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STATE OF THE ART REVIEW
Heart failure and cardiomyopathies

Small non-coding RNA therapeutics for cardiovascular disease

Ajay M. Shah * and Mauro Giacca *

King's College London, British Heart Foundation Centre of Research Excellence, School of Cardiovascular and Metabolic Medicine and Sciences, The James Black Centre, 125 Coldharbour Lane, London SE5 9NU, UK

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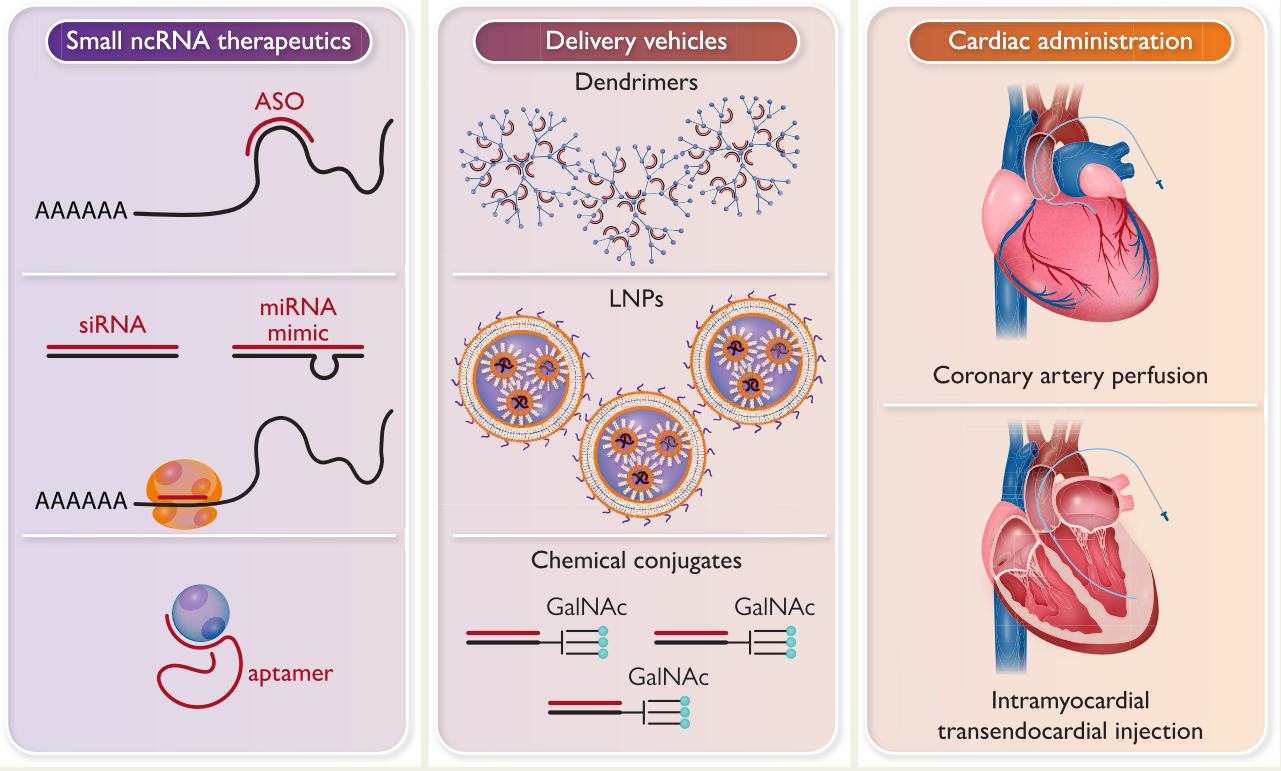
Graphical Abstract



Types of small non-coding RNA therapeutics (left panel); some of the methods for *in vivo* delivery (centre); and routes for administration to the heart (right). ASO, antisense oligonucleotides; GalNAc, *N*-acetylgalactosamine; LNPs, lipid nanoparticles; miRNAs, microRNAs; siRNA, short interfering RNAs.

* Corresponding authors. Emails: ajay.shah@kcl.ac.uk (A.M.S.), mauro.giacca@kcl.ac.uk (M.G.)
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Abstract

Novel bio-therapeutic agents that harness the properties of small, non-coding nucleic acids hold great promise for clinical applications. These include antisense oligonucleotides that inhibit messenger RNAs, microRNAs (miRNAs), or long non-coding RNAs; positive effectors of the miRNA pathway (short interfering RNAs and miRNA mimics); or small RNAs that target proteins (i.e. aptamers). These new therapies also offer exciting opportunities for cardiovascular diseases and promise to move the field towards more precise approaches based on disease mechanisms. There have been substantial advances in developing chemical modifications to improve the *in vivo* pharmacological properties of antisense oligonucleotides and reduce their immunogenicity. Carrier methods (e.g. RNA conjugates, polymers, and lipoplexes) that enhance cellular uptake of RNA therapeutics and stability against degradation by intracellular nucleases are also transforming the field. A number of small non-coding RNA therapies for cardiovascular indications are now approved. Moreover, there is a large pipeline of therapies in clinical development and an even larger list of putative therapies emerging from pre-clinical studies. Progress in this area is reviewed herein along with the hurdles that need to be overcome to allow a broader clinical translation.

Keywords Antisense oligonucleotides • Cardiovascular disorders • MicroRNAs • RNA therapeutics • Short interfering RNAs

Clinical need for new cardiovascular therapies

Cardiovascular disease (CVD) remains the leading cause of death globally despite major advances in prevention and invasive treatments. Symptomatic ischaemic heart disease (IHD), sudden death, and heart failure (HF) in survivors of acute myocardial infarction (MI) remain major problems. Indeed, much CVD, such as IHD, cardiomyopathy, HF, and peripheral vascular disease, is ultimately relatively intractable. Current therapies generally only delay disease progression. With few exceptions, they target a limited number of disease pathways identified decades ago. Furthermore, the overall approach is largely 'one-size-fits-all' with relatively little stratification, e.g. by disease stage or mechanism.

Conceptual progress in understanding the pathomechanisms of CVD, including appreciation of the role of non-coding RNAs (ncRNAs) in these processes, and technological advances, now permit the development of an innovative class of therapies based on small nucleic acids.

Here, we discuss the different types of ncRNA-based therapies (*Graphical Abstract*), emerging clinical and advanced pre-clinical data in CVD, and the challenges that need to be overcome to maximize the potential of this exciting new therapeutic modality.

Types of small, non-coding nucleic acid therapeutics

Small non-coding nucleic acid therapeutics that have reached clinical application fall into three categories (*Figure 1*): (i) short antisense oligonucleotides (ASOs) that pair with and inhibit messenger RNAs (mRNAs), microRNAs (miRNAs), long ncRNAs (lncRNAs), and circular RNAs (circRNAs); (ii) positive effectors of the RNA interference (RNAi) pathway [small interfering RNAs (siRNAs), miRNAs]; and (iii) conformational small RNAs (aptamers) that target proteins. Other therapeutic ncRNAs (e.g. miRNA sponges), lncRNAs, and circRNAs, which have longer dimensions and are less advanced in clinical development, or CRISPR guide RNAs for gene editing are not discussed.

Antisense oligonucleotides

Antisense oligonucleotides are usually 17–22 nt long and chemically modified to decrease degradation by endogenous nucleases and improve cellular uptake. Pairing with the antisense cellular RNA is followed by steric inhibition of target RNA function and/or induction of target RNA degradation.

