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Future Therapeutic Directions in Reverse Cholesterol Transport

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Abstract

Despite a robust inverse association between high-density lipoprotein (HDL) cholesterol levels and atherosclerotic cardiovascular disease, the development of new therapies based on pharmacologic enhancement of HDL metabolism has proven challenging. Emerging evidence suggests that static measurement of HDL levels has inherent limitations as a surrogate for overall HDL functionality, particularly with regard to the rate of flux through the macrophage reverse cholesterol transport (RCT) pathway. Recent research has provided important insight into the molecular underpinnings of RCT, the process by which excess cellular cholesterol is effluxed from peripheral tissues and returned to the liver for ultimate intestinal excretion. This review discusses the critical importance and current strategies for quantifying RCT flux. It also highlights therapeutic strategies for augmenting macrophage RCT via three conceptual approaches: 1) improved efflux of cellular cholesterol via targeting the macrophage; 2) enhanced cholesterol efflux acceptor functionality of circulating HDL; and 3) increased hepatic uptake and biliary/intestinal excretion.

Keywords

Reverse cholesterol transport; High-density lipoprotein; Lipid metabolism

Introduction

High-density lipoprotein cholesterol (HDL-C) levels have been recognized as a strong inverse predictor of cardiovascular risk since the 1970s [1]. Population-based studies suggest that an increment of even 1 mg/dL in HDL-C is associated with a 3–4% reduction in mortality from cardiovascular disease [2]. However, despite intense efforts in both the private sector and academia, the development of new therapies based on pharmacologic enhancement of HDL metabolism has proven challenging. A number of interventions are linked to increases in HDL-C levels, but robust associations with improved clinical outcomes remain sparse [3, 4•]. Recent research findings that provide novel insight into lipid

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metabolism, particularly with regard to reverse cholesterol transport (RCT), are likely to prove critical to ongoing drug development efforts.

The mechanistic and causal basis of HDL's apparent atheroprotective effects has not been definitively proven and is of central importance to the field, particularly with regard to drug development. Although likely pleiotropic in nature, HDL's promotion of RCT is thought to play a key role in the attenuation, and potential regression, of atherosclerosis (Fig. 1) [5]. RCT involves the return of excess cholesterol from peripheral tissues to the liver and ultimately biliary excretion into the intestines [6]. Although macrophage RCT represents only a small fraction of total flux through the pathway, it may warrant particular focus given the importance of cholesterol-laden macrophage foam cells in the development of atheromatous lesions [7, 8].

Static, mass-based measurement of HDL-C has inherent limitations as a metric of overall functionality, particularly with regard to flux through the RCT pathway. Increased HDL levels should thus be viewed as neither necessary nor sufficient as a surrogate for therapeutic efficacy. Indeed, a validated murine assay that quantifies macrophage RCT has proven a better predictor of atherosclerosis measures than HDL concentration [9]. Therefore, careful attention to the subtleties of HDL particle heterogeneity and the RCT pathway offers a way forward in the development of HDL-centric therapeutics.

This article reviews methodologies of quantifying RCT and strategies for augmenting macrophage RCT via three conceptual approaches: 1) increased efflux of cholesterol from macrophage cells; 2) enhanced acceptor functionality of circulating HDL; and 3) improved hepatic uptake and biliary/intestinal excretion (Table 1).

Metrics of Reverse Cholesterol Transport in Mice and Men

Quantification of dynamic flux through the macrophage RCT pathway, although methodologically challenging, would be immensely valuable to the assessment of HDL metabolism in the setting of pharmacotherapy. One approach has been to study efflux of cellular cholesterol ex vivo [10]. These assays allow investigation of both cell-based and acceptor-based interventions. For example, stimulation of liver X receptor (LXR) activity has been shown to promote macrophage cholesterol efflux ex vivo, primarily via the ATP-binding cassette A1 (ABCA1) pathway [11]. Infusion of reconstituted HDL particles, by contrast, was shown to improve the acceptor function (efflux capacity) of plasma from human patients with type 2 diabetes [12•]. Major current limitations of ex vivo cholesterol efflux assays include limited data linking efflux capacity to coronary disease, methodologic variability, and an inability to assess the terminal components of the RCT pathway. However, if validated and standardized, cellular efflux assays may prove highly useful in screening novel therapeutics.

