Translational and Therapeutic Approaches to the Understanding and Treatment of Dyslipidemia

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Circulating lipoproteins containing cholesterol and triglycerides are major risk factors for many cardiovascular diseases (CVD). Many therapeutic strategies targeting lipid metabolism have been successfully used to treat dyslipidemia and subsequently reduce cardiovascular risk. Most notably, statins, which effectively lower low-density lipoprotein-cholesterol (LDL-C), have been widely used and have been shown to substantially reduce cardiovascular events. More recently, a new class of LDL-lowering drugs, the proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, has been developed with highly promising results. Many other therapeutic approaches aiming to modulate lipids are also being considered. These include antisense oligonucleotides (ASOs) designed to inhibit well-characterized gene targets involved in dyslipidemia, modulators of high-density lipoprotein (HDL) function and reverse cholesterol transport (RCT) pathways, and triglyceride-lowering drugs. We herein highlight recent basic, translational, and clinical studies published in *ATVB* that further elucidates the mechanisms and therapeutic potential of these approaches.

PCSK9: A New Promising Therapeutic Target

LDL-receptor (LDLR) is a cell surface receptor that was found to be mutated in patients with familial hypercholesterolemia (FH).[1] FH is a genetic disorder typically characterized by hypercholesterolemia, ectopic cholesterol deposition, and premature CVD. Hepatic LDLR is essential for the uptake of circulating LDL-C, which cannot be effectively cleared in FH patients. Conversely, increasing LDLR levels, particularly in the liver, enhances LDL-C clearance. Recent evidence identified PCSK9, a protein secreted by the liver, as a mediator of LDLR degradation by interacting with its extracellular domain and promoting its trafficking to the lysosome, rather than allowing normal recycling to the cell surface.[2,3] PCSK9 gain-of-function mutations in humans were associated with decreased LDLR levels, and thus increased circulating LDL-C, causing autosomal dominant hypercholesterolemia.[4,5] In contrast, loss-of-function mutations in PCSK9 were associated with lower LDL-C levels and reduced CVD events.[6] Therefore, PCSK9 became a promising therapeutic target.[7] This section highlights the endogenous and exogenous modulators of PCSK9 levels, its pleiotropic effects, and the clinical implications of PCSK9 mutations and circulating levels.

Endogenous Modulation of PCSK9 Levels

There has been a growing interest in identifying endogenous regulators of PCSK9 expression. Insulin resistance, the hallmark of type 2 diabetes mellitus, is a well-established risk factor for CVD. Humans with type 2 diabetes mellitus and obesity displayed elevated PCSK9 levels.[8] In a recent article, Miao et al[9] explored the role of insulin in the regulation of PCSK9 production. In vitro experiments using rat hepatocytes suggested that insulin promoted the degradation of LDLR in a PCSK9-dependent manner. However, in vivo studies using murine models revealed that decreased levels of PCSK9 in insulin-deficient states are generally not associated with an increase in LDLR protein levels, suggesting that insulin may act independently of PCSK9 to increase LDLR protein expression. In obese subjects, intestinal insulin resistance has been demonstrated to modify lipid and lipoprotein homeostasis. Veilleux et al[10] studied insulin-sensitive and insulin-resistant obese subjects and showed that insulin-resistant subjects had reduced expression of intestinal PCSK9, possibly reflecting defects in intracellular cholesterol transport and metabolism. Taken together, these articles highlight the complexity of PCSK9 regulation and bring forth the implication of other players that are potentially interacting with insulin to control PCSK9 and LDLR levels. A better understanding of this interaction may facilitate the development of improved treatments for dyslipidemia in obese and type 2 diabetes mellitus patients with insulin resistance.

