STATE-OF-THE-ART REVIEW

Angiopoietin-Like 3

From Discovery to Therapeutic Gene Editing

Xiao Wang, PhD, Kiran Musunuru, MD, PhD, MPH, ML

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CME/MOC/ECME Objective for This Article: Upon completion of this activity, the learner should be able to: 1) discuss the role of cholesterol testing to stratify cardiovascular risk; 2) compare different pharmacologic strategies for treatment of hyperlipidemia; and 3) discuss the potential role for ANGPTL3 directed therapies for hyperlipidemia.

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Angiopoietin-Like 3

From Discovery to Therapeutic Gene Editing

Xiao Wang, PhD, Kiran Musunuru, MD, PhD, MPH, ML

HIGHLIGHTS

- Individuals with ANGPTL3 loss-of-function mutations have reduced cholesterol levels, triglyceride levels, and risk of coronary heart disease, making ANGPTL3 a potential therapeutic target.
- An antisense oligonucleotide inhibitor of ANGPTL3 and a monoclonal antibody against ANGPTL3 have been advanced into clinical trials, with encouraging results to date.
- A distinct approach to targeting ANGPTL3 would be therapeutic gene editing in patients to induce permanent loss of function mutations mimicking those in individuals with naturally occurring cardioprotective mutations.

SUMMARY

Hyperlipidemia is a major causal risk factor for atherosclerosis and coronary heart disease (CHD). Angiopoietin-like 3 (ANGPTL3) has emerged as a promising molecular target to reduce CHD risk due to its regulation of all 3 major lipid traits: low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglycerides. Here, the authors review the discovery of ANGPTL3, the role of ANGPTL3 in lipoprotein metabolism, and the genetic association between naturally occurring ANGPTL3 loss-of-function mutations and CHD. In light of the favorable consequences of ANGPTL3 deficiency, various therapeutic strategies to target ANGPTL3 are currently in development, including a monoclonal antibody, an antisense oligonucleotide, and gene editing. (J Am Coll Cardiol Basic Trans Science 2019;4:755–62) © 2019 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Over the last few decades, tremendous progress has been made in understanding and decreasing the incidence of coronary heart disease (CHD). Low-density lipoprotein cholesterol (LDL-C) has been established as a major causal risk factor for atherosclerosis and CHD. Remarkable efforts have been made to develop LDL-lowering therapies, and statins have been proven to be an effective means of reducing the risk of CHD. However, even with the use of statin therapy, there remains a large residual risk of CHD, particularly in patients with familial hypercholesterolemia (1). In light of these observations, an intensive search for new molecular targets to further reduce CHD risk is ongoing. In 2015, the efficacy of alirocumab and evolocumab, 2 human monoclonal antibody-based drugs that target proprotein convertase subtilisin/kexin type 9 (PCSK9), was confirmed with respect to the reduction of LDL-C levels in patients (2,3).

Despite advances in the development of lipid-lowering therapies, clinical trials have shown that a substantial risk of cardiovascular disease persists even when receiving currently recommended medical therapy. For example, in 1 trial involving patients who had an acute coronary syndrome, the lowering of LDL-C levels to a median of 54 mg/dl with the use of a statin plus ezetimibe was found to prevent only a slightly higher proportion of events than treatment with a statin alone; the difference in absolute risk associated with the 2 regimens was only 2 proportion points, and approximately one-third of both sets of patients had a major cardiovascular event within 7 years (4). A similarly small reduction in the absolute risk of coronary events was found among patients whose levels of LDL-C were reduced to 30 mg/dl with the use of a PCSK9 inhibitor (5). In addition, researchers have continued to search for additional molecular targets that are particularly relevant for the treatment of patients with homozygous familial hypercholesterolemia (hoFH) who have genetic deficiency of the LDL receptor. Since the efficacy of both statins and PCSK9 antibody therapies largely depends on functional LDL receptors, patients with hoFH show limited responses to both therapies.