To date, nine ASOs have been approved in Europe and the USA (*Table 1*). The first of these, fomivirsen (https://www.accessdata.fda.gov/drugsatfda_docs/nda/98/20961_vitravene.cfm), was a 21-mer ASO against cytomegalovirus (CMV) for the treatment of CMV retinitis; its commercialization ended in 2002 due to the development of more effective antivirals. Three of the approved ASOs have a specific cellular mRNA target [mipomersen against apolipoprotein B (ApoB), inotersen against transthyretin, and volanesorsen against ApoC3]. The remaining five ASOs are alternative splicing modulators of the dystrophin (eteplirsen, golodirsen, viltolarsen, and casimersen) or the SMN2 (nusinersen) pre-mRNAs. Recent reviews have discussed the development of these products.[2,3] None of these ASOs target miRNAs, lncRNAs or circRNAs, but at least 10 other ASOs are in advanced Phase 2/3 clinical development,[3] including molecules targeting miRNAs.

The original idea of therapeutic ASOs dates back three decades,[4] with over 100 Phase 1 studies performed and 25% reaching Phase 2/3.[5,6] Clinical success, however, was only rendered possible by the progressive identification of chemical modifications that improve stability and reduce immune recognition. Classic DNA ASOs undergo DNA:RNA pairing but are then relatively rapidly degraded by RNAse H. This problem can be circumvented by substituting non-bonding oxygen atoms in the phosphate groups of ASOs with sulphur atoms (phosphorothioates, PS) or introducing chemical modifications in the 2′ position of the sugar molecule of nucleotides (e.g. 2′-*O*-methyl-, 2′-*O*-methoxy-ethyl-, or 2′′-fluoro-nucleotides; 2′-OMe, 2′-MOE, or 2′-F, respectively; *Figure 2A*; reviewed in Lennox and Behlke[7] and van Rooij and Olson[8]).

An effective modification to increase stability of ASOs is the inclusion of RNA nucleotides in which the ribose moiety contains an extra bond between the 2′ oxygen and 4′ carbon (2′-*O*,4′-*C*-methylene bridge).[9] These locked nucleic acid (LNA) nucleotides confer remarkable thermal stability against enzymatic degradation while maintaining base-pairing specificity.[10] However, for mRNA and larger ncRNA targeting, substrate duplexes with either LNA or 2′-*O*-alkylated RNA nucleotides are unable to recruit RNAse H and thus inhibition can only occur through steric hindrance.[11,12] This issue can be solved using a GapmeR approach, where a central continuous stretch of RNase H-recruiting nucleotides (e.g. PS DNA) is flanked by modified nucleotides. This arrangement improves ASO stability while still permitting RNAse H recruitment to the central DNA–RNA duplex for degradation. A gap of at least seven to eight DNA nucleotides is necessary

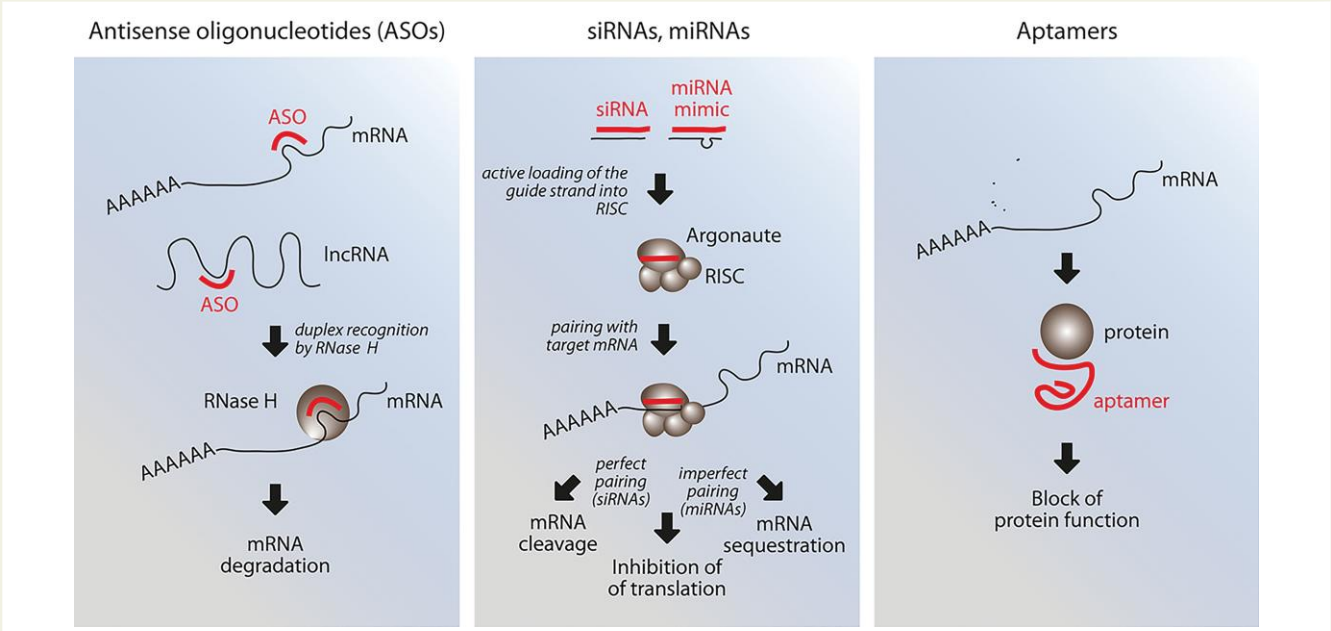

Figure 1 Schematic representation of the three main classes of small, non-coding RNA therapeutics. Not shown here for simplicity is also the possibility of using antisense oligonucleotides to target circular RNAs (reviewed in Holdt *et al.*[1]). ASO, antisense oligonucleotide; lncRNA, long non-coding RNA; mRNA, messenger RNA; RISC, RNA-induced silencing complex.

for activation of RNAse H.[13–15] The GapmeR design also permits the use of shorter ASOs (typically, 16-mers),[14–16] with increased target knockdown efficacy, the possibility of targeting several miRNA family members sharing the same seed sequence and neighbouring regions, and most relevant, reduced innate immune activation.

The first small nucleic acid therapeutic to be approved (fomivirsen) was a DNA ASO in which all bonds were phosphorothiates. A significantly more effective GapmeR design is present in mipomersen (this drug, however, showed significant liver toxicity and was withdrawn), inotersen, and volanesorsen (*Figure 2B*).

The modulation of pre-mRNA splicing and the inhibition of miRNA processing do not require RNAse H-mediated duplex degradation. In the case of splicing, steric block ASOs selectively exclude or retain a specific exon (exon skipping and inclusion, respectively) by masking a splicing signal in the pre-mRNA or to affect RNA structure thus preventing access to cis splicing signals by the spliceosome (reviewed in Neil *et al.*[17]). Five steric block ASOs that modulate splicing have reached clinical approval. Nusinersen is an 18-mer ASO for spinal muscular atrophy formed of 2′-MOE nucleotides linked by PS bonds. Other approved ASOs are for Duchenne muscular dystrophy and consist of phosphorodiamidate morpholino oligomers in which the nitrogen bases are connected to a morpholino ring rather than to the deoxyribose ring and a phosphorodiamidic bond substitutes the natural phosphodiester bond (*Figure 2A*). A schematic representation of eteplirsen, one of these ASOs, alongside that of nusinersen, is shown in *Figure 2C*.

