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Review

MOLECULAR METABOLISM

VLDL receptor gene therapy for reducing atherogenic lipoproteins

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ABSTRACT

Over the past 40 years, there has been considerable research into the management and treatment of atherogenic lipid disorders. Although the majority of treatments and management strategies for cardiovascular disease (CVD) center around targeting low-density lipoprotein cholesterol (LDL-C), there is mounting evidence for the residual CVD risk attributed to high triglyceride (TG) and lipoprotein(a) (Lp(a)) levels despite the presence of lowered LDL-C levels. Among the biological mechanisms for clearing TG-rich lipoproteins, the VLDL receptor (VLDLR) plays a key role in the trafficking and metabolism of lipoprotein particles in multiple tissues, but it is not ordinarily expressed in the liver. Since VLDLR is capable of binding and internalizing apoE-containing TG-rich lipoproteins as well as Lp(a), hepatic VLDLR expression has the potential for promoting clearance of these atherogenic particles from the circulation and managing the residual CVD risk not addressed by current lipid lowering therapies. This review provides an overview of VLDLR function and the potential for developing a genetic medicine based on liver-targeted VLDLR gene expression.

Keywords Lipid disorders; VLDL; VLDL receptor; Triglycerides; lipoprotein(a); Gene therapy

1. INTRODUCTION

Lipid disorders affect over 105 million people in the US alone [1]. Although many patients are asymptomatic, left untreated, chronic elevations in atherogenic lipoproteins can lead to clinical cardiovascular disease (CVD) events, namely myocardial infarction and stroke [2]. In addition, extreme elevations of plasma triglyceride (TG) levels can result in acute pancreatitis [3], and as with CVD, a potentially fatal outcome. There has been extensive research devoted to the study of this diverse class of disorders and, in particular, to improving treatments that are aimed at controlling and reducing pathologic lipoprotein levels.

Multiple strategies have been pursued for managing atherogenic lipid disorders, most notably low-density lipoprotein cholesterol (LDL-C) lowering, which exhibits the highest level of evidence for preventing atherosclerotic CVD [4–6]. For this reason, current treatment guidelines for CVD risk reduction center around the reduction of LDL-C, with the intensity of lowering dependent on the overall assessment of CVD risk [7]. There is abundant evidence for the atherogenic properties of TG-rich lipoproteins, which include very low-density lipoprotein (VLDL) and intermediate-density lipoprotein (IDL), as well as lipolytic remnants of VLDL and chylomicrons [8,9]. While genetic evidence has pointed to plasma triglyceride (TG) as an independent CVD risk factor [10], there has yet been no consensus on targeting elevated levels of these lipoproteins for CVD prevention. However, TG reduction is recommended for pancreatitis protection in individuals with severe hypertriglyceridemia (TG $>$ 500 mg/dL) [11].

2. CURRENT GAPS IN THE MANAGEMENT OF HIGH-RISK LEVELS OF TG-RICH LIPOPROTEINS AND LP(A)

2.1. TG-associated residual risk with statin treatment

There have been several studies that highlight the importance of TG lowering for meaningful CVD risk reduction with statins, the most widely used drug class for LDL-C reduction and CVD prevention. Subgroup analyses in statin clinical trials have shown that patients with high TG and low high-density lipoprotein cholesterol (HDL-C) have worse CVD outcomes than those with isolated elevation of LDL-C. For example, an analysis of the Scandinavian Simvastatin Survival Study (4 S) trial found that the CVD event rate in patients with hypercholesterolemia was highest in the subgroup with high TG and low HDL-C and that this group had a more favorable effect with simvastatin treatment than those with isolated elevated LDL-C levels [12]. Likewise, the Pravastatin or Atorvastatin Evaluation and Infection Therapy-Thrombolysis in Myocardial Infarction 22 trial (PROVE IT-TIMI 22), which assessed the impact of on-treatment TG levels on coronary heart disease (CHD) risk after an acute coronary syndrome, found that on-treatment TG $<$ 150 mg/dl was independently associated with a lower risk of recurrent CHD events [13]. These data suggest that reducing TG can be a means of treating residual risk and further reducing CVD events beyond LDL-C lowering.

