment with atorvastatin plus exercise training act synergistically to stabilize atherosclerotic plaques by increasing its content in elastin and collagen and by influencing the MMP/TIMP equilibrium in the absence of significant changes in body-weight [54]. A small study in patients with CVD indicates that this synergistic effect is also occurring in humans [55].

4. HDL AND ATHEROSCLEROSIS REGRESSION

Historically, therapeutic efforts have focused on slowing or ceasing the progression of atherosclerosis. However, there has been increasing evidence over the past 15 years that atherosclerotic plaques can regress, and HDL has been centrally implicated in this regression. Indeed, the inverse relationship between plasma HDL-C levels and the risk of atherosclerotic cardiovascular disease has long been recognized from epidemiological studies [56,57]. However, a number of more recent, larger-scale trials have failed to show that elevated HDL-C reduces the risk of cardiovascular disease (CVD) [58–62]. Moreover, use of Mendelian randomization analysis to investigate the relationship between HDL-C and coronary heart disease has demonstrated that increasing plasma HDL-C does not uniformly translate into reductions in risk of myocardial infarction [63]. This raises two fundamental questions: first, is HDL as cardioprotective as previously assumed, and secondly, do plasma levels of HDL-C reflect the multitudinous functional activities of HDL? To address the first of these questions, we have evaluated the limited evidence from clinical studies in which plasma HDL and HDL-C levels have been manipulated, and the impact on atherosclerosis or CVD events assessed (Table 1). The second question has been addressed, in part, in the previous section, namely that HDL function can be independent of HDL-C, a point we will re-visit in this section.

Several important points can be made from the studies outlined above. First, atherosclerotic regression can be achieved in humans [64–67,74,75], and this is associated with a reduction in clinical events [64,66]. However, numerous large-scale niacin and cholesteryl-ester transfer protein (CETP) inhibitor trials have failed to demonstrate plaque regression [67,70–72]. More importantly, they failed to reduce the incidence of major cardiovascular events, despite significantly raising plasma HDL-C levels [58–62]. Simplistically, it may be suggested that a threshold level for plasma HDL-C was not met in these studies. As noted above, it seems more likely that HDL-C is not an accurate biomarker for HDL functionality. As such, pharmacological strategies solely raising 'good cholesterol' levels without accounting for HDL modification and dysfunction are unlikely to prove efficacious. This brings us to the second question, of whether HDL-C levels reflect the protective properties of HDL.

The composition and biologic properties of HDL particles are markedly heterogeneous [76], and the influence of size, charge, or protein composition of HDL on its pleiotropic atheroprotective effects is poorly understood. As such, HDL-C levels alone are a poor metric of in vivo HDL function. Indeed, Khera et al. [77] demonstrated that HDL's cholesterol efflux capacity (a key HDL anti-atherogenic activity [78]) was inversely associated with carotid intima-media thickness and angiographically confirmed coronary artery disease (CAD), independent of HDL-C levels. Significantly, cholesterol efflux capacity is also inversely associated with the incidence of atherosclerotic cardiovascular disease [79,80]. This association persisted after adjustment for HDL-C level [79]. Together these data suggest that biomarkers of HDL function are likely to be a more reliable therapeutic target for cardiovascular disease prevention than measurement of the cholesterol level. Moreover, it is clear that the development of specific, targeted HDL-based therapies requires a detailed mechanistic understanding of how HDL particles mediate atherosclerosis regression. For this, we turn to pre-clinical models.

While controversy is ongoing, there is promising evidence for the feasibility of HDL-mediated CAD regression in humans. These findings build on well-established data validating that atheromata can regress in pre-clinical models (reviewed in [91]). Despite significant differences between murine and human HDL metabolism (e.g., no subspecies of HDL in mice nor any CETP activity), we and others have developed several mouse models to allow for mechanistic investigations into the influence of HDL on plaque size and composition. Using such models to improve understanding of the processes underlying plaque resolution may help to shed light on the discrepant evidence from HDL-regression clinical trials.

One such model has been the extension of work beginning in 2001. In that year, we published an aortic transplantation model of atherosclerosis regression, which has greatly advanced the knowledge of the processes underpinning plaque resolution [81]. In this model, atheromata develop in apolipoprotein E-deficient ($apoE^{-/-}$) mice, and a segment of the atherosclerotic aorta is transplanted into normolipidemic wild type (WT) recipients [82]. With normalization of the plasma lipoprotein profile, almost complete regression of the atherosclerotic lesions occurs. Using this model, Feig et al. demonstrated the primacy of HDL as a mediator of atherosclerosis regression [83]. Transplantation of plaque-laden aortic arches from $apoE^{-/-}$ mice into mice transgenic for the human apoAI gene ($hA1/apoE^{-/-}$), which have normal HDL-C and HDL function, as well as high non-HDL-C, still resulted in >50% reduction in plaque macrophage ($CD68^{+}$ cell) content only 1 week after transfer. In contrast, there was little change in the plaques after transfer to hypolipidemic $apoA1^{-/-}$ mice [83]. This strongly implied that it was the normalization of functional HDL levels, not the reduction in non-HDL cholesterol, which promoted the regression of atherosclerosis. The reduction in plaque macrophages following transplantation into a normolipidemic environment was associated with the induction of the chemokine receptor CCR7 in the macrophages and their emigration from plaques [84,85]. The mechanism by which HDL alters macrophage CCR7 gene expression remains unclear, but likely involves a SRE-transcriptional mechanism, as both the human and mouse promoters have this site and it appears to be active [86].

