Cholesterol Lowering biotechnological strategies: from monoclonal antibodies to antisense therapies. A pre-clinical perspective review.

S. Bellosta[1] (Orcid: 0000-0001-5344-7824), C. Rossi[1] (Orcid: 0000-0002-6403-2533), A.S. Alieva[2] (Orcid: 0000-0002-9845-331X), A.L. Catapano[1,3] (Orcid: 0000-0002-7593-2094), A. Corsini[1,3] (Orcid: 0000-0002-9912-9482), A. Baragetti[1,3] (Orcid: 0000-0002-6216-1999)

Affiliations:

[1] Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Via Balzaretti 9, 20133 Milan, Italy.

2 Almazov National Medical Research Centre, Saint Petersburg, Russia.

[3] IRCCS MultiMedica, via Fantoli 16, 20138, Milan, Italy.

Corresponding author:

A. Corsini, Ph.D.

Full Professor of Pharmacology,

Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, via Balzaretti 9, 20133 Milan

e-mail: alberto.corsini@unimi.it

Key words: Angptl3, Car-T therapy, LDLR, Lp(a), mAb, PCSK9

Acknowledgments

The authors' research is supported by: EXTRALIPO "New lipid-oriented pharmacological and chemical approaches to discriminate and unravel extracellular vesicles biological functions" Bando SEED – PSR 2019 (SB); Russian Ministry of Science and Higher Education (Agreement No. 075-15-2020-901 dated 13.11.2020) (ASA); H2020 REPROGRAM PHC-03-2015/667837-2 (ALC); Fondazione Cariplo 2015-0524 and 2015-0564 (ALC); ERANET ER-2017-2364981 (ALC); Ministry of Health-IRCCS MultiMedica GR-2011-02346974 (ALC); PRIN 2017H5F943 (ALC); "Cibo, Microbiota, Salute" by "Vini di Batasiolo S.p.A" AL_RIC19ABARA_01 (AB), "Post-Doctoral Fellowship 2020" by "Fondazione Umberto Veronesi" 2020-3318 (AB).

Abstract

In recent years, the leverage on available genetic information and a better understanding of the genetic bases of dyslipidemias has led to the identification of potential new avenues for therapies. Additionally, the development of new technologies has represented the key for developing novel therapeutic strategies targeting not only proteins (e.g. the monoclonal antibodies and vaccines) but also the transcripts (from Antisense Oligonucleotides (ASOs) to small interfering RNAs) or the genomic sequence (gene therapies).

These pharmacological advances have led to successful therapeutic improvements, particularly in the cardiovascular arena since we are now able to treat rare, genetically driven, and previously untreatable conditions (e.g: familial hypertriglyceridemia or hyperchylomicronemia).

In this review, the pre-clinical pharmacological development of the major biotechnological cholesterol lowering advances will be discussed, describing facts, gaps, potential future step forwards and therapeutic opportunities.

Introduction

The biotechnological option for anti-cholesterol drugs: an overview.

Statins, fibrates and ezetimibe represented for decades the most effective cholesterol-lowering small molecules for treating dyslipidemias. Historically large trials demonstrated that statins reduce up to -23% the risk of developing cardiovascular diseases (CVD) [1] representing, so far, the first choice in the management of hypercholesterolemia. Later on, the IMPROVE-IT trial reinforced the concept of "the lower, the better", showing that the reduction provided by statins can be further extended by adding ezetimibe [2]. Furthermore, recent data from the REDUCE-IT demonstrated that the addition of a specific omega-3 formulation (Icosapent ethyl) lowered the CVD risk in patients still on statin treatment [3]. Thus, the most recent guidelines from both the European Society of Cardiology/European Atherosclerosis Society and the American College of Cardiology/American Heart Association integrated all these evidence supporting the earliest and most effective reduction of plasma Low-Density Lipoprotein Cholesterol (LDL-C) levels proportionally to the degree of residual CVD risk [4].

Genome Wide Association Studies (GWAS) and Mendelian randomization approaches discovered new genetic mediators of cholesterol and lipid metabolism, including Proprotein Convertase Subtilisin Kexin Type 9 (PCSK9), Angiopoietin Like-3 (Angptl3) and Apolipoprotein CIII (ApoCIII). In addition to these genetic linkage, further studies identified other key players, such as Lipoprotein(a) (Lp(a)), that are causally associated with cardiovascular disease. Altogether these findings contributed to a significant step forward in the understanding of complex pathophysiological frameworks of common dyslipidemias, of some genetic conditions associated with elevated cardiovascular risk (e.g. heterozygous Familial Hypercholesterolemia (FH)) and of rarer conditions (such as FH in homozygosity or Familial Chylomicronemia).

Monoclonal antibody-based therapies are well known therapeutic choices among clinicians, as they allow a therapeutic intervention against different targets. However, they are costly, and need frequent injections thus lowering patients' compliance. Vaccination could circumvent at least some of these drawbacks. As alternatives, new biotechnological

tools have been developed to provide powerful lipid-lowering activity. These advancements have been developed with the precise aim of targeting either the DNA or the transcript of the final protein. Adeno-associated viral (AAV) vectors, Clustered Regularly Interspaced Short Palindromic Repeats associated to Cas9 (CRISPR-Cas9), or the "Chimeric Antigen Receptor T cell" (namely CAR-T system) represent the first case. Vice versa, RNA silencing (like those against Proprotein Convertase Subtilisin Kexin Type 9 (PCSK9) and Apolipoprotein (a)), or Antisense Oligonucleotides (ASOs) (like those against Apolipoprotein (a) (apo(a)) and Angplt3) are the main tools developed for the latter (**Figure 1)**.

Biotechnological strategies targeting proteins

Monoclonal antibodies against PCSK9 and Angptl3

In the last decade, the development and use of monoclonal antibodies (mAbs) in multiple therapeutic areas have been a major breakthrough following the development of mAbs against PCSK9 or Angptl3 **(Figure 2).** In the cardiovascular field, mAbs against PCSK9 have been recently marketed and they are very effective at reducing plasma cholesterol levels, and consequently atherosclerosis, in experimental animal models and in humans.

Unlike polyclonal antibodies, mAbs are monospecific antibodies, usually of the Immunoglobulin (Ig)G type, made by identical immune cells that are all clones of a unique parent B cell. Their superiority for therapeutic purposes stands on the elevated specificity of their binding site to the epitope of the target protein and on a relatively long half-life. In fact, IgG serum half-life is modulated by their interaction with the Fc neonatal receptor (FcRN) that, at acidic pH, binds the IgG and prevents lysosomal degradation [5]. In addition, their cellular re-cycling mediated by the reticuloendothelial system (RES) [6] prolongs their half-life [7]. MAbs are eliminated from the circulation following two distinct pathways: an antigen-specific target-mediated disposition, in which antibodies bind their target antigens and are cleared via endocytosis and lysosomal degradation, and a nonspecific elimination through the phagocytic and endothelial cells of the RES [6]. Hepatic and renal function do not significantly affect mAbs metabolism or excretion, therefore their clinical efficacy is not significantly modified in renal or liver disease-affected patients. In addition, mAbs undergo a metabolic disposition that does not appreciably interfere with other small drug elimination from the body [8]. Based on these characteristics, mAb-based therapy has become an effective way for treating many pathologies.

In the search for effective therapeutic interventions to modulate PCSK9 levels and activity, specific neutralizing mAbs against PCSK9 have been developed. These mAbs bind selectively to extracellular PCSK9 thus preventing its interaction with the LDL receptor (LDLR), that is free to recycle on the surface of hepatocytes and bind to new LDL particles, thus leading to the reduction of plasma LDL cholesterol (C) levels. Two fully human mAbs, Alirocumab (a 146 KDa fully human IgG1) and Evolocumab (a 141.8 KDa fully human IgG2), have been approved by FDA and EMA as mAbs binding PCSK9, preventing the degradation of LDLR. This, in turn, promotes the reduction of LDL-C plasma levels (up to 65% for alirocumab and 80% for evolocumab in humans, following an injection every two or four weeks) and the risk of recurrent ischemic cardiovascular events [9–11].

In pre-clinical studies, alirocumab showed a high affinity binding to all species of recombinant PCSK9 proteins tested under neutral (pH 7.4) and acidic (pH 5.5) conditions (KD range 0.58 to 14.5 nM at pH 7.4). Alirocumab has no *ex vivo* Fc effector function activity and does not induce antigen-dependent cell cytotoxicity or complement-dependent cell cytotoxicity in the

presence or absence of PCSK9 since the complex of alirocumab and human PCSK9 is unable to bind C1q, the first step in the activation of the complement cascade [9]. Alirocumab, like all IgG antibodies, can cross the placenta, most probably through an FcRn-mediated mechanism. Concentrations of total alirocumab were detectable in infant monkeys on the seventh day after birth, confirming exposure in utero. Weekly administration of alirocumab did not affect the pharmacokinetics (PK) of daily administered atorvastatin and of its active metabolites. Intravenous or subcutaneous (SC) injection of alirocumab reduces, in a time and dose-dependent manner LDL-C, apolipoprotein B (apoB) and total cholesterol (TC) levels in different rodent models, and blocks the effects of PCSK9 in cynomolgus monkeys and Sprague-Dawley rats [9]. In the ApoE*3Leiden-CETP mice, a well-established mouse model of hyperlipidemia that shows the typical features of human familial dysbetalipoproteinemia, which is characterized by accumulation of remnant lipoproteins and an increased VLDL-C to LDL-C ratio, alirocumab alone dose-dependently reduced TC and TGs and further decreased TC in combination with atorvastatin [12]. Alirocumab decreased also atherosclerotic lesion size, severity, and composition by reducing monocyte recruitment and increasing smooth muscle cell and collagen content. There was no hepatic lipid accumulation, nor changes in fecal output of biliary acids or neutral sterols. A similar approach was used in another study performed in apoE*3Leiden mice that were treated with alirocumab, evinacumab (a mAb inhibitor of Angptl3, see below) and atorvastatin for 25 weeks. The triple treatment reduced plasma TC up to 80%, non-HDL-C up to 91%, and lesion size up to 50% in the thoracic aorta with an improvement of lesion composition [13]. This study also highlighted the therapeutic potential of the coadministration of drugs with different pharmacological effects. Three variants of alirocumab were prepared by inserting two or three amino acid substitutions in the Fc fragment of the mAb. These substitutions improved half-life of mAbs by 2 to 5-fold by enhancing its binding to the FcRN [14]. The lipid-lowering effect was also improved and the mAbs required a less frequent administration and lower doses thanks to the improved recycling of the mAb into the circulation.