One such promising molecular target is angiopoietin-like 3 (ANGPTL3). ANGPTL3 belongs to a

subfamily of angiopoietin-like proteins involved in the regulation of plasma lipid metabolism. ANGPTL3 is secreted from liver and was first identified via positional cloning of a hypolipidemic mouse strain (6). ANGPTL3 is unique in that it regulates all 3 major lipid traits: LDL-C, high-density lipoprotein cholesterol (HDL-C), and triglycerides. The primary action of ANGPTL3 is to inhibit lipoprotein lipase (LPL), which hydrolyzes the triglycerides carried in triglyceride-rich lipoproteins in the circulation (7). ANGPTL3 also inhibits endothelial lipase to modulate HDL-C metabolism (8). The mechanism by which ANGPTL3 regulates LDL-C remains unclear (9).

DISCOVERY OF ANGPTL3

The *Angptl3* gene was identified by positional cloning of the mutation responsible for the hypolipidemia phenotype in KK/San mice, a sub-strain of KK mice that have moderate obesity, abnormally high levels of plasma insulin (hyperinsulinemia), glucose (hyperglycemia), and lipids (hyperlipidemia). There is a 4-bp insertion in exon 6 of *Angptl3* in KK/San mice, introducing a premature stop codon. KK/San mice have lower levels of triglycerides, total cholesterol, and non-esterified fatty acids in the circulation compared to KK mice. Using adenovirus expressing human or mouse ANGPTL3 rescued the lower levels of triglycerides, total cholesterol, and non-esterified fatty acids in KK/San mice. The same treatment also increased triglycerides and total cholesterol levels in wild-type (WT) C57BL/6 mice (6). These data proved that disruption of *Angptl3* is responsible for the hypolipidemia in KK/San mice and that ANGPTL3 regulates circulating triglycerides and total cholesterol levels in mouse.

ANGIOPOIETIN-LIKE PROTEINS. ANGPTL3 belongs to a family of 8 angiopoietin-like proteins (ANGPTL1 to ANGPTL8) that share a similar structure and carry out related functions. Seven of 8 angiopoietin-like proteins (ANGPTL1 to ANGPTL7) contain a signal peptide, an N-terminal coiled-coil domain, a linker region, and a C-terminal fibrinogen-like domain (FLD) (10). ANGPTL8 differs from the other angiopoietin-like proteins in that it lacks a C-terminal FLD (11). Similar to other angiopoietin-like proteins, ANGPTL3 undergoes cleavage; the cleavage site is at amino acid residues 221-Arg-Ala-Pro-Arg-224, which yields separate fragments containing the coiled-coil domain and the FLD (12). ANGPTL3 is found in the plasma as full-length and truncated forms. ANGPTL3 is cleaved intracellularly by furin (also known as PCSK3) and extracellularly mainly by PACE4 (also known as

PCSK6) (13). The truncated form of ANGPTL3 is more active, and cleavage enhances the ability of ANGPTL3 to inhibit lipoprotein lipase and regulate plasma levels of triglycerides both in vitro (11) and in vivo (12).

Similar to ANGPTL3, another 2 members of the angiopoietin-like protein family—ANGPTL4 and ANGPTL8—are involved in the regulation of plasma lipid metabolism. ANGPTL4 is highly expressed in the liver and adipose tissue and upregulated by fasting and hypoxia (14,15). ANGPTL4 forms dimers and tetramers before secretion and undergoes cleavage at a canonical proprotein convertase cleavage site, 161-Arg-Arg-Lys-Arg-164, after secretion (16). The N-terminal fragment remains oligomerized after cleavage, binds transiently to LPL, and converts LPL from catalytically active dimers to inactive monomers to decrease its activity (17). *Angptl4* knockout mice have lower triglyceride levels and modestly lower cholesterol levels (18). However, when *Angptl4* knockout mice were fed a high-fat diet, they showed reduced viability associated with lipogranulomatous lesions, which raises a significant safety concern with respect to the proposed targeting of ANGPTL4 for the treatment of dyslipidemia and atherosclerosis (18,19).

ANGPTL8 is an atypical member of the angiopoietin-like protein family because of its lack of a C-terminal FLD, but it does share structural homology with the N-terminal domains of ANGPTL3 and ANGPTL4 (20,21), and it can inhibit LPL and thereby regulate triglyceride metabolism (11).