A robust quantitative in vivo assay has been developed in mice that permits detailed assessment of the tissue-specific macrophage RCT pathway. In brief, the technique involves injecting radiolabeled and cholesterol-loaded macrophage "foam cells" into the intraperitoneal space [13]. The radioactive tracer can then be followed as it moves from the implanted cells into the bloodstream, is taken up by the liver, and is excreted into the feces. This assay has provided critical insight into experimental situations where perturbations of HDL levels have seemingly paradoxical effects on atherosclerosis. For example, overexpression of hepatic scavenger receptor class B type 1 (SR-B1) is associated with markedly decreased HDL levels but decreased atherosclerotic burden [14, 15], an effect likely mediated by enhanced hepatic uptake of HDL and increased RCT flux [16]. Recent efforts have demonstrated effects of several therapeutic interventions on murine macrophage RCT, providing important insight in to their potential utility.

However, species-specific differences in lipid metabolism preclude direct extension of animal findings to efficacy in humans. A direct human analog of the macrophage RCT pathway would thus be highly useful to ongoing translational research efforts. Measurement of fecal cholesterol excretion, the terminal event of RCT, has been proposed as a potential proxy for total centripetal flux [17]. One small human study demonstrated increased fecal steroid excretion after infusion of apolipoprotein (apo) A-I precursor liposome complexes [18]. Another strategy applies principles of steady-state isotope dilution in tracking efflux of cholesterol from tissues into plasma, as detailed in a recent review [19•]. These metrics, although conceptually intriguing, remain limited in overall sensitivity and particularly in their ability to assess macrophage-specific RCT. Ongoing research efforts may yield robust assays, ideally macrophage-specific, of human RCT in vivo.

Increasing Macrophage Cholesterol Efflux

Cholesterol efflux, the first step of the RCT pathway, plays a critical role in maintaining intracellular cholesterol homeostasis. Peripheral tissues gain cholesterol via synthetic pathways and direct uptake of circulating lipoproteins but are largely unable to catabolize it. The toxic buildup of cholesterol in arterial foam cells is thought to play a key role in the initiation and progression of atherosclerotic plaque development, and potentially plaque rupture as well. Once assumed to be a largely passive process, recent research has demonstrated that cholesterol efflux occurs largely via ABCA1 and ATP-binding cassette G1 (ABCG1). The two transporters differ in their acceptor specificities, with ABCA1 responsible for efflux to lipid-poor apoA-I and ABCG1 promoting efflux to mature HDL particles.

Animal studies have confirmed that both ABCA1 and ABCG1 facilitate macrophage RCT [20]. Mice deficient in both of these proteins exhibit dramatic increases in foam cell accumulation and atherosclerosis, reinforcing the concept that the macrophage cholesterol efflux pathway is antiatherogenic in vivo [21••, 22]. Similarly, loss of function ABCA1 mutations in humans, which underlies Tangier disease, is associated with inability to lipidate apoA-I particles and cholesterol build-up in peripheral tissues [23–25]. Among patients with rare ABCA1 mutations, an inverse relationship was noted between ability to efflux cellular cholesterol and carotid intimal-media thickness [26]. These findings have fostered interest in upregulating cholesterol efflux activity as a means of increasing RCT.