Evolocumab is pharmacologically active in all species and binds PCSK9 with an equilibrium dissociation constant of 4 pM for humans and cynomolgus, and 160 pM for mice, and a half-maximal inhibition (IC_{50}) of about 2.1 nM [15]. In mice, evolocumab reduced TC in a time- and dose-dependent manner [16]. In cynomolgus monkeys, after evolocumab injection, PCSK9 levels decreased by 97% in 15 minutes, and remained at this level for 3 days, returning to base-line only after 10 days [16]. In a mouse model of thrombosis, evolocumab reduced the PCSK9 pro-thrombotic effects, thus suggesting the use of PCSK9 inhibition to prevent thrombotic complications [17]. More recently evolocumab was able to prevent a high-fat high-cholesterol diet-induced hyperlipidemia and atherosclerotic plaque formation in a genetically modified hamster model [18].

Both alirocumab and evolocumab display nonlinear PK due to their target-mediated elimination, a small volume of distribution (3.5 L and 3.3 L respectively [14,15]) indicating a limited tissue distribution, and an absolute bioavailability after SC injection of 75% for alirocumab and 82% for evolocumab [19]. Both mAbs did not show any gender differences, unforeseen accumulation or time-dependent changes in pharmacokinetics after multiple doses [9].

To overcome the limitations caused by the need for multiple mAb injections, more recently a DNA-encoded mAb (DMAB) targeting PCSK9 has been engineered. A single intramuscular injection in mice led to a robust production of DMAB within days, with peak levels by 1-week after administration, that lasted for up to 2 months, leading to a reduction of TC by up to 10% and of non-HDL-C by up to 29% [20]. DMAB levels could be boosted by repeated administrations.

The mAb-based technology has been also used for interfering with Angptl3, an inhibitor of the Lipoprotein Lipase (LPL), that has been approved in U.S. for the treatment of homozygous FH patients [21]. Peripheral and endothelial lipases hydrolyze

TGs contained in VLDL favoring their conversion into LDL. While in a physiological condition the resulting increased amount of LDL is efficiently taken up by hepatocytes, in the presence of hypercholesterolemia or FH, due to deficiency of LDLR, LDL accumulates in the circulation. Evinacumab is a mAb that inhibits Angptl3, thus favoring the activity of peripheral lipases contributing to the reduction of LDL-C levels. Evinacumab displays anti-atherogenic activity in rodents [13,22], and potent and consistent TG and LDL-C lowering effects (by 40% in healthy volunteers [23] and up to 49% in subjects with homozygous FH [24]). In a recent phase III study on 59 homozygous FH patients (of whom 94% on statins, 77% on PCSK9 inhibitor, 75% on ezetimibe, 25% on lomitapide, and 34% undergoing apheresis, as standards of care), the -47.1% in the reduction of LDL-C with evinacumab (as compared to standard of care only) was maintained for the 24 months observational period [24]. It is of note that in this study the effect of evinacumab was comparable between subjects with complete LDLR deficiency (null-null mutations) as well as in patients with a partial LDLR deficiency (that is those with either two non-null alleles or one null and one non-null allele). This observation might further imply that the inhibition of Angptl3 activity in the periphery increases the uptake of VLDL remnants by hepatocytes and, in turn, reduces the pool of LDL even when the LDLR is not present. This possibility opens the way towards new therapeutic opportunities, since the inhibition of the circulating form of Angplt3 might provide an added value, even beyond anti-PCSK9 mAbs, for the treatment of the most sever genetically determined FH. The pharmacodynamics properties of this compound include i) a high affinity to the binding sites of the circulating form (about KD=0.26±1.28 nM in human, non-human primates, rats and mice) [25] and ii) a low plasma IC_{50}= 2.9±9.6 nM [25].

By contrast to the inhibition of angptl3, that of angptl4 using mAbs is currently less advanced, and its hypothetical clinical use still under scrutiny [26].

Vaccination against PCSK9

Vaccination against PCSK9 may also provide a promising approach for the treatment of hypercholesterolemia allowing a better compliance. Many different experimental pre-clinical approaches have been tested. In the first one, mice were immunized with human recombinant PCSK9, using a DNA-oligonucleotide as an adjuvant, causing a 40% reduction of TC [27]. In another study, a panel of short non immunogenic peptides mimicking PCSK9 epitopes were linked to a potent carrier protein, the keyhole limpet hemocyanin (KLH), used as a source of foreign T helper epitopes thus imparting immunogenicity to the conjugate. In mice and rats, this vaccine elicited a high-titer antibody response against PCSK9 that persisted for almost one year with a half-life of about 4 months, much longer than the half-life of mAbs [28]. TC levels were reduced by up to 30%, and LDL-C up to 50% in treated animals. The same peptide-based vaccine reduced systemic and vascular inflammation and atherosclerotic lesion size in the aorta of APOE*3Leiden.CETP transgenic mice [29]. In another study in apoE KO mice, the peptide vaccine was administered three times at 2-week intervals maintaining anti-PCSK9 antibody peak levels for up to 24 weeks with no activation of T-cells, and reducing plasma TC, VLDL and chylomicron levels [30]. Unfortunately, the downside of this technical approach is the induction of undesirable anti-KLH antibodies which can mask peptide epitopes impairing antibody production. To circumvent this problem, the KLH was camouflaged with serum albumin creating a hybrid nanocarrier with PCSK9 peptide anchored onto the surface [31]. The production of nontarget antibodies was almost eliminated and the LDL-C lowering potential of the vaccine was improved.

A different approach exploited the ability of virus-like particles (VLPs) to display on their surface a battery of self-antigens in a highly dense, repetitive format thus inducing a strong antibody response against self-antigens with production of IgG. In

this way, VLP display could be used to target self-molecules that are causing a series of chronic pathologies. In mice, VLP-based vaccines, targeting five regions of PCSK9 predicted to be involved in LDLR binding, caused a significant reduction of total and free cholesterol and of TG levels (up to −60%). In macaques, the vaccination caused a reduction of TC by −19%, LDL-C by −32%, TG by −15%, and of apoB by -30% [32]. A modified VLP-based vaccine raised by using a peptide spanning prodomain and catalytic domain of PCSK9 (named PCSK9Qβ-003) decreased plasma PCSK9 levels, and upregulated hepatic LDLR, SREBP-2, HNF-1 and HMGCoA reductase expression in LDLR KO mice [33]. Interestingly, the same vaccine improved also renal lipid accumulation and fibrosis by regulating fatty acid beta-oxidation in LDLR KO mice [34] and attenuated atherosclerotic plaque progression by modulating reverse cholesterol transport and inhibiting inflammatory cell infiltration and apoptosis in apoE KO mice [35].

An immunogenic fused PCSK9-tetanus (IFPT) peptide was incorporated on the surface of nanoliposomes and injected four times at bi-weekly intervals in atherosclerotic mice. It reduced PCSK9 plasma levels by 59%, TC by 45%, LDL-C by 52% and VLDL-C by 19% after the fourth vaccination. The reduction was long-lasting with a LDL-C decrease by up to 42% after 16-weeks and an increased production of IL-10 and IFN-gamma [36].

In addition, the combination with genome-editing technologies, such as CRISPR-Cas9, led to a vaccine variant that may give better response in patients. Researchers generated a CRISPR construct that introduced a site-specific nonsense mutation in PCSK9 gene similar to the loss of function (LOF) mutations identified in humans. This approach took advantage of the fact that the CRISPR-Cas9 tool is particularly efficient in the liver, where it can edit a much higher proportion of cells than in other tissues. A single injection of an adenovirus containing CRISPR-Cas9 system directed against PCSK9 may silence more than 50% of hepatic PCSK9 genes leading to a 90% decrease of PCSK9 plasma levels and a 35-40% reduction of LDL-C in mice [37]. No off-target mutagenesis was detected in 10 selected alternative sites.

Biotechnological strategies targeting DNA

Viral vectors

Gene therapy and DNA editing are two independent strategies developed to induce permanent changes in the DNA sequence, either ameliorating or repressing a gene function. Gene therapy consists in the insertion of a genetic sequence required for coding for a specific functional protein that is lacking in a pathological condition. The insertion is done by injecting a biological (e.g. viral) or non-biological (lipid-, peptide- or polymer-based carriers) vector which will integrate its genetic information into the genome of the patients in whom it is lacking since inception. This approach was at the basis of a gene therapy using the adeno-associated viral (AAV) vector "alipogene triparvovec" for the treatment of severe hypertriglyceridemia, a genetic condition in which LPL deficiency causes a massive elevation of plasma TG levels, due to impaired lipolysis of circulating chylomicrons and triglycerides-rich lipoproteins (TGRLs). The rationale of adding extra-copies of functionally LPL directly into the muscle tissue of an LPL deficient patient paved the road for the development of a nonreplicating and nonintegrating AAV vector of serotype 1 (AAV1), engineered to deliver a naturally occurring gain-of-function (GOF) LPL gene variant (S447X), carried by 20% of the general population and associated with an increased TGRL turnover. AAV1-LPL^{S447X} was produced by baculovirus-based production in insect cells [38], giving rise to the so called "alipogene tiparvovec". After a single administration, the adenoviral genome persisted in the myocytes for up to one year and

was responsible for the reduction by 90% of TGs in mice and -77% in humans. Commercial concerns, technical difficulties (e.g: multiple serial injection under anesthesia regimens), insufficient PK data and criticism regarding immune-inflammatory safety have been raised (accumulation of circulating T cells-mediated antibodies against the AAV1 capsid and lymphocytic infiltration in the spleen [39,40]) and lead to withdrawal of this drug in 2012.