***ANGPTL3* GENETICS AND PLASMA LIPIDS.** Genome-wide association studies and exome sequencing studies have identified associations between loss-of-function genetic variants in the *ANGPTL3* gene and low levels of plasma LDL-C, HDL-C, and triglycerides (22,23). The *ANGPTL3* coding regions were sequenced in 3,551 individuals in the Dallas Heart Study, and a total of 35 nonsynonymous sequence variations (nonsense, missense, frameshift, and splice-site mutations) were identified. An excess of sequence variants in the lowest quartile for plasma triglyceride levels (14 vs. 5 variants) approached the nominal significance threshold (p = 0.06). In vitro functional studies revealed that all *ANGPTL3* missense variants that were associated with low plasma triglyceride levels interfered either with the synthesis or secretion of the protein or with the ability of the ANGPTL3 protein to inhibit LPL activity (24).

ABBREVIATIONS AND ACRONYMS

ANGPTL3 = angiopoietin-like 3

ASO = antisense oligonucleotide
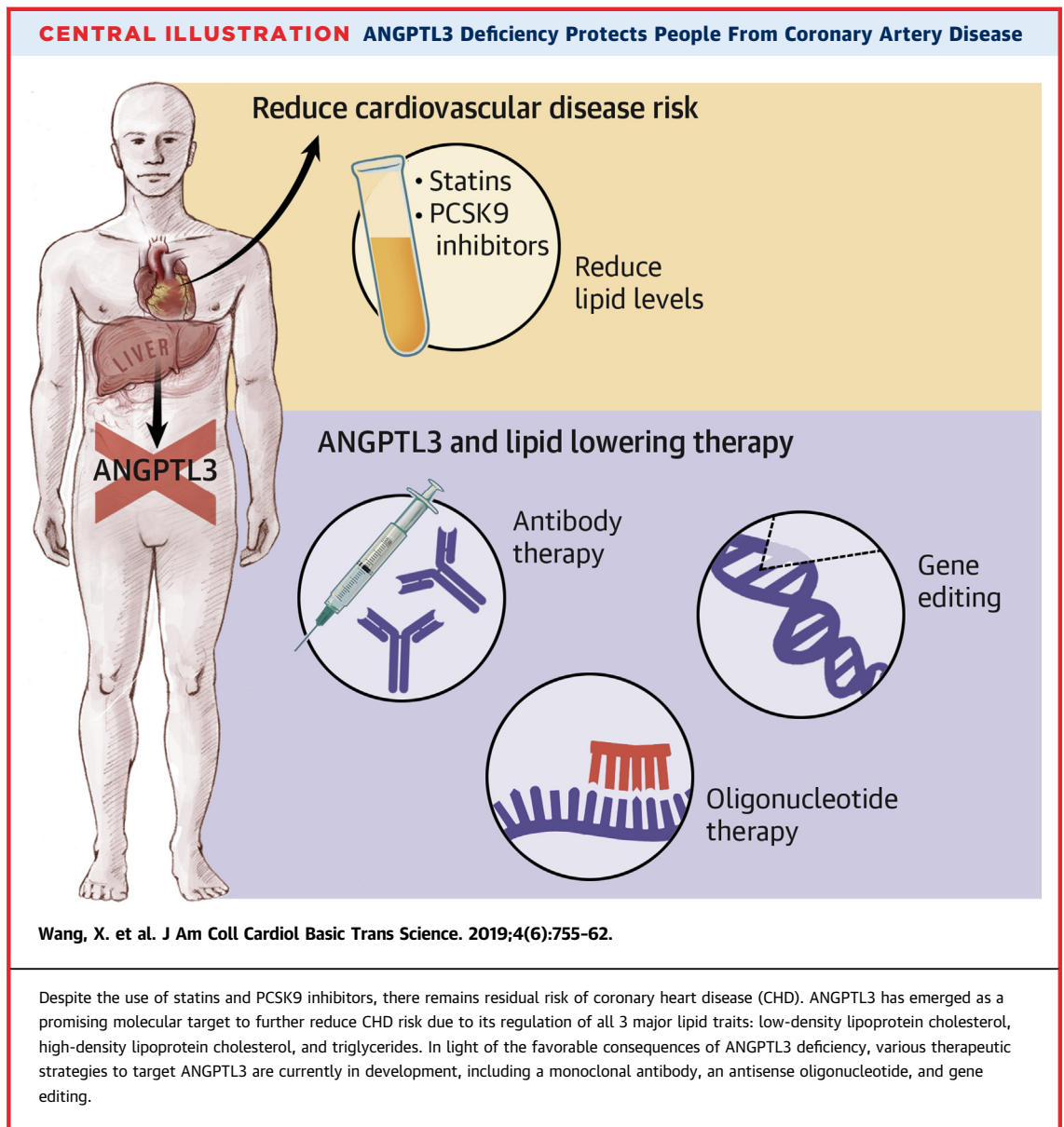
CHD = coronary heart disease