LXR Agonism

Liver X receptors (LXR), including LXRα and LXRβ, serve as the major regulators of cellular ABCA1 and ABCG1 expression via binding with the heterodimer retinoid X receptor [27]. Accordingly, pharmacologic LXR agonism has been shown to promote macrophage RCT and decrease atherosclerosis in mouse models [28–30]. However, development of first-generation LXR ligands has been hampered by their induction of hepatic steatosis. Stimulation of hepatic LXRα induces increased fatty acid biosynthesis via increased expression of sterol response element binding protein-1c (SREBP-1c), resulting in fat accumulation [31]. Furthermore, the drugs have been shown to increase low-density lipoprotein cholesterol (LDL-C) levels in some species [32].

Recent studies have highlighted the primary role of the macrophage in mediating LXR agonists' antiatherogenic activity [33, 34]. Because LXRα is the predominant subtype in the liver, selective agonism of LXRβ may help overcome the undesirable hepatic effects seen with nonselective stimulation. Indeed, LXRβ-selective agonists have been developed that retain the ability to promote macrophage cholesterol efflux [35]. As such, LXR agonism remains a highly plausible and conceptually attractive therapeutic target, particularly if it can be accomplished with selective targeting of the macrophage or intestine.

A proof-of-concept study was performed in humans using LXR-623 (Wyeth, Madison, NJ), a nonselective small molecule agonist of LXR [36]. The compound was associated with increased expression of ABCA1 and ABCG1 in blood. However, adverse central nervous system-related effects were noted in more than half of patients, leading to termination of the study. Although these were likely off-target effects of the specific compound, additional study will be needed to confirm that these events were not due to modulation of central nervous system LXR activity.

Improving HDL Acceptor Number and Function

HDL particles are highly heterogenous with regard to size, lipid composition, and protein cargo. Multiple approaches seek to enhance the efflux capacity of circulating plasma in vivo via oral or infusion therapies.

Increased apoA-I Expression

A large body of evidence suggests that apoA-I, which makes up 70% of HDL protein, is antiatherogenic. Increased apoA-I expression increases plasma HDL levels, promotes RCT, and leads to decreased atherosclerosis in several animal models [13]. One study documented atherosclerotic plaque regression, an elusive “holy grail” of modern cardiology, with hepatic apoA-I overexpression [37]. Small molecules that promote hepatic apoA-I expression in humans may recapitulate these strikingly beneficial effects. One such compound, RVX-208 (Resverlogix, Calgary, AB, Canada), recently entered early-phase clinical trials [38]. Serum from human patients treated with RVX-208 exhibited increased cholesterol efflux capacity despite an only modest increase in HDL-C levels [39]. It will be of interest to follow the progress of this and other approaches to upregulation of apoA-I expression that may develop.

A second mechanism of increasing apoA-I production is via peroxisome proliferator-activated receptor α (PPARα) agonism. Although PPARα stimulation has multiple effects on lipid metabolism, including increasing ABCA1 expression, most promising is its stimulation of apoA-I production [40]. Although currently prescribed fibrates are week PPARα agonists, several agents with substantially increased potency and selectivity have been developed. LY518764 (Eli Lilly and Company, Indianapolis, IN) has been shown to increase apoA-I production rate by 31% in patients with metabolic syndrome [41]. Intriguingly, these patients exhibited an equivalent increase in apoA-I catabolic rate, resulting in no change in steady state apoA-I or HDL-C levels. A second study noted potential safety concerns and modest increases in HDL and apoA-I levels [42]. However, the increased apoA-I turnover may in fact promote macrophage RCT, as has been demonstrated in mice treated with a different potent PPARα agonist [43]. Although PPARα is currently not considered an optimal target for new therapies, available data suggest that PPARα agonists more potent than fibrates have the potential to substantially promote macrophage RCT through both hepatic apoA-I and macrophage ABCA1 upregulation.