CRISPR-Cas9-based approaches against Pcsk9 and Angptl3

DNA editing, acting on the DNA at the level of the cross-over in meiosis, represents a step-forward contributing to the phenotypic difference of the probands as compared to the rest of consanguineous family members. This ambitious aim has been recently achieved by developing the CRISPR-Cas9 system. This system, since its discovery in 2012, is based on the same evolutionarily conserved mechanisms by which bacteria produce a transcript for protection against exogenous DNA. In fact, the system harnesses the CRISPR DNA fragments that specific prokaryotic organisms (for example *Streptococcus Pyogenes* [41,42]) use to guide the activity of the Cas9 enzyme to recognize and cleave specific complementary DNA strands to the CRISPR sequence. Upon finding an RNA harboring the spacer sequence, the CRISPR-associated Cas9 proteins will recognize and cut foreign pathogenic DNA and introduce specific mutations at a desired location in the host genome [42]. Previous classical viral vector-based techniques (e.g.: alipogene triparvovec) introduced constructs integrating at specific target sites by homologous recombination and relied on viral vector delivery of therapeutic transgenes that could cause insertional oncogenesis and immunogenic toxicity. As compared to these approach, CRISPR-Cas9, by generating targeted double strand breaks and by controlling the specificity of homology directed repairs (HDR) system, provides superior specificity for the target site as compared to classical viral vectors. CRISPR-Cas9 no longer requires modular protein assembly but merely the modification of few nucleotides of the targeting RNA guide. The simplicity and efficacy of the resulting CRISPR/Cas9 system make effective gene editing broadly accessible and versatile towards a vast number of genes. Also, recent development harnessing Cas-9 based incorporations of alternative RNA-aptamer-binding molecules, opened new avenues in the possibility of CRISPR-mediated transcriptome modulation and cell reprogramming [43].

CRIPR-Cas9 represents a promising approach to modulate key proteins involved in plasma lipid metabolism, due to its durable effects without the need for repeated administrations. The experience with this technique in lipidology is at an initial stage, with Angptl3 gene editing being the pioneering approach contributing to the improvement of CRISPR-Cas9 based strategy **(Figure 3)**. Angptl3 targeting represented a step forward also for CRISPR-Cas9 itself being the first based Cas9 editor (namely BE3), a last generation version designed to overcome undesired effects of this technology, such as on-target mutagenesis from DNA double-strand breaks, off-target mutagenesis from insufficient specificity of guide RNA (gRNA), or inefficient on-target mutagenesis due to low-rate homology-directed repair. After injection of the BE3 with Angptl3-targeting gRNA at the codon Gln-135 of the *ANGPTL3* locus (BE3-Angptl3) (as compared to the BE3 with no gRNA), mice showed highly efficient editing, with +35% increase in ANGPTL3 edited alleles (with no evidence of off-target mutagenesis), associated with 50% reduction in circulating angptl3 protein, 35% reduction in fasting TG, and 20% reduction in cholesterol levels. In hyperlipidemic LDLR knockout mice, the injection of BE3-*Angptl3* significantly reduced TG by 56% and cholesterol by 51% [44]. However, little is known about its safety profile; of interest, the significant reduction in hematopoietic stem cells content in bone marrow, observed in ANGPTL3 full knock-out mouse [45], was not observed following the use of this gene-editing system.

Following Angptl3, also PCSK9 became targetable by CRISPR-Cas9. An initial approach used gRNA to the codon Trp-159 of the *PCSK9* locus ("BE3-pcsk9 w159") [46] **(Figure 3)**, with significant LDL-C reductions in mice, although lower as compared to the Angplt3-based CRISPR-Ca9 approach. Vice versa, more recently CRISPR base guides and editors, that were loaded onto lipid nanoparticles to specifically reach the liver, provided an almost total PCSK9 lowering effect (-90%), resulting in the reduction of up to -60% in LDL-C in nonhuman primates that lasted for up to 8 months after a single injection [47].

CAR-T

CAR-T therapy consists in the infusion of autologous T-cells that have been engineered to express a chimeric antigen receptor (CAR) on their cell surface **(Figure 1)**. The resulting cell will express a significant amount of engineered transmembrane receptors harboring an extracellular ScFV domain, that will be specific for the target protein, and an intracellular CD3 ζ domain with tyrosin-kinase activity, that will be responsible for the CAR-T activation upon recognition of the target. In hypercholesterolemia, the CAR-T option has been conceived for the reconstitution of the LDLR in case of genetic deficiency. In fact, the re-injected autologous engineered CAR expressing leukocytes home back to the hematopoietic niche and locally foster the establishment of a new cell lineage expressing the LDLR.

The long-term effect of these options is still debated and the oscillatory concentration of circulating reinjected T cells leaves unresolved the question whether the expansion of the genetically modified CARs is proportional to the kinetics of CAR-T cells engraftment. Furthermore, the homing capacity of CAR-T engineered T cells could be a critical problem, in light of recent data testing the use of such treatment for solid tumors [48]. This aspect might be even more relevant for anti-atherosclerotic therapies which require large extraction of the cells at the site of action. Finally, detailed PK studies are also required for these strategies to interpret the favorable, but variable, efficacy profiles among individuals.

As for the safety profile, as compared to adenoviruses, today patient-derived autologous cells are known to have the advantage of a lower genotoxic potential and lower risk of insertional oncogenesis as compared to the inoculation of a heterologous engineered viral or HIV-derived lentiviral DNA vectors.

Biotechnological strategies targeting gene transcripts

Intervening on RNA has so far required the development of gene silencing tools (**Figure 4**). By silencing gene expression (not its structure) these strategies rapidly and effectively modulate the functionality and activity of downstream transcripts. Small interfering RNAs (siRNAs) are a very promising example of this approach since they lead to the degradation of different targets (not only mRNA, but also transcription factors, non-coding RNAs or microRNAs), when guided to their target mRNA by a specific RNA-induced silencing complex (RISC). This in turn represses gene activity and prevents the coding of altered proteins associated diseases. Low stability in circulation and appropriate targeting susceptibility to endonucleases were initial siRNA pitfalls, but further biotechnological refinements (either by inclusion into lipid nanoparticles, to enhance penetration in the nucleus of hepatocytes, or by synthesis of glycoconjugates to favor siRNA resistance against endonucleases) improved these unfavorable PK properties.

By contrast to siRNA, the principle of the antisense oligonucleotides (ASOs) approach, is to inoculate a specific single-stranded DNA molecule (sequences of 15 up to 100 nucleotides) complementary to a specific mRNA sequence,

preventing its ribosomal translation and promoting its degradation by enzymes with RNase H activity. Thanks to their structure and favorable PK profile (high affinity for circulating proteins), ASOs rapidly distribute in the body, reaching maximal hepatic concentrations in 3-4 hours following SC injection. These PK advantages led to a significant impulse in the use of ASOs, with a valuable success against a higher number of players in lipid metabolism as compared to siRNAs. In fact, a siRNA against PCSK9 (Inclisiran) has reached the market while ASOs against the atherogenic Apo(a), Angptl3 and Apolipoprotein CIII (apoCIII) are under development. However, thrombocytopenia represents a common side effect of different ASOs [49], that has been also encountered in randomized trials against these proteins. In a recent conference-abstract, two CpG-rich phosphorothioate deoxyoligonucleotide (PS ODN) sequences ASOs (818290 and 120704) and two non-CpG 2'-MOE containing sequences ASOs (104838, causing liver and spleen sequestration of platelets, and 501861) were titrated both in whole blood and in platelet-rich plasma [50]. Of note, only the two CpG-rich PS ODN ASOs triggered a spontaneous platelet aggregation in platelet-rich plasma, which was reverted by the injection of a tyrosine-kinase inhibitor [51]. By contrast, platelet aggregation was observed in all four ASOs tested. Thereby, these data suggest that the effect of ASOs on platelet stability is independent of the presence of the 2^{I}-MOE sequence and that cellular mechanisms controlled by the activity of tyrosine-kinase should be better deciphered.