2.2. CVD risk in trials of TG-lowering drugs

Clinical trials of TG-lowering with fibrate drugs, in particular fenofibrate, have had limited success in reducing CVD risk, though subgroup

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Abbreviations:

AAV	adeno-associated virus
apo(a)	apolipoprotein a
apoB	apolipoprotein B
apoB48	apolipoprotein B48
apoB100	apolipoprotein B100
apoC3	apolipoprotein C-III
apoE	apolipoprotein E
CHD	coronary heart disease
CRISPR	clustered regularly interspaced short palindromic repeats
CVD	cardiovascular disease
DHA	docosahexaenoic acid
DSBs	double-stranded breaks
EPA	eicosapentanoic acid
ER	endoplasmic reticulum
FGF21	fibroblast growth factor 21
GalNAc	N-acetyl-D-galactosamine
HDL-C	high-density lipoprotein cholesterol
IDL	intermediate-density lipoprotein
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LDL-C	low-density lipoprotein cholesterol
LNP	lipid nanoparticle
Lp(a)	lipoprotein (a)
LRP-1	low-density lipoprotein receptor-related protein 1
MHC	major histocompatibility complex
NAFLD	nonalcoholic fatty liver disease
PPARa	peroxisome proliferator-activated receptor alpha
PPARβ/δ	peroxisome proliferator-activated receptor beta delta
PCSK9	proprotein convertase subtilisin/kexin type 9
siRNA	small interfering ribonucleic acid
T2DM	type-II diabetes mellitus
TG	triglyceride
VLDL	very low-density lipoprotein
VLDLR	very low-density lipoprotein receptor

analyses have suggested CVD reducing benefit in subgroups with high TG and low HDL-C [14–16]. However, the recent PROMINENT trial of pemafibrate, a more potent fibrate drug, in patients with type 2 diabetes mellitus, mild-to-moderate hypertriglyceridemia, and low levels of HDL-C while treated with statins, found no effect on the primary CVD endpoint despite significant reductions of TG, VLDL cholesterol, remnant cholesterol, and apolipoprotein C-III [17]. There was however no reduction of plasma apoB-100, a measure representing levels of all atherogenic particles, and it was suggested in an accompanying editorial that to be effective for CVD risk reduction, a TG-lowering therapy should have a mechanism for increasing clearance of VLDL remnant particles, rather than, as appeared to be the case for pemafibrate, converting them to LDL [18]. Notably, induction of hepatic VLDLR expression by fenofibrate was found to be required for its TG-lowering effect in mouse models [19], although this study did not assess fenofibrate's effect on VLDL remnant clearance or plasma apoB levels.

Trials of the omega-3 fatty acids eicosapentanoic acid (EPA) and docosahexaenoic acid, which can reduce plasma TG levels, have also not consistently been found to reduce CVD risk [20]. While the Japan EPA Lipid Intervention Study (JELIS) found that EPA (1800 mg/day) in addition to treatment with a statin reduced CVD events in the statin-treated patients [21], the more recent STRENGTH trial of Epanova—a mixture of EPA and DHA— was stopped early because no significant reduction in a composite outcome of major adverse CVD events was seen [22]. In contrast, the REDUCE-IT trial of icosapent ethyl, an EPA-derived drug, showed overall reduction in myocardial infarction in statin-treated patients, but the effect was not found to be related to plasma lipid levels [23,24] and the interpretation of the results has been questioned due to the use of mineral oil as a control [25–28].

While treatment with an anti-sense inhibitor of ANGPTL3 (Vupanorsen) has been shown to lower TG levels [29], it is only currently approved for LDL lowering in homozygous familial hypercholesteremia [30]. Furthermore, another TG-lowering treatment currently in development is the inhibition of apoC3 synthesis with an anti-sense oligonucleotide or siRNA [31–34], but as yet there are no studies of CVD outcomes with these agents. An overall consideration with regard to assessing the cardiovascular benefit of TG-lowering drugs is that TG levels may need to be lower than those achieved in study trials. For example, it has been reported that even for individuals with low to moderate CVD risk and normal LDL-C levels, TG levels $\geq$150 mg/dl were significantly associated with the presence of arterial inflammation, a significant contributor to development of atherosclerotic CVD [35].