HDL-C = high-density lipoprotein cholesterol

hoFH = homozygous familial hypercholesterolemia

LDL-C = low-density lipoprotein cholesterol

PCSK9 = proprotein convertase subtilisin/kexin type 9

CENTRAL ILLUSTRATION ANGPTL3 Deficiency Protects People From Coronary Artery Disease

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Despite the use of statins and PCSK9 inhibitors, there remains residual risk of coronary heart disease (CHD). ANGPTL3 has emerged as a promising molecular target to further reduce CHD risk due to its regulation of all 3 major lipid traits: low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglycerides. In light of the favorable consequences of ANGPTL3 deficiency, various therapeutic strategies to target ANGPTL3 are currently in development, including a monoclonal antibody, an antisense oligonucleotide, and gene editing.

Exome sequencing of 2 siblings with combined hypolipidemia, characterized by extremely low plasma levels of LDL-C, HDL-C, and triglycerides, led to the identification of 2 loss-of-function variants in *ANGPTL3* as the cause. The siblings were compound heterozygotes for 2 distinct nonsense mutations (S17X and E129X) [23]. Since the publication of this study, additional mutations in *ANGPTL3* have been identified in combined hypolipidemia subjects without *APOB* gene mutations [25-27].

ANGPTL3 AND CORONARY HEART DISEASE. Numerous animal models, such as inactivation of ANGPTL3 by an antibody [9], inhibition by an antisense oligonucleotide (ASO) [28], and genetic knockout of *Angptl3* [29], support that ANGPTL3 regulates plasma lipid levels. In humans, individuals bearing 2 nonsense mutations in *ANGPTL3* have familial combined hypolipidemia. The individuals with complete ANGPTL3 deficiency showed no evidence of coronary atherosclerotic plaque [30]. Predicted loss-of-function (LOF) variants (nonsense, frameshift, and splice-site) in *ANGPTL3* are rare in the general population, found in only ∼1 in 300 individuals in the United States, limiting the power of association analyses seeking to link LOF *ANGPTL3* variants to coronary artery disease risk [30]. Accordingly, a mouse

model was used to functionally classify *ANGPTL3* missense variants as LOF versus neutral. WT human *ANGPTL3* and each *ANGPTL3* missense variant were reconstituted in the livers of *Angptl3* knockout mice by adenoviral expression. Missense variants were defined as LOF if they conferred <25% of WT activity as assessed by percent change in circulating triglyceride and cholesterol levels induced by heterologous gene expression. Eleven rare missense variants predicted to be damaging by 5 in silico prediction algorithms underwent functional validation in a mouse model, of which only 2 (p.Asp42Asn and p.Thr383Ser) were functionally validated as LOF. With the inclusion of nonsense, frameshift, and splice-site mutations along with the 2 LOF missense variants, there was a 34% lower risk of CHD among carriers of an *ANGPTL3* LOF mutation compared with non-carriers in a meta-analysis of 21,980 patients with CHD and 158,200 control individuals (30). Subsequently, a different group reported that individuals carrying LOF variants in *ANGPTL3* had a 41% lower risk of CHD than non-carriers (31).