siRNA to modulate PCSK9 expression

Inclisiran is a SC delivered and a long-acting synthetic siRNA that binds to PCSK9 mRNA favoring its degradation, thus leading to PCSK9 plasma levels reduction, increase of LDLR expression and reduction of hypercholesterolemia. Inclisiran is an oligonucleotide conjugated to a trivalent N-acetylgalactosamine (GalNAC) carbohydrate residue that ensures a liver specific uptake by the asialoglycoprotein receptors (ASGPRs) selectively present on the extracellular surface of hepatocytes [52]. The three GalNAC molecules are attached to the 3' terminal of the siRNA by using a triantennary spacer thus increasing the affinity for ASGPR [53]. Thanks to the GalNAC residues, the majority of the dose is delivered to the liver. This modification allows also for an increased potency, reduces off-targets effects and reduces dose requirements thus lowering production costs [54,55]. Following an acidification of the endosome, the GalNAC-siRNa complex dissociates from the ASPGR. Part of the oligonucleotides remains trapped inside the endosomes, serving as an intracellular deposit for the drug, and only a small amount is released slowly over time and loaded into RISC [56,57]. The binding to RISC guides the siRNA to PCSK9 mRNA blocking its translation and targeting it to RISC-mediated degradation (**Figure 5**). This strand-RISC complex has a short plasma half-life, but a prolonged functional half-life in hepatocytes (it remains intact and is able to block and degrade repeatedly PCSK9 mRNA molecules up to several weeks [55,57]). Additional phosphorothioate, 2'-O-methyl nucleotide and 2'-fluoro nucleotide modifications have improved the stability against nucleases and biological activity [53]. After a SC injection, inclisiran reaches its maximal concentration and area under the curve in a linear relationship with its dose [58]. Peak plasma levels of inclisiran occur about 4 hours after first administration [59]. No drug is detectable in plasma within 24 to 48 hours after dosing, with principal elimination through the urinary route [60,61]. This PK profile support the efficacy of the compound, since the intravenous injection of this siRNA in animal models causes reduction of up to -70% in PCSK9 protein at a dose of 1 mg/kg of body mass, and up to -85% at the dose of 3 mg/kg that lasted almost one month, with parallel reduction of -60% in LDL-C, and a slow return to baseline levels in three to four months [62,63]. The promising pre-clinical results were maintained also in humans where inclisiran injection resulted in the reduction of PCSK9 levels up to 84%, and of LDL-C up to 52% [64]. Inclisiran has a good safety profile, with myalgia, headache, fatigue, nasopharyngitis,

back pain, dizziness, diarrhea, and hypertension as the most common adverse events reported [65]. By contrast, inclisiran did not affect liver or kidney functions, creatine kinase, high-sensitivity C-reactive protein, or platelet levels [66] and rarely caused symptoms of immune system activation [67]. Anti-inclisiran antibodies were present at low tit er, mostly transiently, in about 2% of the patients treated with the drug, but they were not associated with any changes in pharmacological activities or clinical parameters [64]. Inclisiran did not cause any sign of peripheral neuropathy, a phenomenon generally observed with other siRNAs [68]. This evidence may be explained by the peculiar structure of the GalNAC conjugation with synthetic strands bound in inclisiran, that strongly limit its peripheral tissue activity.

siRNAs to modulate apo(a) expression

Specific RNA targeting has been recently directed against the apo(a), a single molecule characterized by repeated copies of a proxy structure of the plasminogen kringle (KIV), covalently bound to the apolipoprotein B-100 (apoB-100) of an LDL by a disulfide bond. Lysine-binding sites present in some of the kringles are the functional clues for the molecular pathology of Lp(a), as the KIV10 contains both a strong leucine-binding site that allows the interactions with fibrin and binding sites for oxidized phospholipids (oxPL). This structure forms the Lp(a) that i) promotes the adhesion of circulating cells on vascular endothelial barrier; ii) activates endothelial remodeling promoting metabolic reprogramming, and iii) induces inflammatory activation of tissue resident macrophages in atherosclerotic lesions, through oxPL locally released. Up to 90% of plasma Lp(a) levels are genetically driven by the *LPA* locus in humans, being the primary contributor for inter-individual differences in apo(a) length. Apo(a) is determined by the copy number of KIV2 region, and it is inversely related to circulating lipoprotein levels. These fundamental observations rationalized the development of a liver-specific inhibition of Lp(a) expression to blunt its hepatic production, rather than targeting its circulating form **(Figure 4)**.

In this attempt, a brand-new RNAi therapeutic compound ("Olpasiran" or "AMG 890") has been designed. The long-range goal was a larger time-window with less-frequent dosing schedules [69]. After a single injection of the maximal dose (225 mg), concentrations below 10 ng/ml can be found in plasma 24 hours later, in line with the PK profile of other small interfering molecules. In transgenic mice and non-human primates, three weekly SC injections resulted in 85% up to 98% reduction of Lp(a) level lasting for more than one month, while in humans with elevated Lp(a) similar reduction rates remained stable over six months. In terms of safety, no particular concerns of toxicity were reported [70].

Additional data are still required, and a further development of this promising biotechnological approach will bolster the cardiovascular drug pipeline in the near future.

ASOs to modulate apo(a) expression

So far, promising experiences have been gathered by studies where the AOS approach was tested against apo (a). When a human apo(a)-specific ASO was tested in either a mouse model expressing a human LPA construct (8K-LPA mouse), a mouse model expressing the human APOB and the human LPA construct (8K-LPA), or a mouse model expressing the natural human apo(a) gene in a yeast artificial chromosome (12K-LPA) [71]), the greatest efficacy was observed in the third model, in which the hepatic apo(a) mRNA expression was reduced by up to 87% compared with the 52% reduction observed in the first and second mouse models. Accordingly, circulating protein levels were reduced by 84% and oxPL content by 65%. These findings translated at clinical level, since a randomized clinical trial showed that this ASO, at the maximal dose of 20

mg weekly, robustly reduced circulating Lp(a) by 70% up to 92% in patients with elevated Lp(a) and in those with atherosclerotic cardiovascular disease [72].

These important pharmacological effects are supported by the PK profile of these compounds. Both -APO(a)Rx and APO(a)-LRx oligonucleotides, administrated subcutaneously, are characterized by the implementation of phosphodiester groups and deoxyribose or 2^{I}-O-methoxyethil-modified ribose ("2^{I}-MOE ASOs") at their extremities. This provides significant pharmacokinetic and pharmacodynamics advances, including an increased affinity for the target, hepatic tissue specificity, a reduced susceptibility to cellular endonuclease activity, an elevated protein binding and a delayed urinary elimination [73]. Together these properties allowed a significant reduction (up to one log10-fold) of the required dose to halve basal Lp(a) levels, while increasing its bioavailability (compared with the ASO lacking the 2^{I}-MOE modification).

Due to the potential involvement of Lp(a) in fibrinolysis, a possible effect of such treatments in platelets count has been controversially discussed in the literature with some in favor [74–78] and other against [79,80]. The efficacy of these compounds on the remodeling of the atherogenic oxidized phospholipids characterizing Lp(a) still remains to be analyzed [81].

ASO to modulate Angptl3

The development of the ASO against Angtpl3 (IONIS-ANGPTL3-LRx) showed robust efficacy in pre-clinical models, where it provided important anti-atherosclerotic effects in LDLRKO mice reducing TG up to 63% as compared to untreated animals. Silencing Angtpl3 mRNA also exerted anti-cholesterol effects that appeared to be independent from the residual activity of the LDLR. In fact, a dual Angptl3 and PCSK9 siRNA injected in heterozygous LDLRKO mice induced cumulative LDL-C reduction as compared to what observed with the single siRNAs silencing either PCSK9 or angptl3 alone. In addition, Angptl3 RNA silencing reduced VLDL size and lipid content [82] and did not provide robust LDL-C lowering in mice with genetic deficiency of endothelial lipase [83]. These observations suggest that cholesterol-lowering and LDL reduction might more likely depend on an enhanced catabolic rate of all cholesterol rich lipoproteins. In parallel, encouraging data came from clinical observations, where in phase 1 studies anti-Angplt3 ASO provided dose-dependent reductions in TGs (63%). IONIS-ANGPTL3-LRx demonstrated a similar PK profile as compared to those against apo(a). In fact, this ASO showed a half-life of about 3-5 weeks and a linear, dose-dependent, increase of maximum plasma concentration within 10-60 mg doses, after a rapid systemic distribution [84].

ASO to modulate ApoCIII expression

ApoCIII is a glycoprotein principally produced in the liver and in the intestine and it increases TG levels by an "LPL-dependent" mechanism, where apoCIII inhibits LPL-mediated lipolysis of remnant cholesterol particles (RLPs). LOF genetic mutations in *APOC3* locus result into a reduction up to -43% in TGs, and the heterozygous APOC3 genetic inhibition results into higher conversion of VLDL to LDL and, to a minor extent, in poorly increased hepatic uptake of RLPs. APOC3 LOF variants result into an extremely reduced risk of coronary calcifications, confirming that specific inhibition of apoCIII might exert peculiar anti-atherosclerotic effects by rescuing the activity of lipases and reducing cholesterol content in RLPs. Such strategies have been initially developed in 2015, and recently the first mRNA antisense oligonucleotide showed massive TG reduction in patients affected by Familial Chylomicronemia Syndrome (FCS), who display TGs> 1000 mg/dl even in fasting due to a complete absence of LPL.

The ASO sequence was coupled to a N-Acetylgalactosamine moiety (namely AKCEA-APOCIII-LRx or "Volanesorsen") to improve specific uptake in the liver and increase its potency by at least fifteen folds as compared to the non-conjugated compound. The compound proved its efficacy in primates [85] as well as in rodents, where fasting hepatic APOC3 mRNA inhibition in LDLR deficient mice reduced TGs by about 40% [85]. Clinically, apoCIII inhibition by ASO was effective in FCS patients, where a -70% reduction in TG content of RLPs under an apoC-III targeted therapy clearly demonstrates that the benefit from such treatment comes also from the rescue of a second, "LPL-independent", mechanism, by which apoCIII reduces the cholesterol content of remnant particles produced by the liver. However, such reduction were not achieved nor in double-blind, placebo-controlled, dose-escalation study in healthy subjects [86], neither in patients with diabetes-associated atherogenic dyslipidemia [86].

Volanesorsen rapidly distributes systemically, it has a long half-life and exhibits a consistent hepatic extraction useful for its pharmacodynamics interaction (it is eliminated via urinary route in 24 hours after injections, when the hepatic concentration is 50% lower than that in kidneys) [85,87].