One potential mechanism bridging PCSK9 and the function of insulin in the regulation of lipoprotein metabolism suggests an important role for estrogen in regulating this pathway. Hussain et al[11] showed that G-protein–coupled estrogen receptor (GPER) activation increased LDLR and decreased PCSK9 mRNA and protein levels. In addition, a common missense GPER mutation, P16L, was associated with higher plasma LDL-C levels in women, suggesting that this variant might play an important role in lipoprotein metabolism mediated by estrogen.[11]

Like PCSK9, another negative regulator of LDLR is the inducible degrader of LDLR (IDOL, gene symbol *MYLIP*). In a study using an adenoviral vector to overexpress Idol (Ad-Idol), Sasaki et al[12] explored how IDOL and PCSK9, both LDLR degraders, affect each other. In mice and hamsters, hepatic IDOL overexpression increased PCSK9 levels, which in turn resulted in enhanced degradation of LDLR. Whether the increased PCSK9 levels were simply caused by the reduced availability of LDLR, which was directly degraded by IDOL, remains to be determined. The authors suggested that targeting IDOL, in concert with PCSK9, could become potential therapeutic targets against hypercholesterolemia and CVD in humans.

Exogenous Modulation of PCSK9

Modulation of PCSK9 was shown to be an important new tool for atherosclerosis research.[13] Two recent studies demonstrated that overexpression of a gain-of-function mutant of PCSK9 led to induction of atherosclerosis without germline genetic engineering.[14,15] With a single injection of adenoassociated virus particles for targeted gene transfer to the liver, Roche-Molina et al[15] showed that adenoassociated virus–mediated overexpression of D374Y gain-of-function mutant form of *PCSK9* (*PCSK9^DY*) resulted in chronic hypercholesterolemia and atherosclerosis in wild-type mice. This exciting development circumvented the need to breed mice into *Ldlr*-deficient mice for atherosclerosis studies, allowing the investigation of potential genetic interactions of PCSK9 modulation and its contribution to atherogenesis.

The clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated (Cas) 9 genome editing technology has become an emerging therapeutic approach. Using adenoviruses bearing *Streptococcus pyogenes* Cas9 and a guide RNA targeting a sequence in the first coding exon of the human *PCSK9* gene, Wang et al[16] showed that this targeting strategy reduced circulating levels of human PCSK9 protein by 52% in chimeric liver-humanized mice bearing human hepatocytes. In addition, the minimal off-target effects reflected the favorable safety profile of CRISPR–Cas9 therapy in targeting the *PCSK9* gene in human hepatocytes in vivo. This in vivo genome editing technology is likely to be further developed in the coming years, but the initial assessment of its efficacy and safety is encouraging.

Pleiotropic Effects of PCSK9

PCSK9 is also thought to exert pleiotropic effects, independent of its action on LDLR degradation.[17,18] A recent study suggested that PCSK9 enhanced the degradation of CD36, a major receptor involved in transport of long-chain fatty acids and involved in triglyceride storage in mouse liver and adipose tissue.[19] Using PCSK9 gain-of-function and loss-of-function mouse models, Demers et al[19] proposed a tissue-selective regulation of CD36 by PCSK9 and illustrated that deletion of PCSK9 allowed greater adipose expression of CD36 and enhanced liver lipid accumulation. This finding, however, raises concerns whether the PCSK9-dependent regulation of CD36 is species specific, as up until now, antibodies blocking PCSK9 have not been shown to affect liver function, liver lipid accumulation, or glucose metabolism in human studies.[20]

Clinical Implications of PCSK9 Mutations and Circulating Levels

An increasing number of studies have investigated the clinical implications of specific mutations and circulating levels of PCSK9. Saavedra et al[21] performed an observational cross-sectional cohort study of FH patients in a French Canadian population. In that cohort, the presence of the R46L loss-of-function missense mutation among FH patients (3% of the FH population) was associated with lower plasma concentration of LDL-C (11%) compared with noncarrier FH patients. The PCSK9 R46L mutation was also associated with protection against CVD. These results suggest that PCSK9-lowering therapies could counteract the damaging effects of the inherited LDLR mutation in FH patients.

Despite the established links between PCSK9 levels, plasma lipids and lipoprotein metabolism, a recent study has reported a lack of association between vascular function and structure and PCSK9 levels. Zhu et al[22] suggested that circulating PCSK9 level was not a suitable biomarker of atherosclerosis. Studying a cohort of 1527 healthy middle-aged men, the group examined the relationship between circulating PCSK9 levels and measures of vascular health, subclinical atherosclerosis, and adverse cardiovascular events. The results suggested that PCSK9 level was not associated with these parameters.

