

NEW BIOTECHNOLOGICAL TREATMENTS FOR LIPID DISORDERS

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ABSTRACT

Disorders of lipid and lipoprotein metabolism play a central role in the pathogenesis of atherosclerotic cardiovascular diseases (CVDs). Despite the widespread use of efficacious lipid-modifying therapies, the residual risk of CVD remains unacceptably high. The purpose of this manuscript is to review the application of new technologies in the treatment of lipid disorders. New therapies work mostly at the gene expression level and are, therefore, different from traditional small-molecule drugs that work mainly by inhibiting already synthesized proteins. We will briefly lay out the function of the gene products targeted by the new agents. Then, we will organize our review of new biotechnological treatments by the molecular approach, namely: monoclonal antibodies, antisense oligonucleotides, small-interfering RNAs, and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated 9 (Cas9)-based genome editing. The paper concludes with the description of the current clinical studies and the perspectives for the use of these agents. (REV INVEST CLIN. 2018;70:244-54)

Key words: Monoclonal antibodies. Antisense oligonucleotides. Small-interfering RNAs.

INTRODUCTION

Disorders of lipid and lipoprotein metabolism play a central role in the pathogenesis of atherosclerotic cardiovascular diseases (CVDs). Despite the widespread use of efficacious lipid-modifying therapies, the residual risk of CVD remains unacceptably high¹. Besides suboptimal control of non-lipid risk factors, our ability to reduce CVD is also constrained by intolerance or poor adherence to statin therapy², limited availability and access to therapies for severe, genetic dyslipidemias³, and inconsistent evidence about

the benefits of impacting the high-density lipoprotein (HDL)/triglycerides (TG) axis⁴.

New and promising pharmacological agents employ tools from biotechnology and molecular biology to have a positive impact on lipid metabolism. These new treatments for lipid disorders work mostly at the gene expression level and are, therefore, different from traditional small-molecule drugs that mainly act by inhibiting already synthesized proteins. We will briefly lay out the function of the gene products targeted by the new agents. Then, we will organize our

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review of new biotechnological treatments by their molecular approach, namely: monoclonal antibodies (mAb), antisense oligonucleotides (ASO), small-interfering RNAs, and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated 9 (Cas9)-based genome editing.

MOLECULAR TARGETS

APOB

The *APOB* gene encodes apolipoprotein B (apoB), the structural apo of chylomicrons (although in the chylomicron-specific version only the initial 48% of the apoB mRNA is translated), very low-density lipoproteins (VLDLs), intermediate-density lipoproteins (IDLs), and LDL. Agents aimed at limiting the hepatic production of apoB can reduce the concentrations of all these atherogenic lipoproteins.

PCSK9

The *PCSK9* gene encodes proprotein convertase subtilisin/kexin type 9, an enzyme whose physiological role is to impede the recycling of LDL receptors (LDLr) from endocytic vesicles to the plasma membrane of hepatocytes. Thus, PCSK9 effectively reduces the number of LDLr present on the membrane and increases plasma LDL concentrations. In 2003, a new type of autosomal dominant familial hypercholesterolemia was identified⁵. The mutated gene mapped to the short arm of chromosome 1 and was later recognized as the gene for PCSK9⁶. It was soon discovered that gain-of-function mutations in *PCSK9* result in a large decrease in the hepatic expression of LDLr through a post-translational mechanism⁷. Subsequently, a genetic study in African-Americans reported an association between two new nonsense *PCSK9* mutations and substantial reductions in plasma LDL cholesterol (LDL-C) and a reduced occurrence of CVD⁸. Other nonsense or missense mutations in *PCSK9* were discovered, all of which were effectively associated with large reductions in LDL-C and reduced CVD^{9,10}. The mechanism by which PCSK9 alters the expression of LDLr was elucidated in 2007: the catalytic domain of PCSK9 binds the LDLr extracellularly. Once the PCSK9-LDL-LDLr complex is endocytosed, PCSK9 prevents the dissociation of LDL and LDLr, channeling the LDLr to lysosomes for its degradation¹¹.