The extraordinary efficacy of Volanesorsen initially corresponded, despite an overall acceptable safety profile, to a significant occurrence of severe thrombocytopenia [86]. In fact, 15 out of 33 patients of the APPROACH trials initially treated with this compound presented thrombocytopenia, with reductions of platelets count up to 100,000 cells/uL, which required dose de-escalation or even the exit from the study for some patients [84]. The conjugation with a GalNAC residue in the AKCEA-APOCIII-LRx demonstrated important reduction in the occurrence of these side effects in healthy humans [88]. Further studies regarding the safety of this biotechnological strategy in contrasting thrombocytopenia is the aim of a planned Phase III trial which will enroll subjects with hypertriglyceridemia and established cardiovascular disease (ISIS 678354; ClinicalTrials.gov Identifier NCT03385239).

Conclusions

In recent years, the learning from genetics led to the development of new approaches in modulating the production or function of the identified targets using state-of-the-art technologies. Potential pharmacological targets, validated by biological and genetic studies can now be modulated by these new biotechnological tools, which allow to intervene also at the genomic or transcriptomic levels. Different experimental approaches have been attempted: besides very promising therapeutic tools such as ASOs, mAbs, siRNAs and CRISPR-Cas9, a more recent approach led to the development of vaccines against PCSK9. However, we must keep in mind that these interesting and potentially very effective interventions still require (except for anti-PCSK9 mAbs) their validation in reducing cardiovascular risk in cardiovascular outcome trials and must demonstrate their long-term safety. An in depth understanding of the pre-clinical aspects of these drugs, including the study of their PK properties, might help in this direction as well as in setting concomitant therapies in high and very-high risk patients.

What will the future hold? To date we have used the classical pharmacological approach of reducing the activity/quantity of key proteins or enzymes. Some of the new technologies (e.g. CRISP-Cas9) can now be exploited to upregulate or modulate the production/activity of targets that in some pathologies are downregulated or even completely knocked-out: a completely new field in pharmacology.

Figure legends

Figure 1 - Summary of biotechnological strategies currently in clinical practice, under development or withdrawn for reducing cholesterol. The figure summarizes the different biotechnological approaches based on their site of injection (Alipogene Triparvovec), their metabolic target (gene editing, gene silencing or antibodies against PCSK9, Apo(a), ApocIII, Angptl3, Angplt4) or their cell target (CAR-T approach).

Figure 2 – Pharmacological effects of the monoclonal antibodies against Angptl3 and Pcsk9. The black line with black turbot at the end indicates an inhibitory effect. LDL= Low Density Lipoproteins; LDL-R= LDL receptor; TGRLs= Triglyceride Rich Lipoproteins.

Figure 3 – CRISPR-Cas9 based gene editing of *ANGPLT3* and *PCSK9* genes.

Figure 4 – Pharmacological effects of the biotechnological anti-Apo(a) approaches. The red line with the red cross at the end indicates the inhibitory effect of Apo(a) ASOs. The blue line symbolizes the Apo(a) RNA-interfering approach.

Figure 5 – Inclisiran; molecular structure and pharmacological mechanism. Panel (A) summarizes the molecular structure of Inclisiran. Different colored dots represent the biotechnological changes in the nucleotide structure. Panel (B) resumes the passages from the entry of Inclisiran in the hepatocyte via the interaction with asialoglycoprotein receptors (ASGPR) up to the degradation of PCSK9 mRNA induced by the siRNA guide of Inclisiran and the RNA-induced silencing complex (RISC).

• **Author Declarations**

O **Ethics approval and consent to participate**

'Not applicable'

O **Consent for publication**

Publication of the article is approved by all authors and tacitly by the responsible authorities of the University of Milan and Multimedica, where the work was carried out. If the article is accepted, it will not be published elsewhere by the authors, including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder.

All authors have read the manuscript and approved its submission to *Cardiovascular Drug therapy*.

O **Availability of data and materials**

'Not applicable'

O **Competing interests**

- A.L. Catapano has received honoraria, lecture fees, or research grants from: Sigma-Tau, Menarini, Kowa, Recordati, Eli Lilly, Astrazeneca, Mediolanum, Pfizer, Merck, Sanofi, Aegerion, Amgen, Genzyme, Bayer, Sanofi and Regeneron Daiichi-Sankyo.

- A. Corsini has received honoraria from Amgen, Astra Zeneca, Daiichi Sankyo, Sanofi, Novartis, DOC, TEVA and Servier, and grants from Daiichi Sankyo and PIAM.

- S. Bellosta, C. Rossi, A.S. Alieva, and A. Baragetti have no relevant financial or non-financial interests to disclose.

O **Funding**

This work has been supported by the Italian Ministry of Health - Ricerca Corrente - IRCCS MultiMedica.

O **Authors' contributions**

Conceptualization, literature search, writing original draft: S. Bellosta, A. Baragetti; Review and editing: C. Rossi, A.S. Alieva, A. Corsini; Supervision: A.L. Catapano.

O **Acknowledgements**

The authors' research is supported by: EXTRALIPO "New lipid-oriented pharmacological and chemical approaches to discriminate and unravel extracellular vesicles biological functions" Bando SEED – PSR 2019 (SB); Russian Ministry of Science and Higher Education (Agreement No. 075-15-2020-901) (ASA); H2020 REPROGRAM PHC-03-2015/667837-2 (ALC); Fondazione Cariplo 2015-0524 and 2015-0564 (ALC); ERANET ER-2017-2364981 (ALC); Ministry of Health-IRCCS MultiMedica GR-2011-02346974 (ALC); PRIN 2017H5F943 (ALC); "Cibo, Microbiota, Salute" by "Vini di Batasiolo S.p.A" AL_RIC19ABARA_01 (AB), "Post-Doctoral Fellowship 2020" by "Fondazione Umberto Veronesi" 2020-3318 (AB); a research grant 2021 from "the Peanut Institute".

O **Authors' information** (optional)

• **Compliance with Ethical Standards**

O **Disclosure of potential conflicts of interest**

- A.L. Catapano has received honoraria, lecture fees, or research grants from: Sigma-Tau, Manarini, Kowa, Recordati, Eli Lilly, Astrazeneca, Mediolanum, Pfizer, Merck, Sanofi, Aegerion, Amgen, Genzyme, Bayer, Sanofi and Regeneron Daiichi-Sankyo.

- A. Corsini has received honoraria from Amgen, Astra Zeneca, Daiichi Sankyo, Sanofi, Novartis, DOC, TEVA and Servier, and grants from Daiichi Sankyo and PIAM.

- S. Bellosta, C. Rossi, A.S. Alieva, A. Baragetti have no relevant financial or non-financial interests to disclose.

O **Research involving Human Participants and/or Animals**

'Not applicable'

O **Informed consent**

'Not applicable'

References

1. Collaboration, C.T.T. (CTT) Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170 000 participants in 26 randomised trials. *Lancet* **2010**, *376*, 1670–1681, doi:10.1016/S0140-6736(10)61350-5.

2. Cannon, C.P.; Blazing, M.A.; Giugliano, R.P.; McCagg, A.; White, J.A.; Theroux, P.; Darius, H.; Lewis, B.S.; Ophuis, T.O.; Jukema, J.W.; et al. Ezetimibe Added to Statin Therapy after Acute Coronary Syndromes. *http://dx.doi.org/10.1056/NEJMoa1410489* **2015**, *372*, 2387–2397, doi:10.1056/NEJMOA1410489.

3. Bhatt, D.L.; Steg, P.G.; Miller, M.; Brinton, E.A.; Jacobson, T.A.; Ketchum, S.B.; Ralph T. Doyle, J.; Juliano, R.A.; Jiao, L.; Granowitz, C.; et al. Cardiovascular Risk Reduction with Icosapent Ethyl for Hypertriglyceridemia. *https://doi.org/10.1056/NEJMoa1812792* **2018**, *380*, 11–22, doi:10.1056/NEJMOA1812792.

4. Packard, C.; Chapman, M.J.; Sibartie, M.; Laufs, U.; Masana, L. Intensive low-density lipoprotein cholesterol lowering in cardiovascular disease prevention: opportunities and challenges. *Heart* **2021**, *107*, 1369–1375, doi:10.1136/heartjnl-2020-318760.

5. Ghetie, V.; Ward, E.S. Multiple roles for the major histocompatibility complex class I-related receptor FcRn. *Annu. Rev. Immunol.* 2000, *18*, 739–766.

6. Tabrizi, M.A.; Tseng, C.M.L.; Roskos, L.K. Elimination mechanisms of therapeutic monoclonal antibodies. *Drug Discov. Today* 2006, *11*, 81–88.

7. Raghavan, M.; Bonagura, V.R.; Morrison, S.L.; Bjorkman, P.J. Analysis of the pH Dependence of the Neonatal Fc Receptor/Immunoglobulin G Interaction Using Antibody and Receptor Variants. *Biochemistry* **1995**, *34*, 14649–14657, doi:10.1021/bi00045a005.

8. Foltz, I.N.; Karow, M.; Wasserman, S.M. Evolution and emergence of therapeutic monoclonal antibodies what cardiologists need to know. *Circulation* **2013**, *127*, 2222–2230, doi:10.1161/CIRCULATIONAHA.113.002033.

9. Regeneron *Alirocumab (SRA236553/REGN727). Investigator brochure*; 2014;

10. Schwartz, G.G.; Steg, P.G.; Szarek, M.; Bhatt, D.L.; Bittner, V.A.; Diaz, R.; Edelberg, J.M.; Goodman, S.G.; Hanotin, C.; Harrington, R.A.; et al. Alirocumab and Cardiovascular Outcomes after Acute Coronary Syndrome. *N. Engl. J. Med.* **2018**, *379*, 2097–2107, doi:10.1056/NEJMoa1801174.

11. Guedeney, P.; Giustino, G.; Sorrentino, S.; Claessen, B.E.; Camaj, A.; Kalkman, D.N.; Vogel, B.; Sartori, S.; De Rosa, S.; Baber, U.; et al. Efficacy and safety of alirocumab and evolocumab: a systematic review and meta-analysis of randomized controlled trials. *Eur. Heart J.* **2019**, doi:10.1093/EURHEARTJ/EHZ430.