ApoA-I/Reconstituted HDL Infusions

Given the substantial challenges of increasing endogenous apoA-I expression, an alternate strategy has been to directly infuse apoA-I or reconstituted HDL (rHDL) into the circulation. These infusions were associated with attenuation, and even regression, of atherosclerosis in rabbit models [44, 45]. Subsequent preclinical studies in humans have noted transient increases in apoA-I levels, total and lipid-poor pre-β HDL concentrations, ex vivo cholesterol efflux capacity, and fecal sterol excretion [12•, 18, 46]. A small human trial studied the effects of rHDL/apoA-I disks on coronary atherosclerosis as visualized using angiography and intravascular ultrasound. The intervention was associated with reduction in

atheroma volume, consistent with plaque regression, when compared with baseline [47]. However, although intriguing, the study was underpowered to detect differences from the placebo group and thus awaits replication in a larger cohort.

Beyond infusions of wild-type apoA-I, several groups have explored the use of apoA-I engineered to harbor the rare, naturally occurring Milano variant. The association of this point mutation with low HDL and apoA-I levels but no dramatic increase in vascular disease has led to speculation that apoA-I Milano may have improved atheroprotective properties [48]. However, this remains controversial, and mice expressing wild-type and apoA-I Milano exhibit no difference in macrophage RCT in vivo [49]. Nevertheless, weekly infusions of apoA-I Milano/phosphatidylcholine disks were associated with reduction from baseline coronary atheroma volume noted on intravascular ultrasound [50]. Although the optimal formulation remains unclear, results in both humans and animal models suggest that infusions of apoA-I/rHDL may increase RCT and improve atherosclerotic burden. A major limitation is the requirement for intravenous infusion, likely limiting its potential use to a peri-acute coronary syndrome timeframe. Similar infusional approaches are currently in clinical use for antithrombotic and antiplatelet agents, including heparin and glycoprotein IIb/IIIa inhibitors, indicating the feasibility of this approach if proven to be effective.

ApoA-I Mimetic Peptides

Substantial research has focused on the development of a small mimetic peptide, ideally with oral administration, that can mimic the effects of infusing full-length apoA-I [51]. One such peptide, D-4F, was engineered to contain only 18 amino acids but retain its lipid-binding properties. Furthermore, the use of only D-amino acids allows the molecule to escape gastrointestinal peptidase breakdown. Oral D-4F was associated with enhanced RCT and decreased atherosclerosis in mouse models [52, 53]. Early-phase clinical investigation of single-dose D-4F demonstrated proof of concept with regard to safety, modest oral bioavailability, and a dose-dependent improvement in measures of isolated HDL anti-inflammatory activity [54]. The future of D-4F and other apoA-I mimetic peptides will likely depend on improving bioavailability and larger-scale evidence of enhanced HDL functionality.

Cholesteryl Ester Transfer Protein Inhibition

Patients with genetic cholesteryl ester transfer protein (CETP) deficiency have markedly increased HDL-C levels, a discovery that stimulated efforts to achieve similar effects with pharmacologic therapy [55]. CETP inhibition therefore represents a strategy of increasing acceptor concentrations for macrophage cholesterol efflux. This concept was reinforced when both genetic deficiency and pharmacologic CETP inhibition were associated with increased cholesterol efflux capacity, primarily via the ABCG1 transporter [56, 57]. However, a major limitation of in vitro efflux assays is the inability to assess terminal components of the RCT pathway. CETP has a well-established role in mediating transfer of cholesteryl esters from HDL to apoB-containing lipoproteins (LDL or very low density lipoprotein) in exchange for triglycerides. Human kinetic studies suggest that this transfer, with subsequent uptake of LDL particles by hepatic LDL receptors, represents the primary pathway for RCT in humans [58]. The impact of CETP on RCT, and thus potentially cardiovascular disease, may therefore vary according to hepatic LDL clearance efficiency. Animal studies have supported this notion, with CETP facilitating RCT in wild-type mice but decreasing RCT flux in LDL-receptor deficient mice [59].