ANGPTL3

The *ANGPTL3* gene encodes angiopoietin-like 3, a protein secreted mostly by the liver that acts as an inhibitor to lipoprotein-lipase (LPL-1) and endothelial lipase¹². *ANGPTL3* inhibition or prevention of its production has the potential to reduce plasma TG. By inhibiting LPL-1, *ANGPTL3* prevents the hydrolysis and clearance of circulating TG from chylomicrons and VLDL¹³. The interaction of *ANGPTL3* with LPL-1 is dependent on the formation of a complex with *ANGPTL8*, a sister protein ubiquitously expressed in various cell types¹⁴. Humans with loss-of-function mutations in *ANGPTL3* present clinically with familial combined hypolipidemia. This phenotype includes reduced plasma total cholesterol, TG, and LDL-C, reduced coronary plaque burden, and a lower risk of CVD¹⁵. The lifetime reduction in CVD risk among patients with loss-of-function *ANGPTL3* variants has been estimated at 41%¹⁶. Subjects homozygous for loss-of-function mutations in *ANGPTL3* also exhibit lower glycemia, insulinemia, and less insulin resistance¹⁷.

APOC3

The *APOC3* gene encodes apoC-III, a small interchangeable apo that interferes with the removal of VLDL, IDL, and LDL by hepatic receptors. Antagonism or blockade of apoC-III increase liver extraction of plasma lipoproteins and reduce plasma cholesterol and TG. ApoC-III antagonizes binding of apoE to the LDLr, promoting accumulation of atherogenic lipoproteins in circulation^{18,19}. Further, the atherogenicity of LDL can be almost totally accounted for by LDL that contains apoC-III²⁰, and HDL that contains apoC-III has a positive, not negative association with CVD risk²¹. Mutations in the *APOC3* gene are associated with lower plasma TG levels, lower CVD risk, and greater longevity²².

LPA

The *LPA* gene encodes apo(a), an inactive serine proteinase with procoagulant activity, through inhibition of tissue-type plasminogen activator. In circulation, apo(a) is present as part of lipoprotein (a) (Lp[a]), a macromolecular structure with similarity to LDL that includes a lipid cargo and an apo(a) molecule covalently bound to apoB. Each molecule of apo(a) has a genetically determined number of kringle-type

domains (usually 2-43), which impact its physical properties²³. Genetic and epidemiological evidence suggests an independent, causal role for Lp(a) as a promoter of atherosclerosis^{24,25}, but the molecular mechanism behind such association is not entirely clear.

Monoclonal Antibodies (mAb)

The development of hybridoma technology in 1975²⁶ has given way to the mass production of mAb against virtually any given protein of interest. A notable feature of mAbs is their long half-life and ability to bind their target with great specificity.

mAb against PCSK9

Binding of the mAb to the active site of PCSK9 or a nearby domain results in increased recycling of LDLr to the cell surface, faster hepatic uptake of LDL, and reduced plasma LDL-C²⁷.

Evolocumab

Evolocumab is a fully human mAb against PCSK9. Evolocumab consists of two heavy chains and two light chains of the lambda subclass; evolocumab does not contain an Fc region.

EFFICACY OF EVolocumab

Patients with heterozygous familial hypercholesterolemia (HeFH)

The RUTHERFORD-2 trial evaluated the efficacy and safety of evolocumab in patients with HeFH. The placebo-corrected reductions in LDL-C at week 12 were 59% for evolocumab 140 mg every 2 weeks and 61% for evolocumab 420 mg every month. Patients in the evolocumab groups also exhibited significant reductions in apoB (49%), Lp(a) (about 30%), non-HDL cholesterol (non-HDLc) (55%), and TG (10-20%) and increases in HDLc (9%)²⁸.

Patients with homozygous familial hypercholesterolemia (HoFH)

50 patients diagnosed with HoFH and high levels of LDL-C despite appropriate lipid-lowering therapy received evolocumab 420 mg or placebo every 4 weeks for 12 weeks. Patients in the treatment group had

significant reductions in LDL-C (31%) and apoB (23%) compared to placebo. Meanwhile, changes in Lp(a), HDLc, and TG did not differ significantly between groups²⁹. In a longer timeframe, TAUSSIG was an open-label, non-randomized trial that studied evolocumab in 106 patients with HoFH over 48 weeks. Patients received 420 mg of evolocumab monthly or biweekly if on LDL apheresis. The LDL-C reduction at week 48 compared to baseline was 23.3%, with simultaneous reductions of 16.2% in apoB and 11.9% in Lp(a), and a 7.6% increase in HDLc³⁰.