2.3. Reduction of elevated Lp(a)

Another important factor contributing to residual CVD risk in statin-treated patients is elevated lipoprotein (a) (Lp(a)), an atherogenic lipoprotein particle whose levels are not lowered by statin treatment, as discussed further below [36]. Modest reductions of Lp(a) can be achieved with high dose nicotinic acid [37] or PCSK9 inhibitors [38], but these effects have not been shown to normalize high-risk Lp(a) levels, and there are no studies to date that have assessed the effects of Lp(a) lowering on CVD outcomes [39,40]. There are newer treatments for Lp(a) currently in development that involve inhibiting apo(a) synthesis with antisense oligonucleotides [41] and siRNA [42], but their effects on CVD risk remain to be tested.

3. HEPATIC EXPRESSION OF THE VLDL RECEPTOR AS A MEANS OF REDUCING LEVELS OF ATHEROGENIC LIPOPROTEINS AND TREATING SEVERE HYPERTRIGLYCERIDEMIA

As reviewed above, there is a strong rationale for reducing residual CVD risk in statin-treated patients by achieving maximal reduction of atherogenic TG-rich lipoproteins and Lp(a). While currently available drugs and those in development have this potential to varying degrees, other therapeutic possibilities should be considered, including the use of a genetic medicine that would preclude the compliance issues that significantly affect the efficacy of lipid lowering drugs in clinical practice [43,44].

An attractive possibility in this regard is a targeted gene therapy for achieving hepatic expression of the VLDL receptor (VLDLR). The VLDLR has been shown to mediate binding and cellular internalization of both TG-rich lipoproteins and Lp(a). VLDLR is not ordinarily expressed in hepatocytes, where lipoprotein particles are cleared from plasma and degraded. In the sub-sections below, we review key features of the VLDLR that provide a rationale for ectopically introducing the *VLDLR* gene into the liver as a means of achieving sustained reductions of pathologic lipoprotein levels and lowering the residual CVD risk resulting from conventional lipid lowering drug therapies.

3.1. Structure of VLDLR

VLDLR is a multifunctional receptor that shares structural homology with members of the LDL receptor (LDLR) gene family [45]. VLDLR has five protein domains: extracellular N-terminal ligand-binding domain with eight cysteine-rich repeats, epidermal growth factor domain, O-linked glycosylation sugar domain, single transmembrane domain, and a cytoplasmic domain with the NPxY motif for signal transduction. Isolation and characterization of cDNAs encoding human *VLDLR* show two forms of the receptor: one full length that resembles the low-density lipoprotein receptor (LDLR), with the exception that LDLR has five cysteine-rich repeats, and a variant form that lacks the O-linked sugar domain [46]. In addition, alternative splicing of *VLDLR* generates multiple transcript variants encoding distinct isoforms, though their protein expression and precise functions have not been established [47].

3.2. Physiological functions of VLDLR

VLDLR is ubiquitously expressed in the heart, skeletal muscle, adipose tissue, endothelium, and brain, as well as in macrophages [45,48]. While not natively present in the liver, hepatic expression can be induced by specific conditions including endoplasmic reticulum stress and treatment with fenofibrate, a PPARa agonist. VLDLR expression is insulin dependent [49] and unlike LDLR, VLDLR expression is not regulated by cellular cholesterol content [50].

VLDLR plays a variety of roles in different tissues. A key function of greatest relevance to this review is its role in lipoprotein uptake, by which it promotes fatty acid β-oxidation in heart and muscle and storage of TGs in adipose tissue. VLDLR also has a role in cellular signaling, notably in the Reelin pathway, which is responsible for the migration of neurons to their proper locations during brain development [51].

While apoE is a ligand for both VLDLR and LDLR binding of lipoproteins, VLDLR differs from LDLR in that it does not bind apoB, the major structural protein of VLDL and LDL particles [52]. Hence it promotes binding and endocytosis of apoE-containing TG-rich lipoproteins. VLDLR up-regulates lipoprotein lipase (LPL)-mediated TG hydrolysis along with the direct uptake of TG-rich lipoproteins in endothelial cell [53]. TG-rich lipoprotein uptake is further increased by the addition of apoE and inactivated LPL [54,55].

3.3. Atherogenic lipoproteins recognized by VLDLR

3.3.1. VLDL

VLDLs are primarily responsible for the transport of endogenously synthesized TG and cholesterol from the liver into the bloodstream and to other areas of the body. The main structural features of VLDL consist of a hydrophobic lipid core (TG and cholesteryl ester) coated by a hydrophilic monolayer composed of phospholipids, free cholesterol, and multiple apolipoproteins, with apoB100 as the primary structural component [56]. In contrast to the accepted model that VLDL is a spherical emulsion-like particle, cryo-electron microscopy-derived 3D structural reconstructions of VLDL reveal a polyhedral shape [57]. This finding suggests that the flat polyhedral surfaces contribute to its binding affinity for VLDLR.