ANGPTL3 AND LIPID-LOWERING THERAPY. In light of the favorable consequences of ANGPTL3 deficiency, an ASO inhibitor of *ANGPTL3* and a monoclonal antibody against ANGPTL3 have been advanced into clinical trials, with encouraging results so far (28,31) (**Central Illustration**). The group that developed the ASO have performed studies in a variety of mouse models and humans. They found that after administration of the ASO, hepatic *Angptl3* mRNA expression and plasma ANGPTL3 protein levels were significantly decreased in the mouse models they tested, including WT C57BL/6 mice, low-density lipoprotein receptor (LDLR) knockout mice($Ldlr^{-/-}$), double-knockout mice ($Apoc3^{-/-}$ and $Ldlr^{-/-}$), heterozygous mice ($Apoc3^{+/-}$ and $Ldlr^{-/-}$), mice with diet-induced obesity, and mice over-expressing human apolipoprotein C-III. The ASO decreased only triglyceride levels, not LDL-C or HDL-C, in C57BL/6 mice on standard chow, whereas in various hypercholesterolemic mouse models, the ASO resulted in reductions in levels of triglycerides (35% to 85%), LDL-C (7% to 64%), and HDL-C (3% to 23%). In addition, the ASO decreased liver triglyceride secretion and accumulation and improved insulin sensitivity, measured by means of intraperitoneal glucose tolerance and insulin tolerance testing in mice with diet-induced obesity. The ASO also slowed the progression of atherosclerosis in LDLR knockout mice.

Subsequently, a randomized placebo-controlled phase 1 clinical trial of an *ANGPTL3* ASO was conducted (28). Forty-four healthy human participants were randomly assigned to receive subcutaneous injections of the ASO or a placebo in a single dose or multiple doses. Among the single-dose groups at day 15, the investigators observed lower levels of ANGPTL3 protein, triglycerides, very low-density lipoprotein cholesterol, non-HDL cholesterol, and total cholesterol in the ASO group compared with placebo group. However, these differences were not significant, probably due to the small sizes of the groups (N = 3 for each group). Among the multiple-dose groups, they observed dose-dependent reductions of ANGPTL3 protein, triglycerides, non-HDL cholesterol, and apolipoprotein C-III in the ASO groups than in the placebo group.

Another group developed a fully human monoclonal antibody with high affinity for ANGPTL3. Intravenous administration of the antibody to normal C57BL/6 mice decreased plasma triglyceride levels by ≥50% and increased the LPL activity (9). Chronic administration of the antibody to dyslipidemic C57BL/6 mice reduced triglyceride, LDL-C, and HDL-C levels in the circulation without any changes in the triglyceride content of the liver, adipose, or heart. The investigators extended their observations to non-human primates by assessing the effect of the antibody on circulating lipid levels in cynomolgus monkeys (9). The antibody produced robust reductions in circulating triglycerides and non-HDL cholesterol in a dose-dependent way. It did not alter the plasma levels of LDL-C, perhaps due to those levels being low at baseline. The investigators also reported that inhibition of ANGPTL3 by the antibody could significantly decrease atherosclerotic lesion size compared to a control antibody in a mouse model of atherosclerosis (31).

A randomized placebo-controlled phase I clinical trial was performed to assess the safety, side effect profile, and pharmacodynamics of the antibody (31). A total of 83 healthy human volunteers with mildly to moderately elevated levels of triglycerides or LDL-C entered a single-ascending-dose trial. The antibody caused dose-dependent reductions in triglyceride levels of up to 76% and LDL-C levels of up to 23%. In addition, a single-group, open-label clinical trial of the antibody was conducted with 9 patients with homozygous familial hypercholesterolemia (hoFH). They received the antibody for 4 weeks, and their LDL-C levels were decreased by ~50% at week 4, although the patients were already taking aggressive lipid-lowering therapy (32).

ANGPTL3 AND THERAPEUTIC GENE EDITING. Another approach to target *ANGPTL3* is via gene

editing to induce permanent LOF mutations in vivo (Central Illustration). Since the first report of successful use in mammalian cells in 2013, CRISPR-Cas9 technology has emerged as a promising gene editor for therapeutic applications due to its efficiency and ease of use (33).

CRISPR-Cas9 introduces a double-strand DNA break at a desired site in the genome. This activates endogenous DNA repair pathways, either non-homologous end-joining (NHEJ) or homology-directed repair (HDR), to fix the break (34). NHEJ is the default repair pathway in all cells, in which the free ends from the DNA break are reconnected. However, NHEJ is an error-prone process and results in introduction of insertions or deletions (indels) at the site of the break. NHEJ can introduce frameshift mutations into a targeted gene, thereby disrupting the gene. By contrast, the cell can accurately repair a DNA break through HDR, which occurs only in proliferating cells. HDR uses a template DNA strand to achieve high-fidelity repair, and if a repair template has matching sequence to the site of the break but also has a custom-made DNA mutation, the mutation can be introduced into the genome. However, introducing a specific mutation at the site with HDR is usually inefficient, and the efficiency in vivo in adult animals is very low (<1%), which limits its use to correct a disease-causing mutation (35). Another major concern about using a gene editor is that it might also cleave the genome at other sites and cause off-target mutations.