Patients with common hypercholesterolemia

The efficacy of evolocumab monotherapy in common (not monogenic) hypercholesterolemia was assessed in MENDEL-2. This was a multicenter trial of 614 patients with LDL-C above 100 mg/dL, and 10-year Framingham risk score estimated CVD risk under 10%, not on statin treatment. In a double-placebo design, patients were randomized to receive evolocumab 140 mg biweekly, evolocumab 420 mg monthly, and ezetimibe 10 mg daily or placebo. After 12 weeks, biweekly evolocumab reduced LDL-C by 57%, and for monthly evolocumab, the reduction was 56%³¹.

Patients who are receiving a statin

The LAPLACE trial evaluated the efficacy of evolocumab 140 mg biweekly, evolocumab 420 mg monthly, and ezetimibe or placebo when added to high-intensity (atorvastatin 80 mg or rosuvastatin 40 mg) or moderate-intensity (atorvastatin 10 mg, rosuvastatin 5 mg or simvastatin 40 mg) statin therapy. After 12 weeks, evolocumab reduced LDL-C by 66-75% in the moderate statin group, and by 63-75% in the high-intensity statin group (both compared with placebo)³².

Patients outside LDL-C goal with different CVD risk profiles

The most common scenario for considering the use of a PCSK9 inhibitor is in patients who do not achieve LDL-C goals despite the use of other currently available therapies. The DESCARTES trial addressed the efficacy of evolocumab in this situation. Patients outside their LDL-C goal according to the Adult Treatment Panel III guidelines were given lipid-lowering treatment for 4-12 weeks with diet alone, diet plus

atorvastatin 10 mg, atorvastatin 80 mg daily alone, or atorvastatin 80 mg plus ezetimibe 10 mg. The 905 patients who still had an LDL-C above 75 mg/dL were randomized to monthly evolocumab 420 mg or placebo. After 52 weeks, evolocumab reduced LDL-C between 48% (atorvastatin 80 + ezetimibe group) and 62% (diet alone group)³³.

Patients with statin intolerance

Use of evolocumab versus ezetimibe in this particular group was studied in the GAUSS-3 trial, which enrolled 511 patients with high LDL-C, who could not tolerate effective doses of at least two different statins due to muscle-related side effects. The reduction in LDL-C at week 24 for monthly evolocumab 420 mg (versus ezetimibe) was 36%³⁴.

IMPACT OF EVOLOCUMAB ON HARD CVD OUTCOMES

OSLER 1 and 2 were long-term extension trials of patients who had completed either a phase 2 or phase 3 clinical trial with evolocumab in a background of standard therapy. The main purpose of the study was to examine the long-term safety of evolocumab, but adverse CVD events were also recorded and analyzed in an exploratory way. After a median follow-up of 11 months, the rate of CVD events was significantly lower in the evolocumab group compared to placebo (HR: 0.47, 95% CI: 0.28-0.78). OSLER also confirmed the findings from Phase II studies regarding the lipid efficacy of evolocumab on LDL-C, non-HDLc, apoB, TG, and Lp(a)³⁵. FOURIER was the pivotal, CVD outcomes trial of evolocumab. More than 27,000 patients with clinically evident atherosclerotic CVD and LDL-C above 70 mg/dL despite statin therapy was randomized to biweekly evolocumab 140 mg, monthly evolocumab 420, or matching placebo. Evolocumab significantly reduced the risk of the primary endpoint (cardiovascular death, myocardial infarction, stroke, hospitalization for unstable angina, or coronary revascularization) (HR: 0.85, 95% CI: 0.79-0.92) and of a secondary endpoint of cardiovascular death, myocardial infarction, or stroke (HR: 0.80, 95% CI: 0.73-0.88)³⁶.

ADVERSE EVENTS WITH USE OF EVOLOCUMAB

OSLER and FOURIER provided reassuring results concerning the long-term safety of this type of agents. The most frequent adverse events were injection site

reactions (4.3% in OSLER, 2.1% in FOURIER)^{35,36}. In FOURIER, evolocumab-binding antibodies were equally prevalent at the end of follow-up in the evolocumab and standard-therapy groups (0.3%). Since PCSK9 inhibitors take LDL-C to values seldom reached with other agents, there has been some concern about potentially adverse neurocognitive consequences of these very LDL-C concentrations. EBBINGHAUS was a substudy of FOURIER, in which formal neurocognitive assessments were administered to patients in the evolocumab and placebo groups of FOURIER (586 evolocumab and 618 placebo patients)³⁷. Changes in neurocognitive scores were numerically very similar and statistically not different between patients in the evolocumab and placebo groups. The same results were observed for the spatial working memory strategy index of executive function (primary outcome), working memory, episodic memory, and psychomotor speed.