Evolving Targets for Lipid-Lowering Therapy by ASOs

ASOs are designed to target key genes in defined pathways, causing degradation of specific mRNA via various mechanisms.[23] The ASO therapeutic approaches open up the entire genome for specific pharmacological targeting and are being successfully used to treat many lipid-related diseases. The first fruitful use of this technology was Mipomersen, an ASO against apoB, that was approved by the Food and Drug Administration in 2013 to treat FH patients.[24] In addition to reducing very low-density lipoprotein assembly and secretion, thus reducing LDL-C levels, Mipomersen was recently shown to lower lipoprotein(a) levels in phase III clinical trials.[25] Many other lipid metabolism targeting drugs are also being tested using ASO technologies. Already well-established programs include ASOs against APOC3, a gene encoding apolipoprotein C-III,[26] currently in phase III clinical trials, as well as ASOs against APO(a), for patients with elevated lipoprotein(a) levels,[27] and angiopoietin-like 3 (ANGPTL3) ASOs to treat various dyslipidemias.

Targeting HDL and RCT

In contrast to LDL-C levels, plasma HDL-cholesterol (HDL-C) levels have historically been inversely associated with CVD risks.[28] However, the evidence that raising plasma HDL-C levels per se will reduce cardiovascular risk has been questioned. Recent failures of several randomized trials in which therapies aimed at increasing HDL-C levels, such as cholesteryl ester transferase protein (CETP) inhibitors[29,30] and niacin[31,32] resulted in no improvement in cardiovascular outcome and have added more fuel to the already heated debate. A key aspect of the HDL hypothesis that is now being considered

has been related to the ability of HDL to promote cholesterol efflux from cells, particularly macrophages, back to the liver, promoting cholesterol excretion in the bile.[33] RCT has been considered as the primary mechanism by which HDL particles protect against atherosclerosis. Cholesterol efflux to plasma, measured ex vivo, is a marker that predicts risks of coronary heart disease better than levels of HDL-C or LDL-C per se.[34] Thus, HDL function rather than HDL-C levels remains a major target for the development of new therapeutic interventions.

CETP Inhibition

CETP is a plasma glycoprotein that transfers CE from HDL to apoB-containing lipoproteins. Inhibition of CETP raises HDL-C levels by reducing the transfer of CE from HDL to atherogenic apoB-containing lipoproteins. Genetic studies have indicated that reduced CETP activity provides cardiovascular protection.[35,36] A growing body of work has provided mechanistic evidence to support the potential of CETP inhibition as a protective strategy against CVD. In a randomized, placebo-controlled, double-blind, fixed-sequence study, CETP inhibition with anacetrapib decreased fractional clearance rates of HDL apoA-I and plasma CETP, which resulted in increased circulating levels of apoA-I and CETP without influencing their production.[37] In New Zealand white rabbit, Des-fluoro-anacetrapib increased plasma HDL-C and apoA-I levels, reduced intimal thickening, and improved regeneration of functional endothelium in balloon injury of abdominal aortas.[38] Because endothelial injury is a key early event in atherosclerotic lesion development, CETP inhibition may exert atheroprotective effects through enhancing the repair of damaged endothelium.

However, pharmacological inhibition of CETP, as a means of enhancing RCT to reduce CVD, has been questioned after failures to show benefit on cardiovascular outcomes. These include torcetrapib in the Investigation of Lipid Level Management to Understand Its Impact in Atherosclerotic Events (ILLUMINATE) trial,[29] dalcetrapib in the Study of RO4607381 in Stable Coronary Heart Disease Patients With Recent Acute Coronary Syndrome (dal-OUTCOMES) trial,[30] and, just recently, evacetrapib in the Assessment of Clinical Effects of Cholesteryl Ester Transfer Protein Inhibition With Evacetrapib in Patients at a High-Risk for Vascular Outcomes (ACCELERATE) study[39] (http://www.medscape.com/view-article/852516). It has been argued that off-target adverse effects of the drug used and defects in the design elements may have masked the benefits in those trials.[40] Anacetrapib is currently being tested in the large phase III cardiovascular outcomes trial, Randomized Evaluation of the Effects of Anacetrapib Through Lipid-Modification (REVEAL) study, which will ultimately determine the effectiveness of CETP inhibition as a strategy to reduce cardiovascular events.[40] Whether a genetically distinct population of patients with CVD may benefit from CETP inhibitor treatment is also a hypothesis that is under investigation.[41]