RNA interference therapeutics (microRNAs and small interfering RNAs)

Endogenous miRNAs are double-stranded RNA molecules of 19–23 nt with 2 nt overhangs at their 5′ ends, which pair with cellular mRNAs and block their function by inducing mRNA degradation or blocking translation[18,19] (*Figure 1*). As pairing with the mRNA target only requires short regions of homology (in particular, a 7 nt seed sequence at position +2/+8 of the miRNA), each miRNA can target tens or hundreds of different transcripts. The delivery of double-stranded RNA molecules having the same structure as natural miRNAs but with perfect complementarity with a given target mRNA (siRNAs) intercepts and exploits the RNAi pathway to obtain more specific, single gene down-regulation.

The first clinically approved siRNA was patisiran in 2018, against the transthyretin mRNA for hereditary transthyretin amyloidosis (HTT-amyloid).[20] This was followed by givosiran, an siRNA against the delta aminolevulinic acid synthase 1 mRNA for acute hepatic porphyria,[21] inclisiran, an siRNA against PCSK9 for familiar hypercholesterolaemia,[22] and lumasiran, an siRNA against the hydroxy acid oxidase 1 mRNA for primary hyperoxaluria Type 1[23] (*Table 1*). The structures of patisiran and inclisiran are shown in *Figure 2D*. As siRNAs need to interact with the cellular RISC complex to exert their function, they usually tolerate less chemical modification than ASOs, in particular in their guide strand (the one that pairs with the mRNA targets).

No miRNA has so far reached clinical approval, yet these molecules have significant therapeutic appeal for several reasons. First, endogenous miRNAs are naturally occurring molecules with a precise and physiological mechanism of action. Second, having evolved to target several different mRNAs, miRNAs can elicit complex yet defined phenotypes [e.g. cardiomyocyte (CM) proliferation[24] or transdifferentiation[25]]. Third, the human miRNome has relatively low complexity (1973 hairpin precursor miRNAs, corresponding to 2675 human mature sequences annotated in miRBase v.22.1[26]); therefore, a synthetic human miRNA mimic library can be screened with relative ease for therapeutic leads. Cell-produced mature miRNAs are single-stranded RNA molecules generated from a hairpin that folds back to form partially double-stranded regions. In principle, either hairpin strand can be used by RISC for RNA interference. In contrast, miRNA mimics have the same sequence as the desired miRNA strand, while the complementary passenger strand, which usually is a perfect complement to

Table 1 **Antisense oligonucleotides and short interfering RNAs approved by the European Medicines Agency and/or the Food and Drug Administration and their main characteristics**

Product (commercial name; developer/manufacturer)	Length	Modifications	Vehicle	Route of administration	Indication	Target organ	Target gene and mechanism	Year of approval
Antisense oligonucleotides (ASOs)								
Fomivirsen (Vitravene; Isis Pharmaceuticals, Novartis)	21-mer	PS	None	Intravitreal	CMV retinitis	Eye	CMV IE-2 mRNA	1998 (FDA), 1999 (EMA); 2002 withdrawn
Mipomersen (Kynamro; Ionis Pharmaceuticals, Kastle Therapeutics)	20-mer	PS, 2′-MOE, GapmeR	None	Subcutaneous	Familiar hypercholesterolaemia (FH)	Liver	Apolipoprotein B (ApoB) mRNA	2013 (FDA); 2019 withdrawn
Nusinersen (Spinraza; Ionis Pharmaceuticals, Biogen)	18-mer	PS, 2′-MOE	None	Intrathecal	Spinal muscular atrophy		Survival of motoneuron 2 (SMN2) pre-mRNA splicing (exon 7 inclusion)	2017 (EMA), 2016 (FDA)
Eteplirsen (Exondys 51, Sarepta Therapeutics)	30-mer	PMO	None	Intravenous	Duchenne muscular dystrophy (DMD)	Skeletal muscle	Dystrophin pre-mRNA splicing (exon 51 skipping)	2016 (FDA)
Inotersen (Tesgedi; Ionis Pharmaceuticals, Akcea Therapeutics)	20-mer	PS, 2′-MOE, GapmeR	None	Subcutaneous	Hereditary transthyretin amyloidosis	Liver	Transthyretin (TTR) mRNA	2018 (EMA), 2018 (FDA)
Golodirsen (Vyondys 53; Sarepta Therapeutics)	25-mer	PMO	None	Intravenous	Duchenne muscular dystrophy (DMD)	Muscle	Dystrophin pre-mRNA splicing (exon 53 skipping)	2019 (FDA)
Viltolarsen (Viltepso, NS Pharma)	21-mer	PMO	None	Intravenous	Duchenne muscular dystrophy (DMD)	Muscle	Dystrophin pre-mRNA splicing (exon 53 skipping)	2020 (FDA) 2020 (EMA)
Volanesorsen (Waylivra; Ionis Pharmaceuticals, Akcea Therapeutics)	20-mer	PS, 2′-MOE, GapmeR	None	Subcutaneous	Familiar chylomicronaemia syndrome (FCS)	Liver	Apolipoprotein C3 (ApoC3I) mRNA	2019 (EMA)
Casimersen (Amondys 45; Sarepta Therapeutics)	22-mer	PMO	None	Intravenous	Duchenne muscular dystrophy (DMD)	Muscle	Dystrophin pre-mRNA splicing (exon 45 skipping)	2021 (FDA)
Small interfering RNAs (siRNAs)								
Patisiran (Onpattro; Anylam Pharmaceuticals)	21-nt ds	2′-*O*-Me	SNALP LNP	Intravenous	Hereditary transthyretin amyloidosis	Liver	Transthyretin mRNA	2018 (EMA), 2019 (FDA)
Givosiran (Givlaari; Anylam Pharmaceuticals)	21-nt ds	PS, 2′-*O*-Me, 2′-F, GalNAc-conjugated	None	Subcutaneous	Acute hepatic porphyria (AHP)	Liver	Delta aminolevulinic acid synthase 1 mRNA	2020 (EMA), 2019 (FDA)
Inclisiran (Leqvio; Novartis Pharmaceuticals)	22-nt ds	PS, 2′-*O*-Me, 2′-F, GalNAc-conjugated	None	Subcutaneous	Primary hypercholesterolaemia or mixed dyslipidaemia	Liver	Proprotein convertase subtilisin/kexi)*n* type 9 (PCSK9) mRNA	2020 (EMA) 2021 (FDA)
Lumasiran (Oxlumo; Anylam Pharmaceuticals)	21-nt ds	PS, 2′-*O*-Me, 2′-F, GalNAc-conjugated	None	Subcutaneous	Primary hyperoxaluria type 1 (PH1)	Liver	Hydroxyacid oxidase-1 mRNA	2020 (EMA), 2020 (FDA)

ASO, antisense oligonucleotide; ds, double stranded; GalNAc, *N*-acetylgalactosamine; PMO, phosphoroamidate morpholino oligomer; PS, phosphorothioate modification; siRNA, short interfering RNA.

the guide strand, is chemically modified to prevent RISC loading.[27] Three miRNA mimics have been tested in cancer trials. The miR-34 mimic, MRX34, for advanced solid tumours[28] showed serious immune-mediated adverse events.[29] MesomiR-1 is a miR-16 mimic for patients with mesothelioma,[30] while remlarsen is a miR-29 mimic administered via intradermal injection for treatment of keloid.[31] Several other therapeutic miRNA applications are likely to follow.