Alirocumab

Alirocumab is a human mAb of the IgG1 isotype, directed against PCSK9. The clinical development program for alirocumab received the name ODYSSEY.

EFFICACY OF ALIROCUMAB

Patients with HeFH

Use of alirocumab in patients with HeFH was tested in the ODYSSEY FH-I and ODYSSEY FH-II trials³⁸. A total of 735 patients with HeFH and inadequate LDL-C control on maximally tolerated lipid-lowering therapy were randomized to biweekly alirocumab 75 mg or placebo, with forced titration to biweekly 150 mg if LDL-C was > 70 mg/dL at week 8. At week 24, LDL-C in the alirocumab group decreased 51-58% versus placebo; the reductions persisted over 78 weeks of follow-up. Similar to evolocumab, significant positive changes were observed in apoB, Lp(a), TG, and HDLc. Some patients with particularly severe forms of HeFH required LDL apheresis to control their LDL-C levels. The ODYSSEY ESCAPE trial assessed whether the use of alirocumab could eliminate the requirement for LDL apheresis in such patients. Sixty-two patients undergoing regular weekly or biweekly lipoprotein apheresis were randomly assigned to receive alirocumab 150 mg or placebo every 2 weeks for 18 weeks. During weeks 7-18 of the study, the apheresis rate was adjusted based on the LDL-C of

each patient; if the LDL-C reduction was > 30%, then apheresis was suspended. Alirocumab-treated patients had a 75% reduction in the standardized rate of apheresis treatments versus placebo³⁹.

Patients who are receiving a statin

Two studies tested the efficacy of alirocumab when combined with maximally tolerated doses of statins, the ODYSSEY COMBO-I and ODYSSEY COMBO-II trials. The difference between the two resides in the comparator: Placebo in ODYSSEY COMBO-I and ezetimibe in ODYSSEY COMBO-II⁴⁰. The placebo-subtracted change in LDL-C with alirocumab was 46%, even though more than 80% of patients in the alirocumab group had remained on the 75 mg biweekly dose. ODYSSEY COMBO-II followed a similar design, but with a longer follow-up (52 weeks), active comparator (ezetimibe), and larger sample size (720 patients). Reductions in LDL-C from baseline were 51% for alirocumab and 21% for ezetimibe. These changes were maintained over the 52-week follow-up⁴¹.

Patients outside LDL-C goal with different CVD risk profiles

A set of studies compared alirocumab with other alternatives aimed at bringing patients into their risk-defined LDL-C goal. Alirocumab proved to be the strategy with the highest probability of bringing patients to goal: 85-90% of all patients on alirocumab reached and maintained their LDL-C levels within goal, compared to 65-70% with ezetimibe, 10-35% with doubling of atorvastatin, and 40-65% with switching to rosuvastatin 40 mg/d⁴².

Patients with statin intolerance

ODYSSEY ALTERNATIVE was a randomized trial focused on the comparative efficacy of alirocumab versus ezetimibe in patients at moderate-to-high cardiovascular risk who could not tolerate at least two different statins⁴³. After a statin rechallenge to confirm intolerance, 361 patients received alirocumab 75 mg every 2 weeks + daily oral placebo, ezetimibe 10 mg/d + biweekly injected placebo, or atorvastatin 20 mg/d + biweekly injected placebo (rechallenge strategy). Alirocumab reduced LDL-C by 45%, compared to only 15% for ezetimibe. Muscular symptoms were significantly less frequent with alirocumab compared to the statin rechallenge group (HR: 0.61, 95% CI: 0.-0.99).