Following the secretion of VLDL into the circulation, TG is hydrolyzed by LPL in peripheral tissues [58], resulting in uptake of free fatty acids and the formation of apoE-enriched remnant lipoproteins. The hepatic uptake of these apoE-enriched remnant lipoproteins is mediated by the binding of apoE to three types of receptors, LDLR, LRP-1, and syndecan, and inhibited by apoC3 [59]. Further intravascular metabolism of these remnants leads to the formation of LDL. The elevated levels of VLDL and VLDL remnant lipoproteins, like LDL, result in an increased risk for cardiovascular disease [60].

3.3.2. Chylomicrons

Chylomicrons are responsible for the transport of exogenous (dietary) TG and cholesterol from the intestines into the circulation [61]. They are larger than VLDL, with a higher TG content, but structurally resemble VLDL except that the primary protein component in chylomicrons in ApoB48 rather than ApoB100. Chylomicron metabolism is similar to that of VLDL, except that the remnants are cleared rapidly by the liver without the intermediate formation of LDL. Importantly, elevated levels of chylomicron remnants, like VLDL remnants, result in an increased risk for atherosclerotic cardiovascular disease. On the other hand, extreme elevations of chylomicron levels as in patients with LPL deficiency and other genetic traits, result in severe hypertriglyceridemia with the concomitant risk for acute pancreatitis, a potentially fatal condition [62–64].

3.3.3. Lp(a)

Lp(a) is an atherogenic particle that consists of an LDL particle covalently attached to apo(a), a protein with high homology to plasminogen by virtue of multiple repeats resembling plasminogen's kringle domain [65,66]. Lp(a) is a major plasma transporter of oxidized phospholipids that, together with its other compositional features, plays a key role in its atherogenesis, including inflammation and thrombosis [67–69]. Allelic variations within the apo(a) gene locus strongly determine plasma levels of Lp(a) [70]. Lp(a) genetic heterogeneity is also manifested by the wide variation in the number of its plasminogen kringle-like repeats. High levels of Lp(a) are associated with increased risk of CHD and stroke, as well as calcific aortic stenosis [71]. Population studies support that Lp(a) levels in the upper tertile are associated with significantly increased CVD risk [72].

Hepatic synthesis and secretion are major determinants of plasma Lp(a) levels, since Lp(a) fractional plasma clearance is low. This is likely related to its relatively low affinity for LDLR, which accounts for the failure of statin therapy to significantly lower Lp(a) levels [73]. VLDLR has a high affinity for Lp(a), and is capable of mediating its cellular uptake, by a mechanism other than the binding to apoE [74]. Thus, the endocytosis of Lp(a) by macrophage-laden VLDLR promotes the formation of lipid-enriched foam cells [74,75]. This mechanism along with VLDLR-mediated uptake of TG-rich particles, contributes to the atherogenic effect of macrophage VLDLR expression. To date, however, the potential for specifically expressing VLDLR in the liver for lowering plasma levels of Lp(a) by promoting its clearance has not been examined.

4. VLDLR AS A THERAPEUTIC TARGET FOR LIPID DISORDERS

Figure 1 illustrates the potential for genetically mediated hepatic VLDLR expression to reduce CVD risk by promoting plasma clearance of atherogenic VLDL remnant and Lp(a) particles.

A number of studies in mouse models have shown reductions in plasma lipid levels and atherosclerosis with liver-directed VLDLR gene therapy via adeno-associated virus (AAV) or helper-dependent adenoviral vector delivery [76–78], though as described below, there have been limitations of this approach in terms of efficacy and safety, and thus, more recently, the use of other gene delivery systems has been explored [79]. However, despite its promise, VLDLR has not been further pursued as a therapeutic target, and the reason appears to be two-fold.