Niacin

Niacin has been used in combination with statins to raise HDL-C levels and reduce residual cardiovascular risk.

However, the impact of niacin on the RCT pathway remains unclear. Ronsein et al[42] suggested that in subjects with a history of CVD, 1-year combination therapy of atorvastatin and niacin raised HDL-C and cholesterol efflux capacity of macrophages, but only marginally increased the concentration of HDL particles (14%). The combination therapy also failed to improve ABCA1-specific cholesterol efflux. Statin alone did not increase HDL particle concentration or macrophage cholesterol efflux capacity, even though it raised HDL-C.[42] In dyslipidemic patients on stable statin therapy, 12 weeks of extended-release niacin/laropiprant therapy reduced CETP activity, moderately improved plasma efflux capacity from macrophages, and increased the ability of HDL particles to deliver CE back to the liver during the postprandial phase.[43] These results suggest that specific subgroups of patients at high cardiovascular risk might benefit from combination therapy of statin and niacin for cardioprotection. However, as the authors pointed out, whether the subtle benefits may translate into improved clinical outcome and whether the benefits were sufficient to overcome the side effects is unclear.[43] The controversy on the benefits of niacin combinations with statins on RCT and reducing cardiovascular events in human continues, and further investigation is needed.

Recombinant HDL Infusion

Another strategy to enhance RCT is the infusion of recombinant HDL, serving as therapeutic strategies to either reduce plaque lipid volume or stabilize vulnerable plaques. Studies of recombinant HDL date back over a decade and evolved with the continuous discovery of improved formulation. CSL112, a formulation of apoA-I and phosphatidylcholine, has completed phase IIa clinical trial.[44] As the first placebo-controlled study, Gille et al[45] showed that single and multiple infusions of CSL112 in healthy subjects caused an immediate elevation of apoA-I and key biomarkers of RCT including increased cholesterol efflux capacity and cholesterol mobilization from tissues. MDCO-216 is a complex formed by palmitoyloleoyl–phosphatidylcholine and recombinant apoA-I Milano, a naturally occurring variant of apoA-I associated with reduced risk of CVD.[46] Kempen et al[46] examined the effects of MDCO-216 on HDL subfractions. In healthy volunteers and in patients with coronary artery disease, MDCO-216 infusion causes immediate dose-dependent decrease in smaller (α-3 and α-4) HDL particles and reciprocal increase in pre-β1, α-1, and α-2 HDL,[46] further confirming the effects previously observed in vitro.[47] The increase in pre-β1 and α-1 HDL both correlated independently with the increase in basal and ABCA1-mediated efflux capacities. These recombinant HDL compounds, currently in clinical trials, will provide further evidence on the hypothesis that therapies targeting HDL function may be more effective in reducing cardiovascular risk than those that simply raise HDL-C levels.

LXR Activation

Enhancing cholesterol efflux from macrophages is also thought to be feasible by driving the expression and activity of the cholesterol transporters ABCA1 and ABCG1.[48] Because both of these sterol transporters are regulated by the nuclear