12. Kühnast, S.; Van Der Hoorn, J.W.A.; Pieterman, E.J.; Van Den Hoek, A.M.; Sasiela, W.J.; Gusarova, V.; Peyman, A.; Schäfer, H.L.; Schwahn, U.; Jukema, J.W.; et al. Alirocumab inhibits atherosclerosis, improves the plaque morphology, and enhances the effects of a statin. *J. Lipid Res.* **2014**, *55*, 2103–2112, doi:10.1194/jlr.M051326.

13. Pouwer, M.G.; Pieterman, E.J.; Worms, N.; Keijzer, N.; Jukema, J.W.; Gromada, J.; Gusarova, V.; Princen, H.M.G. Alirocumab, evinacumab, and atorvastatin triple therapy regresses plaque lesions and improves lesion composition in mice. *J. Lipid Res.* **2020**, *61*, 365–375, doi:10.1194/jlr.RA119000419.

14. Shen, Y.; Li, H.; Zhao, L.; Li, G.; Chen, B.; Guo, Q.; Gao, B.; Wu, J.; Yang, T.; Jin, L.; et al. Increased half-life and enhanced potency of Fc-modified human PCSK9 monoclonal antibodies in primates. *PLoS One* **2017**, *12*, doi:10.1371/journal.pone.0183326.

15. Kasichayanula, S.; Grover, A.; Emery, M.G.; Gibbs, M.A.; Somaratne, R.; Wasserman, S.M.; Gibbs, J.P. Clinical Pharmacokinetics and Pharmacodynamics of Evolocumab, a PCSK9 Inhibitor. *Clin. Pharmacokinet.* 2018, *57*, 769–779.

16. Chan, J.C.Y.; Piper, D.E.; Cao, Q.; Liu, D.; King, C.; Wang, W.; Tang, J.; Liu, Q.; Higbee, J.; Xia, Z.; et al. A proprotein convertase subtilisin/kexin type 9 neutralizing antibody reduces serum cholesterol in mice and nonhuman primates. *Proc. Natl. Acad. Sci.* **2009**, *106*, 9820–9825, doi:10.1073/pnas.0903849106.

17. Qi, Z.; Hu, L.; Zhang, J.; Yang, W.; Liu, X.; Jia, D.; Yao, Z.; Chang, L.; Pan, G.; Zhong, H.; et al. PCSK9 Enhances Platelet Activation, Thrombosis, and Myocardial Infarct Expansion by Binding to Platelet CD36. *Circulation* **2020**, doi:10.1161/circulationaha.120.046290.

18. Wu, Y.; Xu, M.J.; Cao, Z.; Yang, C.; Wang, J.; Wang, B.; Liu, J.; Wang, Y.; Xian, X.; Zhang, F.; et al. Heterozygous ldlr-deficient hamster as a model to evaluate the efficacy of PCSK9 antibody in hyperlipidemia and atherosclerosis. *Int. J. Mol. Sci.* **2019**, *20*, doi:10.3390/ijms20235936.

19. Amgen *Evolocumab (AMG 145). Investigator brochure*; 2014;

20. Khoshnejad, M.; Patel, A.; Wojtak, K.; Kudchodkar, S.B.; Humeau, L.; Lyssenko, N.N.; Rader, D.J.; Muthumani, K.; Weiner, D.B. Development of Novel DNA-Encoded PCSK9 Monoclonal Antibodies as Lipid-Lowering Therapeutics. *Mol. Ther.* **2019**, *27*, 188–199, doi:10.1016/j.ymthe.2018.10.016.

21. FDA EVKEEZ (evinacumab-dgnb) injection, for intravenous use Available online: www.accessdata.fda.gov/drugsatfda_docs/label/2021/761181s000lbl.pdf.

22. Desai, U.; Lee, E.C.; Chung, K.; Gao, C.; Gay, J.; Key, B.; Hansen, G.; Machajewski, D.; Platt, K.A.; Sands, A.T.; et al. Lipid-lowering effects of anti-angiopoietin-like 4 antibody recapitulate the lipid phenotype found in angiopoietin-like 4 knockout mice. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 11766–11771, doi:10.1073/pnas.0705041104.

23. Harada-Shiba, M.; Ali, S.; Gipe, D.A.; Gasparino, E.; Son, V.; Zhang, Y.; Pordy, R.; Catapano, A.L. A randomized study investigating the safety, tolerability, and pharmacokinetics of evinacumab, an ANGPTL3 inhibitor, in healthy Japanese and Caucasian subjects. *Atherosclerosis* **2020**, *314*, 33–40, doi:10.1016/j.atherosclerosis.2020.10.013.

24. Raal, F.J.; Rosenson, R.S.; Reeskamp, L.F.; Hovingh, G.K.; Kastelein, J.J.P.; Rubba, P.; Ali, S.; Banerjee, P.; Chan, K.-C.; Gipe, D.A.; et al. Evinacumab for Homozygous Familial Hypercholesterolemia. *N. Engl. J. Med.* **2020**, *383*, 711–720, doi:10.1056/nejmoa2004215.

25. Gusarova, V.; Alexa, C.A.; Wang, Y.; Rafique, A.; Kim, J.H.; Buckler, D.; Mintah, I.J.; Shihanian, L.M.; Cohen, J.C.; Hobbs, H.H.; et al. ANGPTL3 blockade with a human monoclonal antibody reduces plasma lipids in dyslipidemic mice and monkeys. *J. Lipid Res.* **2015**, *56*, 1308–1317, doi:10.1194/jlr.M054890.

26. Lee, E.C.; Desai, U.; Gololobov, G.; Hong, S.; Feng, X.; Yu, X.C.; Gay, J.; Wilganowski, N.; Gao, C.; Du, L.L.; et

al. Identification of a new functional domain in angiopoietin-like 3 (ANGPTL3) and angiopoietin-like 4 (ANGPTL4) involved in binding and inhibition of lipoprotein lipase (LPL). *J. Biol. Chem.* **2009**, *284*, 13735–13745, doi:10.1074/jbc.M807899200.

27. Fattori, E.; Cappelletti, M.; Lo Surdo, P.; Calzetta, A.; Bendtsen, C.; Ni, Y.G.; Pandit, S.; Sitlani, A.; Mesiti, G.; Carfí, A.; et al. Immunization against proprotein convertase subtilisin-like/kexin type 9 lowers plasma LDL-cholesterol levels in mice. *J. Lipid Res.* **2012**, *53*, 1654–1661, doi:10.1194/jlr.M028340.

28. Galabova, G.; Brunner, S.; Winsauer, G.; Juno, C.; Wanko, B.; Mairhofer, A.; Luhrs, P.; Schneeberger, A.; von Bonin, A.; Mattner, F.; et al. Peptide-based anti-PCSK9 vaccines - an approach for long-term LDLc management. *PLoS One* **2014**, *9*, e114469, doi:10.1371/journal.pone.0114469.

29. Landlinger, C.; Pouwer, M.G.; Juno, C.; van der Hoorn, J.W.A.; Pieterman, E.J.; Jukema, J.W.; Staffler, G.; Princen, H.M.G.; Galabova, G. The AT04A vaccine against proprotein convertase subtilisin/kexin type 9 reduces total cholesterol, vascular inflammation, and atherosclerosis in APOE*3Leiden.CETP mice. *Eur Hear. J* **2017**, *38*, 2499–2507, doi:10.1093/eurheartj/ehx260.

30. Kawakami, R.; Nozato, Y.; Nakagami, H.; Ikeda, Y.; Shimamura, M.; Yoshida, S.; Sun, J.; Kawano, T.; Takami, Y.; Noma, T.; et al. Development of vaccine for dyslipidemia targeted to a proprotein convertase subtilisin/ kexin type 9 (PCSK9) epitope in mice. *PLoS One* **2018**, *13*, doi:10.1371/journal.pone.0191895.

31. Ji, H.; Wu, G.; Li, Y.; Wang, K.; Xue, X.; You, S.; Wu, S.; Ren, T.; He, B.; Shi, X. Self-Albumin Camouflage of Carrier Protein Prevents Nontarget Antibody Production for Enhanced LDL-C Immunotherapy. *Adv. Healthc. Mater.* **2020**, *9*, doi:10.1002/adhm.201901203.

32. Crossey, E.; Amar, M.J.; Sampson, M.; Peabody, J.; Schiller, J.T.; Chackerian, B.; Remaley, A.T. A cholesterol-lowering VLP vaccine that targets PCSK9. *Vaccine* **2015**, *33*, 5747–5755, doi:10.1016/j.vaccine.2015.09.044.

33. Pan, Y.; Zhou, Y.; Wu, H.; Chen, X.; Hu, X.; Zhang, H.; Zhou, Z.; Qiu, Z.; Liao, Y. A Therapeutic Peptide Vaccine Against PCSK9. *Sci Rep* **2017**, *7*, 12534, doi:10.1038/s41598-017-13069-w.

34. Wu, D.; Zhou, Y.; Pan, Y.; Li, C.; Wang, Y.; Chen, F.; Chen, X.; Yang, S.; Zhou, Z.; Liao, Y.; et al. Vaccine Against PCSK9 Improved Renal Fibrosis by Regulating Fatty Acid β-Oxidation. *J. Am. Heart Assoc.* **2020**, *9*, doi:10.1161/JAHA.119.014358.

35. Wu, D.; Pan, Y.; Yang, S.; Li, C.; Zhou, Y.; Wang, Y.; Chen, X.; Zhou, Z.; Liao, Y.; Qiu, Z. PCSK9Qβ-003 Vaccine Attenuates Atherosclerosis in Apolipoprotein E-Deficient Mice. *Cardiovasc. Drugs Ther.* **2020**, doi:10.1007/s10557-020-07041-6.