In addition to stimulating macrophage emigration, regression of atherosclerotic lesions promotes resolution of the maladaptive inflammatory response within the arterial wall [87]. Promisingly, we have

Table 1 — Selected studies using a range of approaches to raise plasma HDL-C or HDL particle number, and assessing the impact on plaque pathology or clinical outcomes.

First author & year (Trial name)	Intervention	Mean change in HDL (%)	Outcome measures	Main findings
Brown et al. 1990 (FATS)[64]	Niacin — Colestipol Lovastatin — Colestipol Placebo or Colestipol alone	+43 +15 +5	QCA Clinical events (death, MI, revascularization)	Greatest increase in HDL in niacin — colestipol group was associated with angiographic atherosclerotic regression at 30 months in 39%, and a 73% reduction in clinical events
Cashin-Hemphil et al. 1990 (CLAS)[65]	Niacin — Colestipol Placebo	+37 +2	QCA	At 4 years, significantly more drug-treated patients showed nonprogression than in the placebo group (52% versus 15%). Regression seen in 18% treatment group, versus 6% in the placebo group
Brown et al. 2001 (HATS)[66]	Simvastatin — Niacin Antioxidants Simvastatin — Niacin — Antioxidants Placebo	+26 +3 +18 +6	QCA Major clinical events (death, MI, stroke, revascularization)	At 3 years, there was a significant 0.4% regression of coronary stenosis in the simvastatin — niacin group. This was associated with 90% reduction in major clinical events
Nissen et al. 2003[67]	IV recombinant ApoA-I Milano/phospholipid complexes (ETC-216) Placebo	— —	IVUS (coronary arteries)	4.2% reduction in atheroma volume in the combined (high- and low-dose) treatment group. No further regression with the higher dose
Taylor et al. 2004 (ARBITER 2)[68]	Once-daily extended-release niacin Placebo (Background statin therapy in both groups)	+21 NS	Change in CIMT	No significant difference in CIMT progression between the niacin and placebo groups at 1 year
Nissen et al. 2006 (ASTEROID)[69]	Rosuvastatin	+15	IVUS (coronary arteries)	6.8% median reduction in total atheroma volume at 24 months
Kastelein et al. 2007 (RADIANCE 1)[70]	Atorvastatin Atorvastatin + Torcetrapib	+2.5 +54	Change in maximum CIMT	No significant difference in annualized change in maximum CIMT between the 2 groups
Bots et al. 2007 (RADIANCE 2)[71]	Atorvastatin Atorvastatin + Torcetrapib	−2 +63	Change in maximum CIMT	Despite substantially raising HDL cholesterol, torcetrapib did not effect the yearly rate of change in maximum CIMT
Nissen et al. 2007 (ILLUSTRATE)[72]	Atorvastatin Atorvastatin + Torcetrapib	−3 +57	IVUS (coronary arteries)	No difference, at 24 months, in the change in atheroma volume in the most diseased vessel segment, between the 2 groups
Tardif et al. 2007 (ERASE)[73]	IV reconstituted HDL (CSL-111) (40 mg/kg or 80 mg/kg) Placebo	— —	IVUS (coronary arteries) QCA	Significant decrease in atheroma volume at 4 weeks in the CSL-111 group when compared to baseline, but not compared to placebo. Significant improvements in plaque characterization index and coronary stenosis score in the CSL-111 versus placebo
Barter et al. 2007 (ILLUMINATE)[58]	Atorvastatin Atorvastatin + Torcetrapib	+2 +72	First major cardiovascular event (death from CHD, nonfatal MI, stroke, hospitalization for unstable angina)	At 12 months, the increase in HDL cholesterol in the group receiving torcetrapib was associated with an increased risk of cardiovascular events and death from any cause
Shaw et al. 2008[74]	IV reconstituted (r) HDL Placebo	+18 −8	Atherectomy to excise plaque from superficial femoral artery 5–7 days after infusion Plaque analysis: lipid content, $CD68^+$ macrophage size, adhesion molecule expression	Reduced lipid content, macrophage size, and adhesion molecule (VCAM-1) expression in plaques from patients receiving rHDL, compared to the placebo group
Lee et al. 2009[75]	Modified-release niacin Placebo (all received statin therapy)	+23 +3	Change in carotid wall area, quantified by MRI	Significant reduction in carotid wall area at 12 months in the niacin group, compared with placebo
Boden et al. 2011 (AIM-HIGH)[59]	Extended-release niacin Placebo (all received simvastatin and ezetimibe, if required for LDL reduction)	+28 +12	Composite death from CHD, nonfatal MI, ischemic stroke, hospitalization for ACS, symptom-driven coronary or cerebral revascularization	No incremental clinical benefit from the addition of niacin to the background statin therapy during the 36-month follow-up period, despite significant increases in HDL cholesterol levels
Schwartz et al. 2012 (dal-OUTCOMES)[60]	Dalcetrapib Placebo (all also received best available care)	+31 — +40 +4 — +11	Composite death from CHD, nonfatal MI, ischemic stroke, unstable angina, cardiac arrest with resuscitation	Despite increasing HDL cholesterol levels, dalcetrapib did not reduce the risk of cardiovascular events over the 31-month follow-up period
Landray et al. 2014 (HPS2-THRIVE)[61]	Extended-release niacin — Laropiprant Placebo	+14 —	First major vascular event (nonfatal MI, death from coronary causes, stroke, arterial revascularization)	Despite the increase in HDL-C levels in patients receiving niacin — laropiprant, there was no significant difference in the incidence of major vascular events between the 2 groups