IMPACT OF ALIROCUMAB ON HARD CVD OUTCOMES

The first study to examine (albeit as a secondary analysis) the impact of alirocumab on CVD events was ODYSSEY LONG TERM⁴⁴. In an observational *post hoc* analysis, the rate of CVD events (death from CHD, nonfatal myocardial infarction, fatal or nonfatal ischemic stroke, or unstable angina requiring hospitalization) was significantly lower with alirocumab (HR: 0.52, 95% CI: 0.31-0.90). The pivotal CVD outcomes trial of alirocumab is ODYSSEY OUTCOMES⁴⁵. More than 18,000 patients with a recent acute coronary syndrome who already received maximally tolerated doses of statins were randomized to alirocumab every 2 weeks or placebo. In the alirocumab arm, the dose was titrated aiming to keep LDL-C between 25 and 50 mg/dL, but above 15 mg/dL. After 48 months, major adverse cardiac events occurred in 9.5% of patients in the alirocumab group and 11.1% of patients within the placebo group (HR: 0.85, 95%CI: 0.78-0.93). This impact took place mostly at the expense of a reduction in total myocardial infarction and ischemic stroke. A somewhat puzzling finding was a significant 0.6% absolute risk reduction on all-cause mortality, but with no effect on cardiovascular mortality.

ADVERSE EVENTS WITH USE OF ALIROCUMAB

The highest rate of injection-site reactions with alirocumab was observed in the ODYSSEY FH trials (12.4% in FH I and 11.4% in FH II), but similar rates were observed with placebo, (11.0% and 7.4%, respectively)^{38,44}. ODYSSEY Outcomes found reassuringly low rates of adverse events with the continued use of alirocumab over 4 years. The only concerning finding was a high rate of development of neutralizing anti-drug antibodies in the alirocumab group (42%), while in the placebo group, only 6% of participants developed such antibodies. These antibodies slightly affected the lipid efficacy of the agent, but apparently did not influence its impact on CVD risk.

Bococizumab

Bococizumab is a humanized (not fully human) antibody against PCSK9, initially developed by Pfizer. Clinical development of bococizumab was halted in

2016 after results of the 10-month SPIRE trial showed a high rate of development of neutralizing anti-drug antibodies, progressive loss of efficacy, and no impact on CVD outcomes⁴⁶.

LY3015014

LY3015014 is a novel humanized mAb of the IgG4 isotype, directed against PCSK9. It has a somewhat different mechanism of action as it allows for the catalytic cleavage of PCSK9, thus reducing the plasma concentrations of intact PCSK9⁴⁷. The IgG4 isotype also confers LY3015014 a longer half-life, making administration every 8 weeks possible. In a 16-week, phase II study of patients with primary hypercholesterolemia⁴⁸, LY3015014 achieved dose-dependent placebo-corrected LDL-C reductions between 22 and 58%, with a maximal reduction observed for the 300 mg monthly dose. No relevant adverse events were reported.

mAb against ANGPTL3

Evinacumab

Evinacumab is a fully human monoclonal antibody of the IgG4 isotype against ANGPTL3. The hinge region of the IgG contains a stabilizing mutation to protect it from forming half-antibodies⁴⁹. In a proof-of-concept study in 83 patients with LDL-C above 100 mg/dL and/or plasma TG between 150 and 450 mg/dL, evinacumab 20 mg/kg administered intravenously induced a 76% reduction in plasma TG, a 23% reduction in LDL-C, and a 18% reduction in HDLc. A single subcutaneous dose of evinacumab induced a 64% reduction in TG after 4 days. With subcutaneous administration, the effects had disappeared by day 15, but with intravenous administration, the effects persisted up to day 15¹⁶. The most common adverse event with evinacumab was headache in about 10% of patients.

In a group of nine patients with HoFH who were already receiving aggressive lipid-lowering regimens with statins, ezetimibe, lomitapide and/or PCSK9 mAbs, evinacumab induced remarkable benefits⁵⁰. Patients achieved reductions between 25% and 90% in LDL-C, with great interindividual variability depending on the type of LDLr mutation (null vs. non-null, homozygous vs. compound heterozygous). Larger

and longer clinical trials with evinacumab are currently in course to test the efficacy of this mAb in diverse patient populations.