Despite being an important determinant of plasma HDL levels, the influence of CETP on RCT and cardiovascular disease remains highly controversial [60]. No increase in fecal sterol excretion was noted in a human study of pharmacologic CETP inhibition, although the

limitations of the methodology preclude definitive conclusions regarding RCT [61]. The field was dealt a major blow when a phase 3 clinical trial of one potent CETP inhibitor, torcetrapib (Pfizer, New York, NY), was terminated after an increase in mortality and cardiovascular events was noted in the treatment group. This occurred despite the predicted effects on patients' lipid profiles, with a 72% increase in HDL-C and 25% reduction in LDL-C [62•]. Unfortunately, torcetrapib was linked to off-target elevations in aldosterone levels and blood pressure, potentially explaining a portion of the adverse effects. Two other CETP inhibitors currently in development, anacetrapib (Merck, Whitehouse Station, NJ) and dalcetrapib (Roche Pharmaceuticals, Basel, Switzerland), show no effects on blood pressure and are likely to provide much-needed insight into the mechanism's utility [63, 64].

Endothelial Lipase Inhibition

Endothelial lipase (EL) plays a major role in modulating both HDL particle composition and catabolism [65]. Like other members of the lipoprotein lipase family, EL is a secreted protein that binds to the endothelial surface. It primarily hydrolyzes phospholipids on circulating HDL particles, resulting in accelerated apoA-I catabolism [66]. Animal studies have noted substantially increased HDL-C levels and decreased atherosclerosis in EL-deficient mice [67]. Decreasing EL activity has thus been proposed as an effective way of increasing plasma levels of HDL acceptors and potentially enhancing RCT.

Human genetic studies have confirmed that variation near the EL gene is an important determinant of plasma HDL-C levels [68, 69]. Furthermore, EL mass is associated with a number of cardiovascular risk factors and increased atherosclerotic burden as assessed by coronary artery calcification [70]. These findings stimulated initial screening efforts to identify small molecule inhibitors of EL [71]. Ongoing efforts in animal models that seek to determine the effects of EL inhibition on HDL phospholipid levels, HDL catabolism, and RCT will inform whether the strategy may ultimately have utility in humans.

Improving Hepatic Uptake and Biliary/Intestinal Cholesterol Excretion

The terminal hepatic and intestinal components of macrophage RCT, although often overlooked, are likely to receive increased attention in coming years. As discussed earlier, diminished hepatic uptake in mice deficient of SRB1 was linked to markedly decreased RCT [16]. Although the physiologic importance of this pathway in humans remains unclear, the finding reinforces the need for additional study of how cholesterol effluxed to HDL particles is ultimately transported into the intestines.

Recent work suggests that cholesterol taken up by hepatocytes is secreted into the bile by the apical membrane sterol transporters ABCG5 and ABCG8. Accordingly, ABCG5/ABCG8 knockout and overexpression studies in mice have noted the predicted effects on biliary cholesterol excretion [72, 73]. Furthermore, humans with mutations in ABCG5 and ABCG8 suffer from sitosterolemia, a genetic disorder involving decreased biliary cholesterol excretion and the accumulation of dietary cholesterol [74]. An additional line of provocative research has called attention to variable rates of intestinal cholesterol absorption as determinants of dynamic RCT flux [75]. Finally, documentation of direct intestinal secretion of cholesterol, bypassing the liver entirely, offers a potentially important revision of the classic RCT model [76].

As with other components of RCT, the in vivo rodent model has facilitated investigations into the importance of terminal RCT components and the effects of various interventions. A recent report noted that acutely induced inflammation impairs macrophage RCT in mice, largely due to decreased flux from liver to feces [77]. Conversely, fish oil, a dietary supplement with an emerging role in cardioprotection [78], increased RCT by ABCG5/

ABCG8-mediated hepatic excretion and decreasing intestinal absorption [79]. Statins and fibrates have been shown to similarly increase ABCG5/ABCG8 expression, although enhanced RCT has not been documented [80, 81]. Ezetimibe, a therapeutic used clinically for its LDL-reducing effects, augments RCT by decreasing intestinal cholesterol absorption via inhibition of the Niemann-Pick C1-like 1 (NPC1L1) transporter [75]. Similar effects with regard to decreased NPC1L1 expression and increased RCT were noted after treatment with a PPARδ agonist [82]. Treatment with a PPARδ agonist was associated with modest increases in HDL levels in humans, although the underlying mechanism remains unclear [83]. Finally, LXR activation, discussed previously with a focus on enhancing cellular efflux, stimulated direct intestinal cholesterol excretion in vivo [84]. Given the adverse hepatic effects of nonselective LXR agonism, intestinal-specific activation has emerged as an additional area of interest.