Aptamers

The SELEX (systematic evolution of ligands by exponential enrichment) method developed in the 1990s permits selection of RNAs that specifically bind proteins or small organic molecules, starting from libraries of oligoribonucleotides with random sequence.[32–34] The selected RNA molecules (or aptamers) exploit the secondary structure of nucleic acids rather than sequence complementarity for binding (*Figure 1*). Aptamers are usually short (25–40 nt) RNA segments that bind their targets at relatively high affinity.[35]

Pegaptanib, a 28-mer modified RNA aptamer conjugated with two polyethylene glycol (PEG) molecules, was the first aptamer to reach clinical approval in 2004.[36] It binds the 165aa isoform of the vascular endothelial growth factor (VEGF) and has an indication by intravitreal injection in age-related neovascular macular degeneration. After initial clinical success, however, pegaptanib has faced competition with the anti-VEGF monoclonal antibodies ranibizumab and bevacizumab.[37]

To date, eight other RNA aptamers and five DNA aptamers have undergone clinical trials in various conditions[35] but most molecules have so far failed to meet clinical safety and efficacy standards.

Delivery vehicles for small, non-coding RNA therapeutics

A major challenge remains the efficiency with which these molecules cross cellular membranes, with the foremost reason for clinical trial termination so far being lack of efficacy.[3] Whether delivered as naked nucleic acid, ligand–oligonucleotide conjugates, or via various nanocarriers, all small RNAs enter cells by endocytosis and need to exit endocytic vesicles to gain access to the inner cellular compartments.[38–40]

Antisense oligonucleotides with a PS backbone enter the endocytic pathway as naked nucleic acids as they can associate with different uptake molecules on the plasma membrane.[41,42] Indeed, all the ASOs approved to date are administered as naked molecules that are injected subcutaneously or intravenously (*Table 1*). Instead, natural nucleic acids with a phosphodiester backbone, including double-stranded RNAs targeting the RNAi pathway (i.e. siRNAs, miRNA mimics), require either conjugation with a ligand or inclusion within a carrier complex for efficient cellular uptake. *Figure 3* shows some of the carrier molecules frequently used for small nucleic acid delivery, with their size compared with that of two viral vectors (AAV and adenovirus).

Uptake ligands

Initial studies indicated that conjugation of ncRNAs with cholesterol favours association with circulating lipoproteins and hepatic uptake, with reduction in exposure to the kidney.[43,44] Cholesterol-conjugated ASOs targeting specific miRNAs were originally named 'antagomirs'.[45] Remlarsen, a miRNA that has reached clinical trials, is a cholesterol conjugate.[31]

The breakthrough technology for naked RNA therapies targeting the liver has been the development of RNA conjugates carrying a trimer of *N*-acetylgalactosamine (GalNAc), which avidly binds the cellular asialoglycoprotein receptor (ASGPR; reviewed in Springer and Dowdy[46]). As this receptor is expressed on hepatocyte plasma membrane, this strategy permits specific liver targeting. Of the 4 approved siRNAs, 3 are GalNac-conjugates (*Table 1* and *Figure 2D*), while >10 other GalNac-conjugated ASOs and siRNAs are in late phase clinical development for liver-directed therapy.[3]

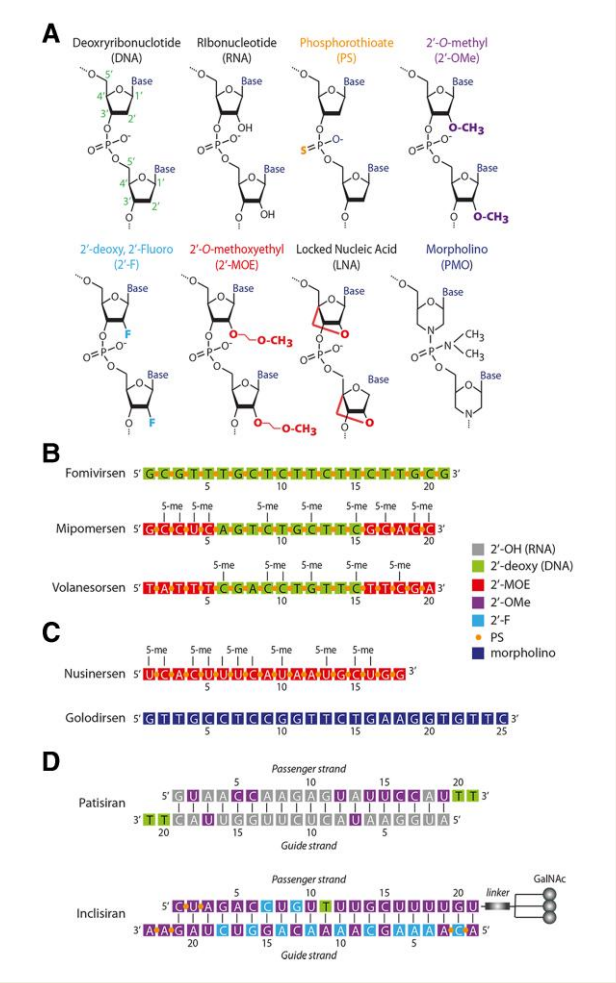

Figure 2 Chemical modifications and structure of selected, clinically approved, non-coding RNAs. (*A*) Chemical modification of ribonucleotides. The abbreviations commonly used to indicate these modified nucleotides are indicated. (*B*) Chemical structure of three clinically approved antisense oligonucleotides. Mipomersen and volanesorsen have a typical GapmeR configuration, in which the first five nucleotides at both the 5′ and 3′ ends are modified, while the central region is formed of oligodeoxynucleotides to allow recruitment of RNAse H to the central target RNA:antisense oligonucleotide DNA duplex. (*C*) Chemical structure of the two splicing modulators nusinersen and golodirsen. The latter is formed of morpholino (phosphoroamidate morpholino oligomer) monomers, same as eteplirsen, vitolarsen and casimersen. (*D*) Chemical structure of two short interfering RNAs. Patisiran is formulated in a lipid nanoparticle, while, in the case of inclisiran, three GalNAc moieties are attached through a linker to the 3′ end of the siRNA passenger strand. Both are designed to target the liver.

Polymers

Small RNAs can be formulated with synthetic or natural polymers. The former include poly(ethylenimine) (PEI), which binds nucleic acids due to its cationic nature. The resulting large size polyplexes are still