To circumvent these problems, CRISPR-Cas9 has been adapted so that it can directly alter specific nucleotides in the DNA sequence without generating double-strand breaks and without the need for a repair template—a phenomenon known as base editing (36-40). A fusion protein with a catalytically impaired Cas9 protein and a cytosine deaminase domain adapted from an RNA-editing or DNA-editing enzyme confers the ability to convert cytosine bases at the CRISPR-Cas9 target site into thymine bases.

The use of base editor 3 (BE3) successfully introduced LOF *Angptl3* mutations into liver cells in mice (41). The investigators first screened potential sites of base-edited nonsense mutations in *Angptl3* in Neuro-2a cells and identified high BE3 activity at the codon Gln-135 site. Then they produced an adeno-viral vector expressing BE3 targeting *Angptl3* Gln-135 and injected the vector into C57BL/6J mice. This resulted in significantly reduced plasma ANGPTL3, triglyceride, and total cholesterol levels (49%, 31%, and 19%, respectively). In hyperlipidemic LDLR

knockout mice, even larger effects were observed. After treatment, triglycerides and cholesterol were reduced by 56% and 51%, respectively. Overall, this proof-of-concept study showed the ability to efficiently introduce LOF mutations in dyslipidemia-associated genes in vivo with significant lipid-lowering effects.

Several aspects make ANGPTL3 an attractive target for gene editing. First, naturally occurring LOF mutations in *ANGPTL3* protect against coronary artery disease without causing serious adverse health consequences (30,31), even in the homozygous or compound heterozygous state (i.e., full knockout of gene function) (23,30). Second, ANGPTL3 is primarily expressed in hepatocytes and secreted into the bloodstream. The liver is an ideal organ for in vivo gene editing because of its accessibility for various delivery methods. Lastly, inhibition of *ANGPTL3* requires only the introduction of inactivating mutations, which is easier to achieve than precisely correcting a specific gene mutation.

Will in vivo gene editing approaches translate into the clinic and provide a potential preventive strategy for CHD in humans? The primary benefit of in vivo gene editing is that it could ultimately yield a 1-shot, long-term therapy that would permanently modify a target gene, removing the need for repeated administration of drugs. Before consideration of translation into the clinic, ethical and regulatory challenges as well as technical issues must be addressed.

The first concern about in vivo gene editing from the scientific community has been the potential for off-target mutagenesis. It has the potential to introduce unanticipated mutations that cause oncogenesis, promoting a different disease than the 1 being treated. Second, unintended on-target mutagenesis requires more characterization before gene editing can be used as a therapy in humans because it causes irreversible genetic changes with possible serious adverse effects. Third, there might be the potential for toxicity or immune responses on delivery, and methods of safe delivery will need to be refined. Adeno-associated viruses are the preferred viral delivery vehicle compared with adenoviral vectors for use in humans because of safety concerns (42,43). However, the size limitation of each adeno-associated virus vector makes it challenging to deliver a gene editor, especially a base editor. A nonviral solution would be the use of lipid nanoparticles to deliver gene editing tools into hepatocytes (44,45).

Despite these challenges, *ANGPTL3* is a promising gene editing target for potential clinical translation.

The best candidates for first-in-human studies would be hoFH patients because they show limited response to current LDL-lowering therapies and the potential benefits might substantially outweigh the risks. With a demonstration of efficacy as well as safety in these selected patients, gene-editing therapies could then be considered for broader groups of patients at risk for CHD.

ADDRESS FOR CORRESPONDENCE: Dr. Kiran Musunuru, Department of Medicine and Department of Genetics, Cardiovascular Institute, Perelman School of Medicine at the University of Pennsylvania, 3400 Civic Center Boulevard, Building 421, 11-104 Smilow Center, Philadelphia, Pennsylvania 19104. E-mail: kiranmusunuru@gmail.com.

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