receptor LXR (NR1H3),[49] LXR agonists have been considered as potential agents to drive RCT. However, broad LXR agonists also have unwanted effects, such as lipid accumulation in the liver caused by increased lipogenesis. This considerable drawback has reduced enthusiasm for targeting LXRs. However, combination therapies that can mitigate the steatotic effects of LXR agonists still hold significant promise. A recent study by Chen et al[50] showed that treating $Apoe^{-/-}$ mice with the LXR agonist T0901317, in combination with a mitogen-activated protein kinase kinase 1/2 (MEK1/2) inhibitor (U0126), can overcome the lipid accumulation caused by the LXR agonist alone in the liver. Furthermore, the study also showed a synergistic effect of the combined LXR agonist with MEK1/2 inhibitor in preventing atherosclerosis development and regressing lesions that had already been established.[50] Other approaches such as selective LXR agonists (such as LXRβ agonists) or cell-specific LXR agonists (eg, macrophage-selective LXR activation) may overcome the hepatic lipid accumulation caused by global LXR activation. In addition to regulating cholesterol efflux, LXRs have recently been shown to modulate phospholipid remodeling via induction of the newly described LXR target gene LPCAT3 (lysophosphatidyl acyltransferase 3).[51] A recent study by Varin et al[52] also showed that LXR activation in macrophages regulated the synthetic program for polyunsaturated fatty acids, which were known to have anti-inflammatory, and therefore atheroprotective effects. Thus, in addition to regulating cholesterol efflux, LXRs may target additional pathways that harbor therapeutic potential.

Effects of Dietary Supplements on HDL and RCT Pathways

The role of dietary and nutritional regulation of HDL and RCT pathways in the control of dyslipidemia has also been increasingly appreciated. Vitamin D deficiency has been associated with increased risk for coronary heart disease. Yucatan microswine fed with vitamin D–deficient (0 IU/d) high-cholesterol diet showed reduced plasma HDL-C levels and lower expression of LXRs and ABCA1/ABCG1 in carotid arteries, accompanied by increased atherosclerotic lesion size and cholesterol accumulation.[53] Supplementation of vitamin D (3000 IU/d) resulted in reduced lesion size.[53] Coenzyme Q10 (CoQ10), a fat-soluble antioxidant vitamin, is often used in conjunction with statins. In murine and human THP-1 monocyte-derived macrophages, CoQ10 enhanced cholesterol efflux by regulating an activator protein-1/miR-378/ABCG1-signaling pathway. Dietary CoQ10 supplementation promoted macrophage RCT and inhibited the progression of atherosclerosis in $Apoe^{-/-}$ mice.[54] These results linked the benefits of CoQ10 with enhanced RCT and reduced atherosclerosis through a novel miR-378 regulatory module.[55] Consumption of olive oil polyphenols has been associated with higher cholesterol content in HDL, but its effects on HDL composition per se in healthy humans are unclear. In a randomized, crossover, controlled trial with 47 healthy European male volunteers, 3-week intervention with polyphenol-rich (366 mg/kg) olive oil enhanced the cholesterol efflux capacity of HDL, increased HDL size, and promoted a greater HDL stability,[56] further

supporting the protective benefits of dietary olive oil polyphenol supplementation against CVD. However, it is important to conduct rigorous basic and clinical studies to establish the scientific validity, efficacy, and safety of combined dietary supplements for treating dyslipidemia with or without pharmacological therapies.

Conclusions

Human genetics and clinical studies strongly suggest LDL as an independent, causal, genetic risk factor for CVD. Over decades worth of research has led to the development of statins and other lipid-lowering drugs that potently reduce LDL-C and consequently decrease atherosclerosis development and CVD risk.[33] The discovery of a new class of LDL-lowering drugs, the PCSK9 inhibitors, addresses much of the unmet need for LDL reduction while also exhibiting pleiotropic effects, drawing significant research interest. Additional therapeutic approaches targeting well-characterized lipid-lowering targets, such as APOB, APOC3, and ANGPTL3, by ASOs hold great promise as novel strategies to modulate plasma lipoproteins. Whereas drugs raising HDL-C levels, such as CETP inhibitors and niacin, have yielded disappointing results in reducing clinical outcomes, further mechanistic studies of their effects on the composition/structure of HDL particles and RCT, as well as therapeutics improving HDL function, remain of interest. Taken together, the studies described in this article have highlighted novel therapeutic targets and approaches to dyslipidemia while bringing forth new perspectives on novel mechanisms of lipid metabolism pathways.

Disclosures

T.Q. de Aguiar Vallim is a consultant for Regulus Therapeutics. The other authors report no conflicts.

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