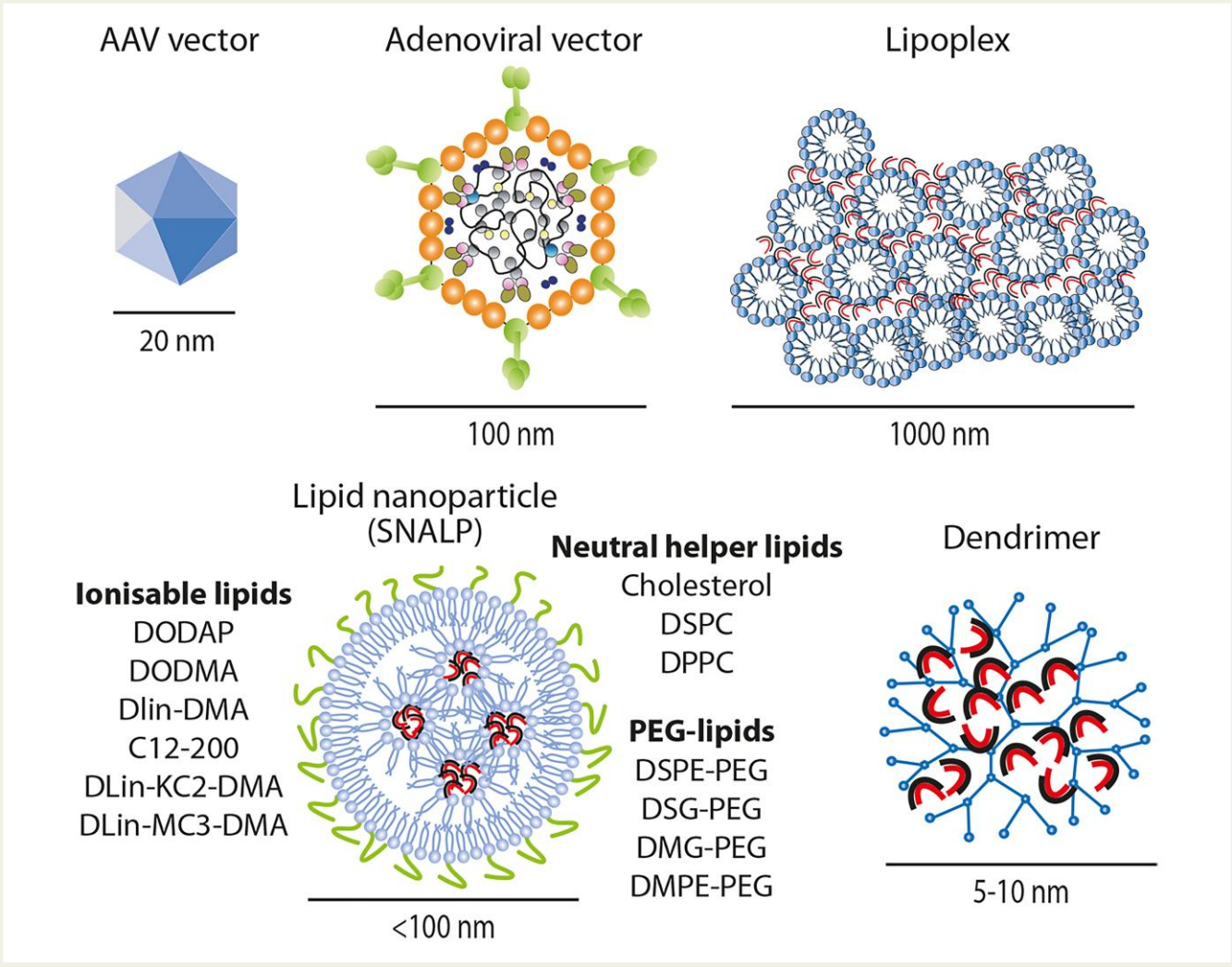

Figure 3 Main delivery vehicles for short interfering RNAs and microRNAs, with the indication of their approximate size. The size of the two most used vectors for cardiovascular applications (based on adeno-associated virus [AAV] and adenovirus) are shown for comparison. For stable nucleic acid lipid particles, some of the commonly used lipids for each of the three lipid categories (ionisable lipids, neutral helper lipids, and polyethylene glycol-lipids) are reported. The RNA molecules are schematically shown as curved duplexes.

positive, interact with negatively charged cell surface polysaccharides and are endocytosed. A pH buffer effect then facilitates endosomal escape. Poly(ethylenimine) is efficient for miRNA delivery[47,48] but its toxicity limits clinical application. Other synthetic polymers used for ncRNA delivery are poly-L-lysine and poly(lactic-co-glycolic acid) (PLGA).[49–51] Among natural polymers, the positively charged chitosan, which is formed of glucosamine and *N*-acetylglucosamine, remains attractive because of its biocompatibility and biodegradability.[52,53]

Several miRNAs have been delivered in animal models using polymers.[3] In the cardiovascular field, intracoronary administration of an antagomiR-92 encapsulated in PLGA microspheres of >9 μm diameter was shown to promote angiogenesis and improve cardiac function after MI in pigs.[54]

Lipids

The most effective methods for small nucleic acid delivery to date are based on lipids (reviewed in Hou *et al.*[55]). A first generation of lipofection reagents was based on nucleic acid entrapment by mixtures of a cationic lipid (e.g. DOTMA or DOTAP) formulated with a neutral co-lipid helper (e.g. DOPE). These lipoplexes are very efficient for cell transfection but their large size (often >1 μm; *Figure 3*) and positive charge results in rapid plasma clearance, toxicity, and inflammation.[56–58] Improved biodistribution can be obtained using neutral lipids, such as the commercial preparation MaxSuppressor In Vivo RNA-LANCEr II—composed of DOPC, squalene oil, polysorbate 20, and an antioxidant—which has been used to deliver miRNAs for cancer and cardiovascular applications.[59,60] The first miRNA to reach clinical experimentation for solid cancer, miR-34, was formulated in a nanoparticle called Smarticle, composed of amphoteric lipids with an overall anionic charge at physiological pH, while becoming cationic in the acidic tumour environment. Drug development was however halted due to serious side effects.[28]

Two breakthrough improvements in lipid-mediated delivery were first, ionisable cationic lipids which are positively charged at acidic pH and complex tightly with negatively charged RNA, while becoming neutral at physiological pH, and second, the development of methods to use these molecules to form small lipid nanoparticles (LNPs) that entrap the RNA.[61] Patisiran (mentioned earlier) is formulated in an LNP.[20] The two other LNPs clinically approved in 2020 were the COVID-19 vaccines pioneered

Table 2 Main non-coding RNA therapies in the pipeline for cardiovascular applications

Product (developer/ manufacturer)	Type (modifications)	Route of administration	Indication	Target organ	Target mRNA or miRNA	Latest clinical studies
Pelacarsen (Ionis Pharmaceuticals)	ASO (PS, 2′-MOE, GalNAc-conjugated)	Subcutaneous	Elevated Lp(a)	Liver	*LPA* mRNA	Phase 3 (NCT04023552)
Olpasiran (Amgen)	siRNA (GalNAc-conjugated)	Subcutaneous	Elevated Lp(a)	Liver	*LPA* mRNA	Phase 2 (NCT04270760)
SLN360 (Silence Therapeutics)	siRNA (GalNAc-conjugated)	Subcutaneous	Elevated Lp(a)	Liver	*LPA* mRNA	Phase 1 (NCT04606602)
Vupanorsen (Ionis Pharmaceuticals, Pfizer)	ASO (GalNAc-conjugated)	Subcutaneous	Hypertriglyceridemia; Familial Chylomicronemia Syndrome (FCS)	Liver	ANGPTL3 mRNA	Phase 2 (NCT03371355) Phase 2 (NCT04516291) Phase 2 (NCT03360747) Discontinued in January 2022
ARO-ANG3 (Arrowhead Pharmaceuticals)	siRNA (liver targeted)	Subcutaneous	Mixed dyslipidaemia	Liver	ANGPTL3 mRNA	Phase 2 (NCT04832971)
MRG-110 (S95010) (miRagen, Servier)	ASO (LNA)	Intradermal	Neovascularization, wound healing	Vasculature	miR-92a-3p	Phase 1 (NCT03494712; NCT03603431)
CDR132L (Cardior)	ASO (LNA)	Intravenous	Heart failure	Heart	miR-132-3p	Phase 1b (NCT04045405)
Teprasiran (Quark Pharmaceuticals)	siRNA	Intravenous	Acute kidney injury	Kidney	p53 mRNA	Phase 2 (NCT02610283) Phase 3 (NCT03510897)
Vutrisiran (Anylam Pharmaceuticals)	siRNA (GalNAc-conjugated)	Subcutaneous	HTT-amyloid with polyneuropathy; TT-amyloid with cardiomyopathy	Liver	Transthyretin mRNA	Phase 3 (NCT03759379) Phase 3 (NCT04153149)

ASO, antisense oligonucleotide; ds, double stranded; GalNAc, *N*-acetylgalactosamine; siRNA, short interfering RNA.

by Moderna (mRNA-1273[62]) and Pfizer/BioNTech (BNT162b2[63]) for the administration of the SARS-CoV-2 Spike mRNA.