Antisense oligonucleotides

ASO are single-stranded nucleic acid molecules (mostly DNA or modified DNA) that bind through Watson-Crick complementarity to cellular mRNAs and induce their degradation or prevent their translation. In doing so, ASO reduce the cellular synthesis of a target protein. ASO are usually 12-20 nucleotides long, antisense, and complementary to the sense sequence of the target mRNA. Multiple chemical modifications can be introduced into the sugar-phosphate backbone of ASO to prolong their stability, improve cellular uptake, or reduce susceptibility to attack by extracellular nucleases^{51,52}. Once inside the cell, ASO bind specifically to regions of the target mRNA forming a DNA-RNA heteroduplex. This complex recruits RNase H1, an endonuclease that cleaves the RNA strand⁵³. ASO-based therapies accumulate in the liver where most of the apo are produced, bind specifically to their target mRNAs and are metabolized by a pathway completely independent of the cytochrome P450. These properties theoretically grant them a lower risk of adverse events or pharmacological interactions⁵⁴. First-generation ASO were created by replacing one of the non-bridging oxygens of the phosphate group in DNA with sulfur to impede nuclease activity, but they possessed cellular toxicity at high doses⁵⁵. Second-generation ASO have modified ribose with a methyl or methoxyethyl group in their 2' Carbon. Mixed modifications that combine changes in the ribose and the phosphate groups attenuate enzymatic degradation and protein binding and enhance affinity to RNA and the endonucleases⁵⁶.

Mipomersen

Mipomersen is a 20 nucleotide long, second-generation ASO directed against the mRNA for apoB. Mipomersen is currently approved only for the orphan indication of HoFH. The key trial in securing the regulatory approval of mipomersen recruited 51 patients with HoFH who were on intensive lipid-lowering therapy excluding lipoprotein apheresis⁵⁷. Twenty-five patients in the mipomersen group and 18 in the placebo group completed the 26-week follow-up.

Mipomersen achieved a somewhat modest reduction of LDL-C by 21% with a concomitant reduction of 24% in apoB. The main caveat, however, was the huge interindividual variability in response to the agent, with changes in LDL-C vs. baseline ranging from +2% to -83%. Studies in HeFH patients have found similarly wide variations, with individual LDL-C responses ranging from +30 to -70% versus baseline⁵⁸. The most common adverse effects of mipomersen, when used continuously for 2 years, are injection-site reactions (98% of patients), flu-like symptoms (65%), and clinically relevant elevations of liver enzymes (13%)⁵⁹. Most studies have also documented significant increases in hepatic fat content (in line with the drug's mechanism -inhibition of lipoprotein production and secretion by the liver)^{3,57,58}. A study of liver biopsies from patients chronically treated with mipomersen showed severe steatosis in most patients (five out of seven), albeit without histological signs of nonalcoholic steatohepatitis. No dedicated studies have been undertaken to evaluate the impact of LDL-C with mipomersen on CVD outcomes, but a pooled analysis that included 233 patients (most of them with familial hypercholesterolemia) from 3 trials lasting 1 year or longer⁶⁰ tried to address this question. The rate of CVD events during the 24 months before randomization in the mipomersen group was compared to MACE after the initiation of mipomersen. CVD events occurred in 9.6% of patients during the 24 months posterior to mipomersen initiation, compared to 61.5% during the 24 months before mipomersen treatment. Thus, mipomersen use was associated with 95% lower odds of CVD events.

IONIS-ANGPTL3-LRx

IONIS-ANGPTL3-LRx is a second-generation, N-acetyl galactosamine-conjugated ASO against ANGPTL3. A proof-of-concept study assessed IONIS-ANGPTL3-LRx in 44 participants with plasma TG 90-150 mg/dL (low-dose group) or > 150 mg/dL (high-dose group). Patients received 10, 20, 40, or 60 mg of IONIS-ANGPTL3-LRx or placebo every week for 6 weeks. Patients in the active treatment groups had reductions between 33% and 63% in plasma TG, between 1% and 33% in LDL-C, and between 10% and 37% in non-HDLc. ApoB and apoC-III were also significantly reduced. No serious adverse events were observed⁶¹.

Volanesorsen

Volanesorsen (or ISIS 304801) is a second-generation ASO complementary to the APOC3 mRNA. This ASO blocks hepatic apoC-III production and secretion, improving removal of circulating atherogenic lipoproteins.