36. Momtazi-Borojeni, A.A.; Jaafari, M.R.; Badiee, A.; Banach, M.; Sahebkar, A. Therapeutic effect of nanoliposomal PCSK9 vaccine in a mouse model of atherosclerosis. *BMC Med.* **2019**, *17*, 223, doi:10.1186/s12916-019-1457-8.

37. Ding, Q.; Strong, A.; Patel, K.M.; Ng, S.L.; Gosis, B.S.; Regan, S.N.; Cowan, C.A.; Rader, D.J.; Musunuru, K. Permanent alteration of PCSK9 with in vivo CRISPR-Cas9 genome editing. *Circ. Res.* **2014**, *115*, 488–492, doi:10.1161/CIRCRESAHA.115.304351.

38. Unger, T.; Peleg, Y. Recombinant Protein Expression in the Baculovirus-Infected Insect Cell System Substrate

specificity of GroEL View project Chapter 13 Recombinant Protein Expression in the Baculovirus-Infected Insect Cell System. *Methods Mol. Biol. 800*, doi:10.1007/978-1-61779-349-3_13.

39. Silva Lima, B.; Videira, M.A. Toxicology and Biodistribution: The Clinical Value of Animal Biodistribution Studies. *Mol. Ther. - Methods Clin. Dev.* 2018, *8*, 183–197.

40. Ferreira, V.; Petry, H.; Salmon, F. Immune responses to AAV-vectors, The Glybera example from bench to bedside. *Front. Immunol.* **2014**, *5*, doi:10.3389/fimmu.2014.00082.

41. Deltcheva, E.; Chylinski, K.; Sharma, C.M.; Gonzales, K.; Chao, Y.; Pirzada, Z.A.; Eckert, M.R.; Vogel, J.; Charpentier, E. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* **2011**, *471*, 602–607, doi:10.1038/nature09886.

42. Jinek, M.; Chylinski, K.; Fonfara, I.; Hauer, M.; Doudna, J.A.; Charpentier, E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science (80-.).* **2012**, *337*, 816–821, doi:10.1126/science.1225829.

43. Zalatan, J.G.; Lee, M.E.; Almeida, R.; Gilbert, L.A.; Whitehead, E.H.; La Russa, M.; Tsai, J.C.; Weissman, J.S.; Dueber, J.E.; Qi, L.S.; et al. Engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds. *Cell* **2015**, *160*, 339–350, doi:10.1016/j.cell.2014.11.052.

44. Chadwick, A.C.; Evitt, N.H.; Lv, W.; Musunuru, K. Reduced blood lipid levels with in vivo CRISPR-Cas9 base editing of ANGPTL3. *Circulation* 2018, *137*, 975–977.

45. Zheng, J.; Huynh, H.D.; Umikawa, M.; Silvany, R.; Zhang, C.C. Angiopoietin-like protein 3 supports the activity of hematopoietic stem cells in the bone marrow niche. *Blood* **2011**, *117*, 470–479, doi:10.1182/blood-2010-06-291716.

46. Chadwick, A.C.; Wang, X.; Musunuru, K. In Vivo Base Editing of PCSK9 (Proprotein Convertase Subtilisin/Kexin Type 9) as a Therapeutic Alternative to Genome Editing. *Arterioscler. Thromb. Vasc. Biol.* **2017**, *37*, 1741–1747, doi:10.1161/ATVBAHA.117.309881.

47. Musunuru, K.; Chadwick, A.C.; Mizoguchi, T.; Garcia, S.P.; DeNizio, J.E.; Reiss, C.W.; Wang, K.; Iyer, S.; Dutta, C.; Clendaniel, V.; et al. In vivo CRISPR base editing of PCSK9 durably lowers cholesterol in primates. *Nature* **2021**, *593*, 429–434, doi:10.1038/s41586-021-03534-y.

48. D'Aloia, M.M.; Zizzari, I.G.; Sacchetti, B.; Pierelli, L.; Alimandi, M. CAR-T cells: The long and winding road to solid tumors review-article. *Cell Death Dis.* 2018, *9*.

49. Henry, S.P.; Narayanan, P.; Shen, L.; Bhanot, S.; Younis, H.S.; Burel, S.A. Assessment of the Effects of 2′-Methoxyethyl Antisense Oligonucleotides on Platelet Count in Cynomolgus Nonhuman Primates. *Nucleic Acid Ther.* **2017**, *27*, 197–208, doi:10.1089/nat.2017.0666.

50. Flierl, U.; Nero, T.L.; Lim, B.; Arthur, J.F.; Yao, Y.; Jung, S.M.; Gitz, E.; Pollitt, A.Y.; Zaldivia, M.T.K.; Jandrot-Perrus, M.; et al. Phosphorothioate backbone modifications of nucleotide-based drugs are potent platelet activators. *J. Exp. Med.* **2015**, *212*, 129–137, doi:10.1084/jem.20140391.

51. Lundberg Slingsby, M.H.; Couldwell, G.; Vijey, P.; Terkovich, B.E.; Noetzli, L.; Okazaki, R.; Thon, J.N.; Henry,

S.P.; Narayanan, P.; Italiano, J.E. Investigating Potential Mechanism(s) By Which ASO-Based Drugs Cause Thrombocytopenia. *Blood* **2018**, *132*, 3747–3747, doi:10.1182/blood-2018-99-116590.

52. Fitzgerald, K.; White, S.; Borodovsky, A.; Bettencourt, B.R.; Strahs, A.; Clausen, V.; Wijngaard, P.; Horton, J.D.; Taubel, J.; Brooks, A.; et al. A Highly Durable RNAi Therapeutic Inhibitor of PCSK9. *N Engl J Med* **2017**, *376*, 41–51, doi:10.1056/NEJMoa1609243.

53. Nair, J.K.; Willoughby, J.L.S.; Chan, A.; Charisse, K.; Alam, M.R.; Wang, Q.; Hoekstra, M.; Kandasamy, P.; Kel'in, A. V.; Milstein, S.; et al. Multivalent N -Acetylgalactosamine-Conjugated siRNA Localizes in Hepatocytes and Elicits Robust RNAi-Mediated Gene Silencing. *J. Am. Chem. Soc.* **2014**, *136*, 16958–16961, doi:10.1021/ja505986a.

54. Warden, B.A.; Duell, P.B. Inclisiran: A Novel Agent for Lowering Apolipoprotein B–containing Lipoproteins. *J. Cardiovasc. Pharmacol.* **2021**, *78*, e157–e174, doi:10.1097/FJC.0000000000001053.

55. Katzmann, J.L.; Packard, C.J.; Chapman, M.J.; Katzmann, I.; Laufs, U. Targeting RNA With Antisense Oligonucleotides and Small Interfering RNA in Dyslipidemias. *J. Am. Coll. Cardiol.* **2020**, *76*, 563–579, doi:10.1016/j.jacc.2020.05.070.

56. Springer, A.D.; Dowdy, S.F. GalNAc-siRNA Conjugates: Leading the Way for Delivery of RNAi Therapeutics. *Nucleic Acid Ther.* **2018**, *28*, 109–118, doi:10.1089/nat.2018.0736.

57. Khvorova, A. Oligonucleotide Therapeutics — A New Class of Cholesterol-Lowering Drugs. *N. Engl. J. Med.* **2017**, *376*, 4–7, doi:10.1056/NEJMp1614154.

58. Fitzgerald, K.; Frank-Kamenetsky, M.; Shulga-Morskaya, S.; Liebow, A.; Bettencourt, B.R.; Sutherland, J.E.; Hutabarat, R.M.; Clausen, V.A.; Karsten, V.; Cehelsky, J.; et al. Effect of an RNA interference drug on the synthesis of proprotein convertase subtilisin/kexin type 9 (PCSK9) and the concentration of serum LDL cholesterol in healthy volunteers: a randomised, single-blind, placebo-controlled, phase 1 trial. *Lancet* **2014**, *383*, 60–68, doi:10.1016/S0140-6736(13)61914-5.

59. Ray, K.K.; Stoekenbroek, R.M.; Kallend, D.; Nishikido, T.; Leiter, L.A.; Landmesser, U.; Wright, R.S.; Wijngaard, P.L.J.; Kastelein, J.J.P. Effect of 1 or 2 Doses of Inclisiran on Low-Density Lipoprotein Cholesterol Levels: One-Year Follow-up of the ORION-1 Randomized Clinical Trial. *JAMA Cardiol.* **2019**, *4*, 1067–1075, doi:10.1001/jamacardio.2019.3502.

60. Levin, A.A. Targeting therapeutic oligonucleotides. *N. Engl. J. Med.* **2017**, *376*, 86–88, doi:10.1056/NEJMcibr1613559.

61. Wright, R.S.; Collins, M.G.; Stoekenbroek, R.M.; Robson, R.; Wijngaard, P.L.J.; Landmesser, U.; Leiter, L.A.; Kastelein, J.J.P.; Ray, K.K.; Kallend, D. Effects of Renal Impairment on the Pharmacokinetics, Efficacy, and Safety of Inclisiran: An Analysis of the ORION-7 and ORION-1 Studies. *Mayo Clin. Proc.* **2020**, *95*, 77–89, doi:10.1016/j.mayocp.2019.08.021.

62. Frank-Kamenetsky, M.; Grefhorst, A.; Anderson, N.N.; Racie, T.S.; Bramlage, B.; Akinc, A.; Butler, D.; Charisse, K.; Dorkin, R.; Fan, Y.; et al. Therapeutic RNAi targeting PCSK9 acutely lowers plasma cholesterol in rodents and

LDL cholesterol in nonhuman primates. *Proc Natl Acad Sci U S A* **2008**, *105*, 11915–11920, doi:10.1073/pnas.0805434105.