Stable nucleic acid lipid particles (SNALPs) are 80–120 nm diameter nanoparticles in which the RNA is surrounded by a lipid bilayer[64] (*Figure 3*). Examples of ionisable lipids essential for SNALP formation include DLin-MC3-DMA (used in patisiran), ALC-0315, and SM102, which are used in the BioNTech/Pfizer and Moderna COVID-19 vaccines, respectively. Other ionisable lipids for LNP formulation that are used experimentally are reviewed in Han *et al.*[65] To form SNALPs, the ionisable lipid is mixed with three other lipids. Two of these helper lipids are usually a phosphocholine-containing lipid (e.g. distearoylphosphatidylcholine [DSPC], dipalmitoylphosphatidylcholine [DPPC]) and cholesterol which, as in cell membranes, provides stability and assists in membrane fusion to facilitate endosomal escape.[66] The fourth lipid is a PEG-derivatized lipid which provides stability to the nanoparticle and prolongs its circulatory half-life. Examples of PEGgylated lipids are distearoyl-rac-glycerol-PEG or 1,2-dimyristoyl-rac-glycero-3-methoxy-PEG.

Dendrimers

These are branched polymers carrying cationic groups that interact with nucleic acids. Similar to PEI, endosomal escape is facilitated by a proton sponge effect after endocytosis. Polyamidoamine (PAMAM) dendrimers in particular were capable of delivering an RNA-triple-helix structure comprising two miRNAs and an antagomiR to breast cancer cells in a mouse model.[67] As with cationic polymers, the main limitation remains toxicity.

Biological carriers

Exosomes[68] are endogenously produced 30–150 nm diameter vesicles spontaneously released by cells that participate in intercellular communication by transferring bioactive material, including small nucleic acids.[69] Methods under development include the enrichment of selected miRNAs into exosomes[70,71] and the inclusion of specific ligands within exosome membranes for cell targeting.[72]

Other non-coding RNA delivery methods

Different inorganic carriers (including gold nanoparticles, quantum dots, carbon nanotubes, nanocrystals, silica- and calcium-based nanoparticles) have also been considered for ASO and RNAi therapeutics delivery.[73] These systems, however, are significantly less efficient than lipid- or polymer-mediated delivery and to date have undergone limited study in animal models.

Clinically approved non-coding RNAs therapies for cardiovascular disease

Several ncRNA therapies for hyperlipidaemia have recently entered the clinical arena (*Table 1*), with a potential advantage of increased patient

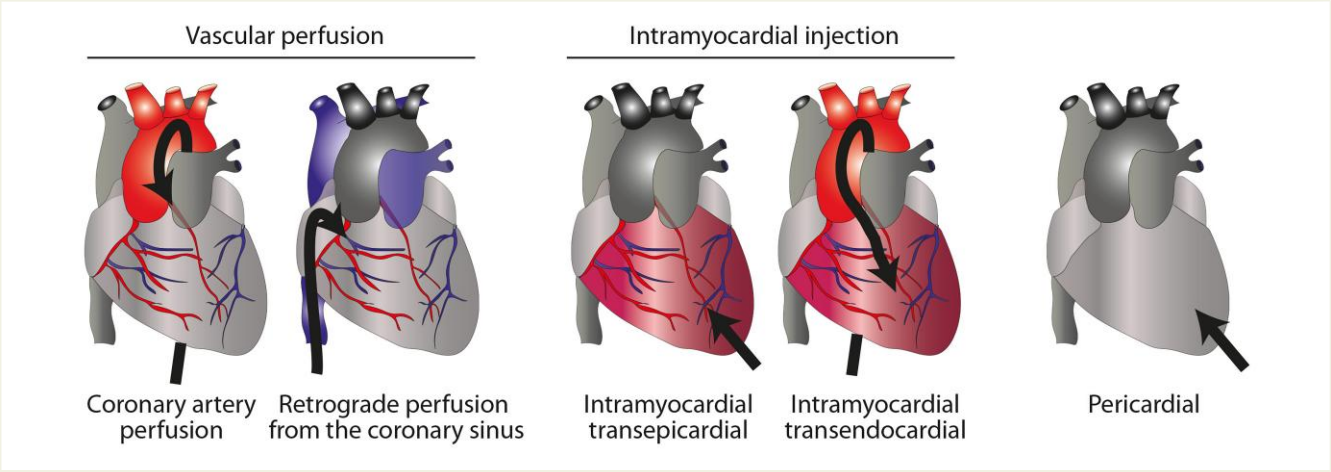

Figure 4 Routes for cardiac delivery. These include injection into the coronary artery as during standard percutaneous coronary intervention or retrograde into the coronary sinus; or intramyocardial, through either direct injection after mini-thoracotomy or during bypass surgery, or after percutaneous catheterization to reach the left ventricle, followed by transendocardial delivery. A fifth route includes administration into the pericardial sac.

compliance due to an infrequent dosing regimen and higher efficacy either as a standalone or combination treatment.

The siRNA inclisiran targets PCSK9, leading to an up-regulation of hepatocyte low-density lipoprotein (LDL) receptors and consequent reduction in plasma LDL-cholesterol levels.[74] A series of randomized placebo-controlled clinical trials (the ORION studies[75]) tested its efficacy in patients with familial hypercholesterolaemia (FH), CVD, or a high risk of CVD, and demonstrated substantial reduction in LDL cholesterol (~50%), total cholesterol, and lipoprotein(a) [Lp(a)]. Inclisiran was subcutaneously injected 3–6 monthly for ≥18 months and had a good safety profile and prolonged effects. While potentially a major advance, clinical studies to determine its effects on CVD endpoints and any long-term adverse consequences are still required. Inclisiran was approved for use in the EU and UK in 2020–21.

Volanesorsen is an ASO-targeting ApoC3 which effectively reduces triglyceride levels in familial chylomicronaemia syndrome.[76] It was tested as a once-weekly subcutaneous injection for 52 weeks in this randomized placebo-controlled trial in 66 patients. Volanesorsen was approved in the EU in 2019 but not by the US Food and Drug Administration (FDA), at least in part due to the high incidence of thrombocytopenia in treated patients. Mipomersen is an ASO administered by weekly subcutaneous injection that targets ApoB-100 and was approved by the FDA in 2013 as an orphan drug to reduce severe hypercholesterolaemia in patients with homozygous FH. It effectively lowers LDL cholesterol[77] but failed to receive EU approval due to a risk of serious liver toxicity and has not been widely used. In the USA, it was withdrawn in 2019. Patisiran, while initially approved for the treatment of HTT-amyloid with neurological manifestations,[78] has significant cardiac relevance. The mutant hepatic transthyretin targeted by patisiran leads to accumulation of amyloid deposits in peripheral nerves, the heart, and other organs. In the initial landmark APOLLO study in HTT-amyloid, patisiran was intravenously injected every 3 weeks for 18 months and led to a slight clinical improvement and marked prevention of disease progression when compared with placebo.[20] Follow-up data indicate safety at ≥5 years in treated patients. An analysis of cardiac structure and function in a subgroup of patients in this study demonstrated significant reductions in left-ventricular wall thickness, improved longitudinal strain, and reduced pro-BNP levels, hospitalization or mortality[79]—strongly suggesting efficacy against cardiac disease. These findings are even more impactful, since it is now recognized that HTT-amyloid is quite prevalent and commonly misdiagnosed as idiopathic HF with preserved ejection fraction (HFpEF).

Another approved transthyretin-targeting ncRNA, inotersen, has shown efficacy against neurological HTT-amyloid in a randomized clinical trial involving weekly subcutaneous injection for 15 months.[80] Its value in cardiac HTT-amyloid remains to be established.