A phase II study tested volanesorsen in patients with untreated severe hypertriglyceridemia (plasma TG 350-2,000 mg/dL, 41 received volanesorsen and 16 placebo), and in patients treated with a stable dose of a fibrate and with plasma TG 225-2,000 mg/dL (20 received volanesorsen and 8 placebo)⁶². Volanesorsen 100-300 mg per week induced dose-dependent reductions in plasma apoC-III over 13 weeks, which translated into a 31.3-70.9% drop in fasting plasma TG. Response to the agent was considerably consistent across all doses, in contrast to the large interindividual variability observed with other ASO. In a pilot study in patients with type 2 diabetes and hypertriglyceridemia⁶³, 15 patients with type 2 diabetes and plasma TG between 200 and 500 mg/dL were randomized 2:1 to receive volanesorsen 300 mg or placebo every week for 15 weeks. The impressive drop in plasma apoC-III (88%) was accompanied by a 69% reduction in plasma TG and a 42% increase in HDLc versus placebo. Moreover, clamp-measured whole-body insulin sensitivity improved by 57% in the volanesorsen group. In a small, but relevant study of three patients with familial chylomicronemia, volanesorsen proved to be similarly effective, inducing profound suppression of apoC-III synthesis and TG reductions between 56% and 86%⁶⁴.

ASO against apo (a)

ISIS-APO(a)_{Rx}

ISIS-APO(a)_{Rx} is a specific ASO that specifically binds a splice site between exons 24 and 25 of the apo(a) mRNA. The agent was selected from a screen of over 2200 ASO complementary to multiple sites across the human apo(a) transcript⁶⁵. In a phase I study on healthy volunteers, ISIS-APO(a)_{Rx} 100-300 mg reduced plasma Lp(a) by 40-78%⁶⁶.

IONIS-APO(a)-LRx

IONIS-APO(a)-LRx is a ligand-conjugated ASO with a triantennary N-acetylgalactosamine complex

covalently attached to allow faster and specific uptake by hepatocytes. In a phase 1/2a trial in healthy volunteers with Lp(a) > 75 nmol/L, multiple doses of 10–40 mg IONIS-APO(a)_{L_{Rx}} administered at days 1, 3, 5, 8, 15, and 22 induced Lp(a) reductions between 66% and 92% at day 36⁶⁷. Isolated case reports suggest that knocking down Lp(a) may have a large clinical impact⁶⁵, but longer and larger clinical trials will be required to confirm this hypothesis.

SMALL, INTERFERING RNAs (siRNAs)

siRNAs are part of the larger family of regulatory RNAs. Specifically, siRNAs are synthetic, double-stranded RNAs that can induce RNAi (RNA interference)⁶⁸. RNAi is a mechanism used by cells to defend themselves against foreign or invasive nucleic acids and to aid to the maintenance of genome integrity. When an unknown dsRNA enters the cell, it is attacked by Dicer, an RNase III endonuclease⁶⁹, giving rise to 21–23 bp-long dsRNA molecules with 3′ overhangs (siRNAs). SiRNAs are then loaded onto the RNA-induced silencing complex (RISC), formed by the siRNA, Dicer and the protein Argonaute2 (Ago2)⁵⁴. Once the RISC is formed, one of the strands (passenger strand) is degraded, and the other (guide strand) is preserved in the complex. The guide strand in RISC binds the target mRNA through Watson-Crick complementarity and leads to its cleavage by Dicer⁷⁰, potentially generating more siRNAs. Through this amplification mechanism, a few siRNAs are able to silence expression of a gene over long periods of time. Therapeutic siRNAs can be chemically modified to decrease susceptibility to endo- and exonucleases, avoid rapid renal excretion, and increase their tissue specificity⁷¹. The internalization of therapeutic siRNAs is achieved by endocytosis due to their negative charge and large molecular weight⁷².

Inclisiran

Inclisiran is a synthetic siRNA directed against PCSK9⁷³. ORION-1 was a dose-finding trial of subcutaneous inclisiran vs. placebo in patients with high CVD risk and high LDL-C despite maximally tolerated statin. Patients had LDL-C concentrations above 70 mg/dL if they had a history of CVD or above 100 mg/dL if they did not have such history. Inclisiran was tested at doses ranging from 200 to

500 mg, and as a single dose at day 1 or 2 doses at days 1 and 90. Plasma LDL-C in day 180 declined by 27.9–41.9% in the single-dose group and by 35.5–52.6% in the two-doses groups. Interestingly, plasma PCSK9 and LDL-C still remained significantly below their baseline levels at day 240. The most common adverse event was injection-site reactions, which happened in 5% of patients on inclisiran. In contrast to studies with ASO-based therapies, no flu-like symptoms were reported in the active treatment groups.