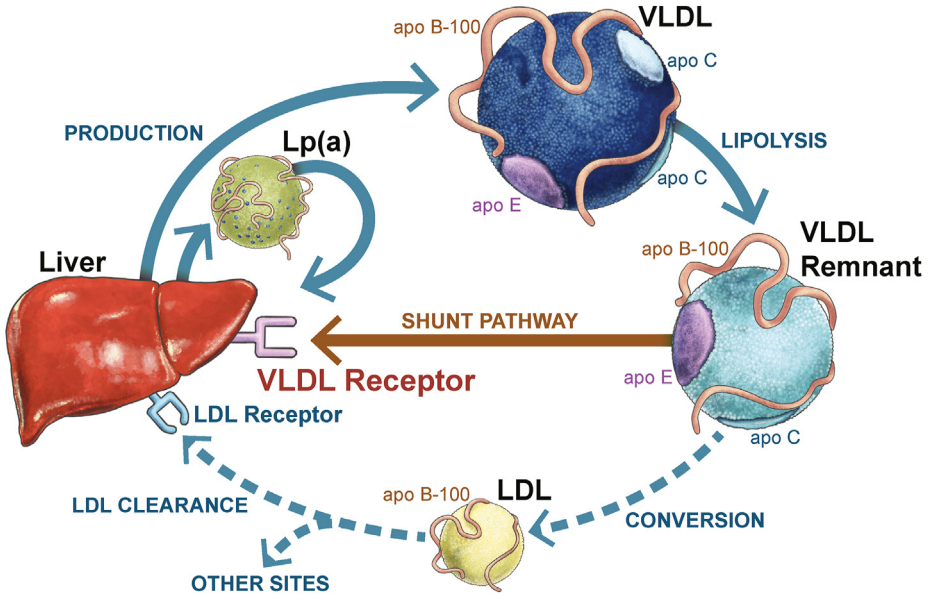

Figure 1: Role of hepatic VLDL receptor expression in the clearance of atherogenic particles. Overview of the pathways by which hepatic VLDL receptor expression may increase plasma clearance of atherogenic VLDL remnant and Lp(a) particles. The VLDL receptor can promote hepatic uptake of VLDL remnants via binding to apoE, and this shunt pathway can limit further remnant processing, resulting in reduced LDL production. Lp(a) has also been shown to be a ligand for the VLDL receptor, and thus hepatic VLDL receptor expression would be expected to increase plasma clearance and reduce concentrations of Lp(a) particles. VLDL: very low-density lipoprotein. LDL: low-density lipoprotein. Lp(a): lipoprotein a. apoC: apolipoprotein C. apoB-100: apolipoprotein B-100. apoE: apolipoprotein E.

First, as reviewed by Rader, there are pitfalls to AAV-mediated gene therapy including a lack of long term expression and safe viral delivery vector systems [80]. Other challenges for use of such a system include: 1) the immunogenicity of the AAV capsid that limits the amount of viral particles dosed; 2) an inability to repeat and titrate treatment to achieve optimal levels of lipids and lipoproteins; and 3) waning durability and clinical efficacy since the AAV transduced gene exists in hepatocytes as an episome, and gene expression declines as the hepatocytes undergo division [81].

Second, previous studies reported that the overexpression of ectopic *VLDLR* in hepatocytes may be pro-inflammatory; thus increasing ER stress and the potential for hepatic steatosis [82]. Additionally, increased VLDLR expression in macrophages was found to promote adipose tissue inflammation and impaired glucose tolerance in obese mice [83]. In another study, it has been reported that VLDLR expression was a factor in adipose tissue inflammation, where this inflammation was only reduced in obese VLDLR-deficient mice fed a high-fat diet [84]. VLDLR has also been found to modulate fibrin-dependent leukocyte transmigration and thereby promote inflammation [85,86].

However, several studies have identified mechanisms for reducing the risk of VLDLR-attributed inflammation and hepatic steatosis. For example, Fuchs et al. discovered that a lack of adipose triglyceride lipase protected mice from endoplasmic reticulum (ER) and hepatic stress in the presence of high TG levels [87]. Zarei et al. observed that FGF21 may protect against hepatic steatosis by attenuating ER stress-induced VLDLR upregulation [88]. Recent results with VLDLR knockout mice have also indicated that VLDLR is not a major factor in fatty liver formation, particularly during protein restriction [89]. Thus, although there is a potential risk of hepatic steatosis resulting from excessive TG uptake in hypertriglyceridemic states [90], it is possible that compensatory mechanisms, such as increased fatty acid β-oxidation or reduced hepatic lipogenesis, may act to mitigate hepatic TG accumulation.