Small non-coding RNA therapies in the clinical pipeline

A series of novel small ncRNA therapeutics have entered clinical study for different CVD applications; *Table 2* lists the most advanced of these.

The ability to readily target the liver has led to a large pipeline of ncRNA therapies to target different aspects of hyperlipidaemia. An important target isLp(a), a major carrier of oxidized phospholipid in human plasma and a causal risk factor for atherosclerotic CVD and aortic stenosis,[81] but not targeted by current small molecule drugs. Lipoprotein(a) contains Apo(a)—the product of the *LPA* gene—covalently bound to ApoB-100. Pelacarsen (formerly AKCEA-APO(a)-L_{Rx}), an ASO-targeting *LPA* mRNA which is subcutaneously injected 2–4 weekly, lowers Lp(a) levels by up to 80%, and is well tolerated apart from injection-site reactions.[82] It is currently in Phase 3 trials in patients with CVD (NCT04023552). Olpasiran, a GalNAc-conjugated siRNA to lower Lp(a), is in Phase 2 studies (NCT04270760), while SLN360 is another GalNAc-conjugated siRNA in Phase 1 trials (NCT04606602).

Another promising hyperlipidaemia target is angiopoietin-like 3 (ANGPTL3), based on findings that loss-of-function variants are associated with substantially lower LDL-cholesterol and triglyceride levels and a reduced CHD risk.[83,84] The ANGPTL3 is mainly produced in the liver and is a circulating inhibitor of lipoprotein lipase and endothelial lipase, thereby modulating muscle-free fatty acid uptake, adipose tissue lipogenesis, and hepatic uptake of LDL-cholesterol and remnant cholesterol.[85] Importantly, the latter effects are LDL-receptor-independent, meaning that ANGPTL3 lowering should be effective in FH patients

with LDL-receptor mutations. Vupanorsen, a GalNAc-modified ASO-targeting *ANGPTL3* mRNA, has been investigated in a Phase 2b study (NCT04516291) in diabetic patients with hepatic steatosis and hypertriglyceridaemia where it achieved 38–58% reductions in triglycerides, ApoC3, and remnant cholesterol.[86] However, despite this trial meeting its primary objectives, at the end of January 2022, drug development was discontinued due to the perceived insufficient efficacy and liver toxicity (https://www.pfizer.com/news/press-release/press-release-detail/pfizer-and-ionis-announce-discontinuation-vupanorsen). Finally, ARO-ANG3, a GalNAc-conjugated siRNA also targeting *ANGPTL3*, has been assessed in a small open-label study in heterozygous FH[87] and is now in a larger Phase 2 trial (NCT04832971).

Two other ASOs in clinical experimentation target endogenous miRNAs, namely miR-92a and miR-132-3p. Antisense oligonucleotide–mediated miR-92a inhibition increases vascularization after MI and hind limb ischaemia,[88] accelerates wound healing,[89,90] improves re-endothelialization,[91] and prevents endothelial dysfunction and atherosclerosis,[92] in murine and porcine models. Catheter-based delivery of an anti-miR-92a LNA ASO in pigs effectively reduced infarct size and improved cardiac function.[93] Analogous results in pigs were also obtained by intracoronary injection of a miR-92a antagomir encapsulated in PLGA microspheres.[54] Based on these findings, a placebo-controlled, dose-escalating study using an intravenously administered anti-miR-92a LNA ASO (MRG-110) was performed in 49 healthy volunteers (NCT03494712). This study demonstrated safety and that a single dose of ASO de-repressed miR-92a target mRNAs in different human peripheral blood cells for 2 weeks.[94] Another Phase 1 study (NCT03603431) investigated intradermal injection of 3-weekly MRG-110 doses to promote angiogenesis and improve wound healing. The results indicate safety and preliminary evidence of neoangiogenesis (https://www.globenewswire.com/en/news-release/2019/10/16/1930277/31683/en/miRagen-Announces-New-Clinical-Data-Showing-MRG-110-Positively-Impacted-Tissue-Repair-and-New-Blood-Vessel-Growth.html).

First-in-human data were also reported for an LNA ASO–targeting miR-132-3p for patients with HF.[95] Pre-clinical studies had indicated that inhibition of miR-132-3p alone is sufficient to prevent HF in mice and pigs by improving contractility and metabolism, and reducing hypertrophy.[96,97] Based on these data, a 16-mer LNA ASO with a PS backbone (named CDR132L) was developed and tested by intravenous injection in a placebo-controlled, dose-escalation Phase 1b study in 28 HF patients with NYHA Classes I–III and left-ventricular ejection fraction >30% (NCT04045405). The study met safety objectives and provided evidence of efficacy based on cardiac function and HF biomarkers.[95] More broadly, the study provided evidence of favourable pharmacokinetics upon a 4-weekly ASO-dosing regimen, which has relevance for future applications of ASOs with a similar chemistry.[98]

Other disease indications being clinically tested include acute kidney injury (AKI) after high-risk cardiac surgery. Teprasiran, a synthetic chemically stabilized siRNA that targets p53, has been tested in a randomized multicentre Phase 2 study in 360 patients (NCT02610283). Teprasiran achieved a 58% relative risk reduction in AKI, with no significant safety issues.[99] A larger Phase 3 trial in >1000 patients completed enrolment (NCT03510897) but was terminated early due to results not meeting efficacy outcomes at Day 90 (NCT03510897).

Finally, a subcutaneously administered GalNAc-conjugated siRNA, vutrisiran, is being developed by the manufacturers of patisiran for the same indication of down-regulating transthyretin mRNA for HTT-amyloid. Vutrisiran is in two large Phase 3 trials in patients with HTT-amyloid with polyneuropathy (NCT03759379) and patients with hereditary or non-hereditary TT-amyloid with cardiomyopathy (NCT04153149).

Novel candidate non-coding RNA therapies from pre-clinical studies

Non-coding RNA therapies for heart failure

Cardiac remodelling is an obvious therapeutic target in HF. The underlying processes, such as CM hypertrophy, altered excitation–contraction coupling, cell death, interstitial fibrosis, microvascular rarefaction, and others, are underpinned by substantial changes in the expression of protein-coding genes, ncRNAs, and re-expression of foetal genes. As such, the targeting of gene expression is an attractive approach to tackle remodelling.

A common approach has been to use 2′-OMe-modified antagomiRs or LNAs to inhibit miRNAs involved in cardiac hypertrophy, with early proof-of-concept for *in vivo* silencing originally reported in mice.[100] The therapeutic antagonism of miRNAs to inhibit hypertrophy and remodelling during experimental HF in mice has been shown for numerous miRNAs, which act on either CMs or fibroblasts. These include miR-133,[100] mir-29,[101] miR-21,[102] miR-23a,[103] miR-199a-5p,[104] miR-24,[105] miR-34a,[106] and miR-25.[107] Other studies reported success with LNA ASOs, e.g. against miR-208a,[108] mir-34,[109] miR-652,[110] miR-154,[111] miR-29,[112] and for miR-21 in pigs.[113] Most of these approaches also reduced fibrosis and cardiac dysfunction. Other anti-miR oligonucleotide applications are reviewed in De Majo and De Windt.[114]

Approaches to target other cell types may also be effective. The inhibition of leucocyte-expressed miR-155 in mice reduced cardiac inflammation, hypertrophy, and dysfunction after chronic pressure overload.[115] Icli *et al.*[116] found that intravenous administration of an LNA targeting miR-26a rapidly induced angiogenesis, reduced MI size, and improved heart function. An RNA aptamer-targeting osteopontin (which drives both fibrosis and hypertrophy) prevented or reversed pressure overload–induced HF in mice.[117] Finally, antagonism of endothelial miR-24 limited MI size by preventing endothelial apoptosis.[118]