Table 1 — (*continued*)

Tardif et al. 2014 (CHI-SQUARE)[62]	CER-001 infusion (recombinant human apoA-I-containing HDL mimetic)	—	IVUS (coronary arteries) QCA Major cardiovascular events	CER-011 infusions did not reduce coronary atheroma burden on IVUS or QCA, compared to placebo
	Placebo	—		No statistically significant difference in the number of major adverse cardiovascular events between the 2 groups

ACS, acute coronary syndrome; **apoA-I**, apolipoprotein A-I; **CHD**, coronary heart disease; **CIMT**, carotid intima-media thickness; **HDL**, high-density lipoprotein; **IV**, intravenous; **IVUS**, intravascular ultrasound; **MI**, myocardial infarction; **NS**, not significant; **QCA**, quantitative coronary angiography.

shown that HDL enhances expression of arginase-1 and the C-lectin receptor, classical markers of the tissue-remodeling and anti-inflammatory M2 macrophage phenotype [88]. Moreover, in a recent study by De Nardo and colleagues, the transcriptional regulator ATF3 was identified as a HDL-inducible target in macrophages, which downregulates expression of Toll-like receptor (TLR)-induced pro-inflammatory cytokines [89]. This novel and apparently cholesterol-independent function of HDL again highlights the need to distinguish between HDL function and plasma levels of HDL-C [90].

Non-surgical models of atherosclerosis regression have also highlighted apoA1, the most abundant lipoprotein in HDL particles, as a translatable approach for CAD resolution. In 1999, Tangirala and colleagues used an adenovirus approach to express human apoA1 (hA1) in the liver of LDL receptor deficient ($Ldlr^{-/-}$) mice, increasing plasma apoA1 levels approximately 3-fold compared to mice injected with control virus [91]. This resulted in 70% reduction in lesion area, as measured by aortic en face analysis, and 46% reduction in aortic root plaque cross-sectional area. However, hA1 expression in $Ldlr^{-/-}$ mice fed an atherogenic diet for a longer period was insufficient to induce reductions in lesion size [92]. Meanwhile, a single, high-dose intravenous injection of apoA-I(Milano) diminished plaque macrophage content by 36% and almost halved plaque lipid content within 48 h in $apoE^{-/-}$ mice [93]. Excluding species-specific effects, atheromata regression has also been achieved through apoA-I(Milano) infusion in a rabbit model [94], and most notably, in people (Nissen 2003 study in Table 1).

Further characterizing the beneficial effects of hA1 administration, we showed a significant decrease in lipid content, macrophage number, and an increase in collagen content in advanced aortic root plaques in $apoE^{-/-}$ mice within one week of their receiving injections of functional human apoA1 [95]. Recapitulating findings from the transplantation model, the decrease in lesional macrophage number was accompanied by induction of CCR7 and increased expression of anti-inflammatory M2 macrophage markers. Intriguingly, *ex vivo* myeloperoxidase-mediated oxidation of hA1 was shown to abolish these beneficial effects, through impairment of RCT in vivo [95]. These data once again emphasize the importance of HDL functionality in the regression of atherosclerosis.

5. CONCLUSIONS

From this brief overview, several HDL functional attributes emerge as important to its amelioration of metabolic disease and promotion of CAD regression. Due to the profound beneficial effect of exercise on CVD health, it is of particular interest to understand the quantitative contribution of the associated increases of HDL functions and the responsible underlying mechanisms in detail for the development of novel synergistic treatment strategies with the goal to reduce CAD progression and to induce plaque regression. Ultimately, HDL-induced changes need to be tested in large-scale placebo-controlled randomized controlled trials.

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CONFLICT OF INTEREST

None declared.

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