5. NOVEL GENETIC MEDICINE APPROACHES TOWARD TARGETING VLDLR

Despite the previous hurdles discussed above, gene therapy targeting hepatic *VLDLR* expression remains a promising modality to effectively treat and manage atherogenic lipid disorders. Thus, upcoming genetic medicine approaches for achieving sustained hepatic expression of *VLDLR* may benefit from incorporating the following features:

5.1. Integrative technology to improve durability of treatment effect

For atherogenic lipid disorders, the lack of durable treatment effects observed with AAV gene therapy in dividing hepatocytes can often be circumvented by using gene integrating or editing technology. However, the main potential safety risks with these methods are related to the site of gene integration. For example, some gene editing methods with CRISPR/Cas9 involve the introduction of double-stranded breaks (DSBs). These DSBs cause extensive genomic rearrangements (chromothripsis) that can drive the rapid acquisition of multiple cancer-causing mutations simultaneously. Chromothripsis can promote tumorigenesis in many tissue types, including ones relevant for therapeutic editing [91,92]. Although some gene editing systems that utilize base editors or prime editors do not introduce DSBs [93—95], these systems are limited in their degree of DNA editing to small genomic regions. The immune response induced by Cas9 protein itself can also be a problem. It is possible that this is due to the presence of certain peptides in Cas9 that may act as MHC-binding epitopes. It should be remembered that Cas9 is a protein of bacterial origin and can have an immunogenic effect in mammals [96].

New generations of gene integration methodology may be able to avoid these concerns and limitations. For example, a transposon-based system that allows genetic material to be transferred to a specific site in a host organism's chromosome may represent a viable methodology for durable and safe integrating gene therapy [97,98].

Currently available non-mammalian transposon systems include fish-derived Sleeping Beauty [79,99,100] and insect-derived PiggyBac [101—103], with newer ones on the horizon. A potential drawback for such methods is that the DNA recognition sequence may be found throughout the human genome and thus the gene would not be targeted to a specific site. This is particularly problematic for the Sleeping Beauty transposon system because the recognition sequence consists of a short dinucleotide sequence (TA), and more recent studies have uncovered the ability of Sleeping Beauty transposon to integrate into non-TA dinucleotides under certain conditions [104,105]. An ideal transposon system would be one that is mammal-derived without an immunogenic effect with the capability to insert genetic material of unlimited size at a site-specific genomic target.

5.2. VLDLR liver-specific targeting via LNP-based delivery

Targeted delivery to the liver is crucial to the success of ectopic VLDLR hepatic expression. While AAV vectors exhibit tropism to specific cell types or tissues by using different capsid proteins, their specificity is not absolute [81,106]. Although lipid nanoparticles (LNPs) lack the tissue tropism of AAV vectors [107—109], their preferential accumulation in the liver, through an apoE-dependent process [110], is ideal for delivering VLDLR to the liver for the treatment of hyperlipidemia. To further enhance the specificity of this targeting, hepatocyte-specific ligands such as N-acetyl-D-galactosamine (GalNAc) can be incorporated into the LNPs to target hepatocyte receptors [111]. In addition, LNP-based delivery systems theoretically offer unlimited packaging capacity [112—114], while viral vectors have a cargo size limitation (4.7 kB for AAVs) [115]. Moreover, non-viral vectors—such as cationic lipids and LNPs—are potentially safer than viral vectors due to the absence of immunogenic viral proteins.

6. CONCLUSION

Hepatic *VLDLR* expression as a therapeutic target is highly promising, exhibiting many features that make it amenable to genetic therapies aimed at reducing levels of TG and Lp(a). Typically, gene therapy is used to correct a genetic defect, where a gene is either not functioning or is overly functional. However, hepatic VLDLR targeting instead represents a genetic medicine approach for lipoprotein lowering therapy that would replace or augment traditional pharmacological (small molecule) drugs that primarily treat the effects of a disordered system or disease state. While further studies will be required to assess the clinical efficacy and potential adverse effects of such a therapeutic agent in humans, its successful development and implementation could present an important opportunity to lower pathologic lipoprotein levels and reduce CVD risk.

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CREDIT AUTHOR STATEMENT

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DATA AVAILABILITY

No data was used for the research described in the article.

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CONFLICT OF INTEREST

RM Krauss is a scientific advisor to SalioGen Therapeutics, Virta Health Corp., Day Two, and Seraphina Therapeutics. JT Lu, JJ Higgins, CM Clary, and R Tabibiazar are full-time employees of SalioGen Therapeutics.

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