THE FUTURE: CRISPR/CRISPR-ASSOCIATED 9 (CAS9)-BASED INVESTIGATIONAL THERAPIES

CRISPR-Cas9 systems originated as a primitive immunity mechanism displayed by some bacterial species. Since the year 2013, they have been harnessed as biotechnological tools for genome editing⁷⁴ in eukaryotic cells and *in vivo*. These systems offer the attractive possibility of “knocking off” or even “switching on” a specific gene in a complete, live multicellular organism.

Synthetic CRISPR-Cas9 systems comprise a protein (Cas9) and an RNA molecule. Cas9 is an enzyme with helicase activity, selective DNA binding activity, and endonuclease activity⁷⁵. The RNA component is called a “guide RNA,” it is about 100 nucleotides long and provides the CRISPR-Cas9 complex with sequence specificity. After binding Cas9, the guide RNA hybridizes to one strand of dsDNA through a so-called “protospacer” sequence contained within its first 20 nucleotides. Meanwhile, Cas9 binds a much shorter sequence in the DNA, adjacent to the protospacer. This protospacer-adjacent motif is different in each type of Cas9 protein. Under this configuration, a Cas9/guide RNA/target DNA complex is formed, and a ds break is introduced in the DNA. In this fashion, systems with the ability to introduce breaks at specific points in virtually any site of a genome can be designed⁷⁶. The most commonly used versions of Cas9 are those from *Streptococcus pyogenes* and *Staphylococcus aureus*⁷⁵.

The versatility of these systems brings about very interesting applications collectively termed “epigenome editing.” If Cas9 is made catalytically inactive

and fused into a chimeric protein with a transcriptional activator domain, the expression of a target gene can be stimulated (CRISPR activation). If catalytically inactive Cas9 is fused to a transcriptional repressor, the result is gene silencing at the transcriptional level (CRISPR interference)⁷⁷. In CRISPR-Cas9-based base editing, Cas9 is fused with a cytosine deaminase domain, resulting in a C-to-U change at the desired place (with the possibility of introducing a stop codon) or in a G-to-A change in the opposite DNA strand through cellular DNA repair systems.

The main concerns with the therapeutic use of CRISPR-Cas9 systems have to do with the potential introduction of unintended changes at genomic sites different from the target and with the choice of an appropriate vector that guarantees efficient delivery of RNAs encoding both the guide RNA and Cas9 (adeno-associated viral vectors and lipid nanoparticles have been attempted)⁷⁵.

A proof-of-concept study in mice demonstrated that an *S. aureus* CRISPR-Cas9 system targeted to break and disrupt the PCSK9 gene could reduce plasma PCSK9 by more than 90% and reduce plasma cholesterol by about 40%, without signs of off-target mutagenesis or liver toxicity⁷⁸. Similar results were found with an *S. pyogenes* CRISPR-Cas9 base edition strategy in adult mice⁷⁹. More recently, an *in vitro* screen for potential sites amenable to introduction of base-edited nonsense mutations in *ANGPTL3* identified a suitable site at codon Gln-135 of the gene. Researchers produced adenoviral vectors containing a CRISPR-Cas9 base editor with a guide RNA targeting Gln-135 of *ANGPTL3* and injected them into 5-week-old mice. 7 days after injection of the adenoviruses, plasma *ANGPTL3* was reduced by 49%, plasma TG by 31%, and plasma cholesterol by 19%⁸⁰. Deep sequencing of liver biopsies revealed no evidence of DNA edition at the top 10 most likely off-target sites. Injection of the same adenovirus into LDLr knockout mice (an animal model of HoFH) reduced TG by 56% and total cholesterol by 51%.

These results have created a great deal of expectations about the prospects for gene editing therapies, but major technical and safety hurdles will need to be overcome before they may become feasible options in the routine management of lipid disorders.

In summary, great advances in the physiology of lipoprotein metabolism have translated into the discovery of new and relevant targets for inhibition. Molecular biology tools aimed at these targets have provided a vast arsenal for the treatment of lipid disorders includes that now includes mAb, ASO, siRNAs and sometime in the future perhaps CRISPR/CAS9-based agents. All these advances will enhance our ability to prevent CVD and improve the lives of our patients.

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