63. Banerjee, Y.; Pantea Stoian, A.; Cicero, A.F.G.; Fogacci, F.; Nikolic, D.; Sachinidis, A.; Rizvi, A.A.; Janez, A.; Rizzo, M. Inclisiran: a small interfering RNA strategy targeting PCSK9 to treat hypercholesterolemia. *Expert Opin. Drug Saf.* **2021**, doi:10.1080/14740338.2022.1988568.

64. Ray, K.K.; Wright, R.S.; Kallend, D.; Koenig, W.; Leiter, L.A.; Raal, F.J.; Bisch, J.A.; Richardson, T.; Jaros, M.; Wijngaard, P.L.J.; et al. Two Phase 3 Trials of Inclisiran in Patients with Elevated LDL Cholesterol. *N. Engl. J. Med.* **2020**, *382*, 1507–1519, doi:10.1056/NEJMoa1912387.

65. Ray, K.K.; Corral, P.; Morales, E.; Nicholls, S.J. Pharmacological lipid-modification therapies for prevention of ischaemic heart disease: current and future options. *Lancet* 2019, *394*, 697–708.

66. Landmesser, U.; Haghikia, A.; Leiter, L.A.; Wright, R.S.; Kallend, D.; Wijngaard, P.; Stoekenbroek, R.; Kastelein, J.J.; Ray, K.K. Effect of inclisiran, the small-interfering RNA against proprotein convertase subtilisin/kexin type 9, on platelets, immune cells, and immunological biomarkers: a pre-specified analysis from ORION-1. *Cardiovasc. Res.* **2020**, doi:10.1093/cvr/cvaa077.

67. Ray, K.K.; Landmesser, U.; Leiter, L.A.; Kallend, D.; Dufour, R.; Karakas, M.; Hall, T.; Troquay, R.P.; Turner, T.; Visseren, F.L.; et al. Inclisiran in Patients at High Cardiovascular Risk with Elevated LDL Cholesterol. *N Engl J Med* **2017**, *376*, 1430–1440, doi:10.1056/NEJMoa1615758.

68. Chi, X.; Gatti, P.; Papoian, T. Safety of antisense oligonucleotide and siRNA-based therapeutics. *Drug Discov. Today* 2017, *22*, 823–833.

69. Dolgin, E. Lp(a)-lowering drugs bolster cardiovascular pipeline. *Nat. Rev. Drug Discov.* 2020, *19*, 154–155.

70. Abstract 17167: Targeting Apolipoprotein(a) With a Novel RNAi Delivery Platform as a Prophylactic Treatment to Reduce Risk of Cardiovascular Events in Individuals With Elevated Lipoprotein (a) | Circulation.

71. Merki, E.; Graham, M.; Taleb, A.; Leibundgut, G.; Yang, X.; Miller, E.R.; Fu, W.; Mullick, A.E.; Lee, R.; Willeit, P.; et al. Antisense oligonucleotide lowers plasma levels of apolipoprotein (a) and lipoprotein (a) in transgenic mice. *J. Am. Coll. Cardiol.* **2011**, *57*, 1611–1621, doi:10.1016/j.jacc.2010.10.052.

72. Tsimikas, S.; Karwatowska-Prokopczuk, E.; Gouni-Berthold, I.; Tardif, J.-C.; Baum, S.J.; Steinhagen-Thiessen, E.; Shapiro, M.D.; Stroes, E.S.; Moriarty, P.M.; Nordestgaard, B.G.; et al. Lipoprotein(a) Reduction in Persons with Cardiovascular Disease. *N. Engl. J. Med.* **2020**, *382*, 244–255, doi:10.1056/NEJMoa1905239.

73. Yu, R.Z.; Grundy, J.S.; Geary, R.S. Clinical pharmacokinetics of second generation antisense oligonucleotides. *Expert Opin. Drug Metab. Toxicol.* 2013, *9*, 169–182.

74. Loscalzo, J.; Weinfeld, M.; Fless, G.M.; Scanu, A.M. Lipoprotein(a), fibrin binding, and plasminogen activation. *Arteriosclerosis* **1990**, *10*, 240–245, doi:10.1161/01.atv.10.2.240.

75. Rouy, D.; Grailhe, P.; Nigon, F.; Chapman, J.; Angles-Cano, E. Lipoprotein(a) impairs generation of plasmin by fibrin-bound tissue-type plasminogen activator: In vitro studies in a plasma milieu. In Proceedings of the

Arteriosclerosis and Thrombosis; Arterioscler Thromb, 1991; Vol. 11, pp. 629–638.

76. Palabrica, T.M.; Liu, A.C.; Aronovitz, M.J.; Furie, B.; Lawn, R.M.; Furie, B.C. Antifibrinolytic activity of apolipoprotein(a) in vivo: Human apolipoprotein(a) transgenic mice are resistant to tissue plasminogen activator-mediated thrombolysis. *Nat. Med.* **1995**, *1*, 256–259, doi:10.1038/nm0395-256.

77. Biemond, B.J.; Friederich, P.W.; Koschinsky, M.L.; Levi, M.; Sangrar, W.; Xia, J.; Büller, H.R.; Ten Cate, J.W. Apolipoprotein(a) attenuates endogenous fibrinolysis in the rabbit jugular vein thrombosis model in vivo. *Circulation* **1997**, *96*, 1612–1615, doi:10.1161/01.CIR.96.5.1612.

78. Boffa, M.B.; Marar, T.T.; Yeang, C.; Viney, N.J.; Xia, S.; Witztum, J.L.; Koschinsky, M.L.; Tsimikas, S. Potent reduction of plasma lipoprotein (a) with an antisense oligonucleotide in human subjects does not affect ex vivo fibrinolysis. *J. Lipid Res.* **2019**, *60*, 2082–2089, doi:10.1194/jlr.P094763.

79. Helgadottir, A.; Gretarsdottir, S.; Thorleifsson, G.; Holm, H.; Patel, R.S.; Gudnason, T.; Jones, G.T.; Van Rij, A.M.; Eapen, D.J.; Baas, A.F.; et al. Apolipoprotein(a) genetic sequence variants associated with systemic atherosclerosis and coronary atherosclerotic burden but not with venous thromboembolism. *J. Am. Coll. Cardiol.* **2012**, *60*, 722–729, doi:10.1016/j.jacc.2012.01.078.

80. Kamstrup, P.R.; Tybjærg-Hansen, A.; Nordestgaard, B.G. Genetic evidence that lipoprotein(a) associates with atherosclerotic stenosis rather than venous thrombosis. *Arterioscler. Thromb. Vasc. Biol.* **2012**, *32*, 1732–1741, doi:10.1161/ATVBAHA.112.248765.

81. Boffa, M.B.; Koschinsky, M.L. Oxidized phospholipids as a unifying theory for lipoprotein(a) and cardiovascular disease. *Nat. Rev. Cardiol.* 2019, *16*, 305–318.

82. Adam, R.C.; Mintah, I.J.; Alexa-Braun, C.A.; Shihanian, L.M.; Lee, J.S.; Banerjee, P.; Hamon, S.C.; Kim, H.I.; Cohen, J.C.; Hobbs, H.H.; et al. Angiopoietin-like protein 3 governs LDL-cholesterol levels through endothelial lipase-dependent VLDL clearance. *J. Lipid Res.* **2020**, *61*, 1271–1286, doi:10.1194/jlr.RA120000888.

83. Wu, L.; Soundarapandian, M.M.; Castoreno, A.B.; Millar, J.S.; Rader, D.J. LDL-Cholesterol Reduction by ANGPTL3 Inhibition in Mice Is Dependent on Endothelial Lipase. *Circ. Res.* **2020**, *127*, 1112–1114, doi:10.1161/CIRCRESAHA.120.317128.

84. Graham, M.J.; Lee, R.G.; Brandt, T.A.; Tai, L.-J.; Fu, W.; Peralta, R.; Yu, R.; Hurh, E.; Paz, E.; McEvoy, B.W.; et al. Cardiovascular and Metabolic Effects of ANGPTL3 Antisense Oligonucleotides. *N. Engl. J. Med.* **2017**, *377*, 222–232, doi:10.1056/NEJMoa1701329.

85. Graham, M.J.; Lee, R.G.; Bell, T.A.; Fu, W.; Mullick, A.E.; Alexander, V.J.; Singleton, W.; Viney, N.; Geary, R.; Su, J.; et al. Antisense oligonucleotide inhibition of apolipoprotein c-iii reduces plasma triglycerides in rodents, nonhuman primates, and humans. *Circ. Res.* **2013**, *112*, 1479–1490, doi:10.1161/CIRCRESAHA.111.300367.

86. Alexander, V.J.; Xia, S.; Hurh, E.; Hughes, S.G.; O'Dea, L.; Geary, R.S.; Witztum, J.L.; Tsimikas, S. N-acetyl galactosamine-conjugated antisense drug to APOC3 mRNA, triglycerides and atherogenic lipoprotein levels. *Eur. Heart J.* **2019**, *40*, 2785–2796, doi:10.1093/eurheartj/ehz209.

87. Post, N.; Yu, R.; Greenlee, S.; Gaus, H.; Hurh, E.; Matson, J.; Wang, Y. Metabolism and disposition of

volanesorsen, a 29-O-(2 methoxyethyl) antisense oligonucleotide, across species. *Drug Metab. Dispos.* **2019**, *47*, 1164–1173, doi:10.1124/dmd.119.087395.

88. Alexander, V.J.; Digenio, A.; Xia, S.; Hurh, E.; Hughes, S.; Geary, R.S.; Witztum, J.L.; Tsimikas, S. Inhibition pf apolipoprotein CIII with GalNac conjugated antisense drug potently lowers fasting serum apolipoprotein CIII and triglycerides levels in healthy volunteers with elevated triglycerides. *JACC* **2018**, *71*, (abstract) doi:10.1016/S0735-1097.