Additional studies are needed to rigorously assess the molecular and genetic underpinnings of hepatic uptake and excretion, as well as the quantitative role of direct intestinal excretion as a proportion of overall macrophage RCT flux. However, the hepato-intestinal components of RCT represent a promising additional target for drug development.

Conclusions

Recent data have reinforced long-standing hypotheses suggesting that HDL's critical role in the RCT pathway may explain its atheroprotective effects. Although this causal relationship has not been definitively proven, the promotion of macrophage RCT remains a promising target for future drug development. Ongoing efforts to systematically assess the impact of therapeutics on robust assays of RCT flux will be of major importance to the field. Ultimately, we look forward to a future in which the complexities of HDL metabolism are overcome by an RCT-focused therapy that is safe and efficacious in the treatment of human disease.

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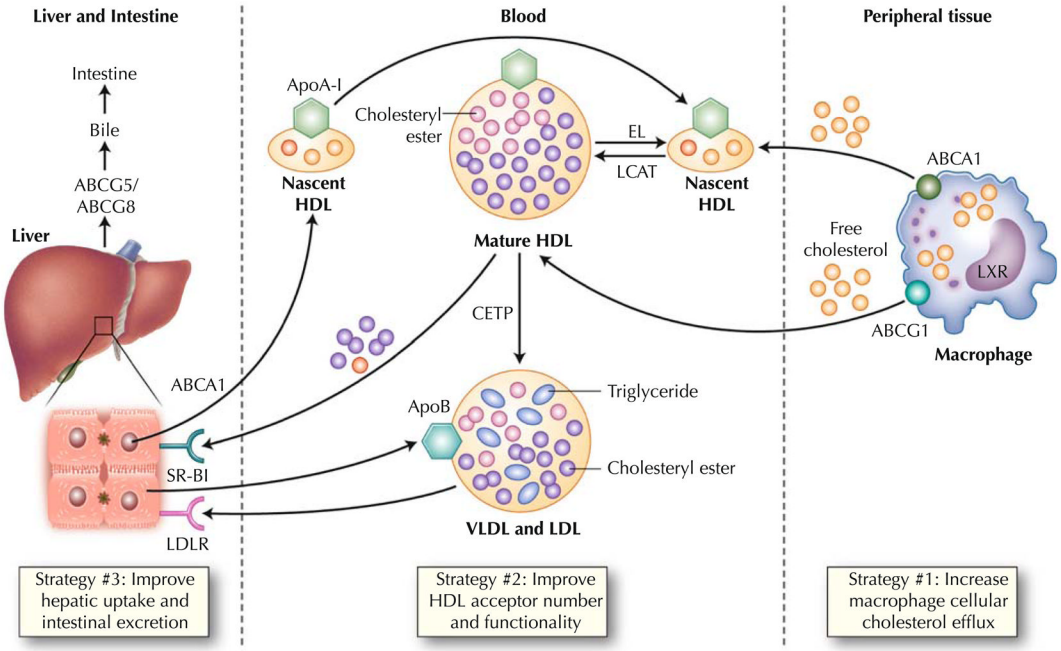

Fig. 1.

Physiology of macrophage reverse cholesterol transport. Both the liver and intestine synthesize apolipoprotein A-I (ApoA-I), which is secreted in lipid-poor form. These particles are lipidated with both phospholipids and free cholesterol via the hepatocyte ATP-binding cassette A1 (ABCA1) transporter to form nascent high-density lipoprotein (HDL). In peripheral tissues, these HDL particles obtain additional free cholesterol via the macrophage ABCA1 transporter. Lecithin cholesterol acyltransferase (LCAT) esterifies free cholesterol to cholesteryl esters, generating mature HDL. These larger HDL particles serve as additional acceptors of cholesterol efflux via the macrophage ATP-binding cassette G1 (ABCG1) pathway. Liver X receptor (LXR) regulates the expression of both ABCA1 and ABCG1 in the macrophage. HDL can be remodeled by lipases such as hepatic lipase and endothelial lipase (EL), which hydrolyze HDL triglyceride and phospholipid, respectively. Mature HDL can transport its cholesterol directly to the liver via the hepatic scavenger receptor class B type 1 (SR-B1) receptor. Alternatively, cholesteryl ester transfer protein (CETP) can mediate transfer of cholesteryl esters from HDL particles to apoB-containing lipoproteins with subsequent uptake in the liver via the low-density lipoprotein receptor (LDLR). This “indirect” pathway is thought to predominate in humans. Hepatic cholesterol is secreted into the bile via the ABCG5 and ABCG8 transporters. Some reenters the circulation via intestinal reabsorption, and the remainder is excreted into the feces. Recent evidence suggests that the intestine may be able to directly excrete cholesterol, bypassing the liver entirely. VLDL—very low density lipoprotein

Table 1

Summary of selected strategies to increase macrophage reverse cholesterol transport

Therapeutic strategy	Effect on HDL Levels	Effect on murine macrophage RCT	Effect on atherosclerosis	Rationale	Limitations	Stage of clinical development
Strategy #1: Increase macrophage cholesterol efflux						
LXR agonism	Variable	Increased	Decreased	Upregulate ABCA1- and ABCG1-mediated efflux; may also increase intestinal secretion	Induction of hepatic steatosis, particularly with nonselective agonists	Early clinical
Strategy #2: Increase HDL acceptor number and functionality						
PPARα agonism	Variable	Increased	Unknown	Stimulate apoA-I production/ turnover; also increases cellular efflux	Nonspecific effects, safety concerns in humans	Early clinical
ApoA-I/reconstituted HDL infusions	Increased	Unknown	Decreased	Promote RCT and enhance plaque stability via short-term infusion	Limited human efficacy data; need for intravenous administration	Early clinical
ApoA-I mimetic peptides	None	Increased	Decreased	Recapitulate beneficial effects of full-length apoA-1 via oral administration	Modest oral bioavailability, no human efficacy data	Early clinical
CETP inhibition	Increased	Variable	Unknown	Prevent transfer of cholesteryl esters from HDL to apoB-containing lipoproteins, increasing HDL levels	Unknown effects on RCT, failure in one large phase 3 clinical trial	Phase 3 clinical trials
Endothelial lipase inhibition	Increased	Unknown	Unknown	Decrease hydrolysis of HDL phospholipids, slowing HDL catabolism	Unknown effects on RCT, paucity of small molecule inhibitors	Preclinical
Strategy #3: Increase hepatic uptake and intestinal excretion						
Ezetemibe	None	Increased	Decreased	Limit intestinal cholesterol reabsorption	Limited data demonstrating improved clinical outcomes in humans	In clinical use
PPARδ agonism	Increased	Increased	Unknown	Limit intestinal cholesterol reabsorption, may improve clearance of postprandial triglycerides	Unknown mechanism of increasing HDL levels, minimal data in humans	Early clinical
Fish oil	Increased	Increased	Decreased	Enhance hepatic cholesterol excretion via ABCG5/ABCG8 transporters, decrease intestinal cholesterol absorption	Unclear if clinical benefit is related to increased RCT	In clinical use

ABC ATP-binding cassette, *apo* apolipoprotein, *CETP* cholesteryl ester transfer protein, *HDL* high-density lipoprotein, *LXR* liver X receptor, *PPAR* peroxisome proliferator-activated receptor, *RCT* reverse cholesterol transport