Long ncRNAs are also potential targets. Nuclear lncRNAs can modify chromatin structure close to their own transcribed regions or at a different site, act as decoys for proteins or regulate mRNA alternative splicing.[119] Cytoplasmic lncRNAs regulate mRNA stability and translation, interact with cytoplasmic proteins and act as miRNA sponges.[120] As most aspects of cardiac function are governed by one or multiple lncRNAs,[121–124] targeting lncRNAs is an attractive therapeutic option. The first examples of therapeutic lncRNA targeting in the cardiovascular field include GapmeR-mediated silencing of the lncRNA Chast, which effectively prevented or attenuated pressure overload–induced pathological cardiac remodelling,[125] lncRNA Meg3, to reduce cardiac fibrosis and improve diastolic function after chronic pressure overload,[126] lncRNA Wisper to inhibit MI-induced fibrosis and cardiac dysfunction,[127] and lncRNA H19 to inhibit aortic aneurysm development[128] or pulmonary arterial hypertension.[129] Other applications of anti-lncRNA antisense technology are reviewed in Hobuss *et al.*,[121] Poller *et al.*,[122] Salamon *et al.*,[123] and Lucas *et al.*[124] It is worth noting, however, that the degree of sequence conservation between lncRNAs of different species is relatively limited,[130–132] rendering the pre-clinical development of effective therapies more problematic.

Non-coding RNAs for cardiac regeneration

A specific therapeutic area gaining momentum at pre-clinical level is the stimulation of endogenous CM proliferation to achieve cardiac

regeneration after MI. As CM proliferation is controlled by both the miRNA and lncRNA networks,[133] a few studies over the last years have explored the possibility of delivering synthetic ncRNAs to achieve cardiac regeneration after MI. Several miRNAs that control CM proliferation were identified in two large screenings that analysed whole-genome miRNA libraries in rodent and human cells.[24,134] Anti-miR LNAs against miR-15b[135] and miR-34a,[136] which both inhibit CM proliferation, were shown to exert a beneficial effect after MI in rodents.

The efficacy of antisense approaches, however, strictly depends on the level of expression of the inhibitory ncRNAs and their relative importance in controlling regeneration. A more tempting pharmacological approach is to impart a proliferative phenotype to CMs with small RNAs, irrespective of whether the administered molecules participate in normal cardiac physiology. A single intramyocardial injection of miR-199a-3p or miR-590-3p mimics using a cationic lipid formulation was sufficient to induce cardiac regeneration after MI in mice.[137] Similar results were also reported with the intramyocardial administration of miR-19a/19b mimics using a neutral lipid delivery reagent,[59] using the same formulation for the daily intravenous administration of miR-302b/c,[60] miR-19a/19b[59] or miR-708[138] mimics, or the intracardiac delivery of cholesterol-modified miR-302b/c mimics using a hydrogel.[139] Of note, the administration of miRNA mimics exerts a transient effect lasting approximately 10 days with miR-199a-3p.[137] This can overcome the adverse effects and safety concerns potentially caused by the continuous expression of pro-proliferative RNAs upon gene transfer of their coding genes using AAV vectors.[140]

Non-coding RNA delivery to the heart

Cardiac-specific delivery may be achieved either through cell targeting upon systemic administration or via direct injection through selective catheterization. While the former approach is still in its infancy, the latter appears readily achievable.

Cardiac cell targeting

An ideal ncRNA therapeutic should fulfil the mandate of the so-called Ehrlich's magic bullet, namely be administered systemically to then reach the target organ in a specific manner. This is achievable for the liver by the conjugation of ASOs and siRNAs with GalNAc, which specifically binds the hepatocyte ASGPR as discussed earlier. No such targeting system yet exists for any cardiac cell type. Virus-mediated gene transfer can achieve tissue-specific efficacy by using CM-specific vectors (e.g. AAV Serotype 9 or 6) and by including CM-specific promoters for transcriptional targeting.[141] Neither possibility is available to synthetic ncRNAs.

After intravenous injection, polymer:RNA or lipid:RNA complex is generally phagocytosed by mononuclear cells in the liver, spleen, lymph nodes, and bone marrow.[142] A first step towards tissue specificity is therefore to reduce interaction with phagocytic cells and increase circulatory half-life, e.g. by PEG surface modification of LNPs and other nanocarriers to provide a hydrophilic shield. Polyethylene glycol-lipids in LNPs also reduce nanoparticle opsonization by ApoE and other circulating lipoproteins, which results in uptake by lipoprotein receptors.[66]

Active cellular targeting could be achieved through inclusion in the LNPs or other nanocarriers of ligands that bind specifically expressed surface molecules. Several peptides have been identified through phage display panning techniques to target CMs[143–148] or the infarcted myocardium.[144,149,150] Additional possibilities for cardiac targeting are a ligand for the angiotensin II Type 1 receptor,[151] antibodies against cardiac troponin T[152,153] or myosin,[154] ligands for activated endothelial cells,[155,156] carbohydrates,[157] or substrates for enzymes present in the infarct area.[158] Despite abundant data in cell culture and rodents, none of these strategies has yet progressed to large animal or clinical testing.

In cancer settings, nanosized particles may accumulate in tumour tissue due to enhanced vascular permeability and retention (EPR effect).[159] A similar EPR effect after MI could allow preferential accumulation of LNPs in the damaged myocardium. While the post-MI EPR effect begins to diminish after 24–48 h and thus might be too short for treatments aimed at preventing adverse remodelling (reviewed in Prajnamitra *et al.*[160]), this time frame may be sufficient for nanoparticles carrying ncRNA payloads that induce acute cardioprotection or stimulate post-MI cardiac regeneration.

Direct cardiac-specific delivery

The heart is amenable to physical targeting by direct injection or catheterization of its main vessels (*Figure 4*).

Intramyocardial injection

This may be performed surgically (mini-thoracotomy or open-chest surgery) through transepicardial injection or percutaneously via transendocardial injection. The surgical approach allows precise delivery and in multiple locations (e.g. the infarct border zone) but is limited by its invasiveness and difficulty in accessing areas such as the interventricular septum. Percutaneous transendocardial delivery via the left-ventricular cavity is less invasive and safe in expert hands.[161,162] Different imaging techniques (e.g. intravascular ultrasound) or periprocedural electromechanical mapping (e.g. NOGA; Biosense Webster) are required to inject nanomedicine products.[163,164]

Intracoronary administration

The simplest and clinically most applicable route for direct delivery of a therapeutic remains antegrade coronary catheterization, which can be safely performed even in patients with marked cardiac dysfunction.[165,166] However, the transit time in the coronary circulation is very short and the endothelial cell barrier needs to be crossed. The latter is normally impermeable to particles >20 nm diameter,[167] much smaller than typical LNPs (80–100 nm) and even larger lipoplexes. Numerous approaches have been proposed to improve antegrade delivery efficacy, such as increasing permeability or coronary sinus occlusion,[168,169] but delivery may also be enhanced early after MI due to the aforementioned EPR effect or the use of closed recirculation systems, by which coronary sinus blood is collected and re-delivered to the coronary artery through an extracorporeal membrane oxygenation system.[170–172]

Higher efficacy may be achieved by retrograde administration via the coronary sinus,[173–176] a relatively simple interventional procedure usually accompanied by transient antegrade blockade of coronary artery flow.[164] Maintenance of high pressure increases efficacy of this approach.[173,177] Potential issues are the presence of cardiac resynchronization devices and the risk of coronary sinus rupture.

Pericardial administration

Percutaneous intra-pericardial delivery could offer broad access to the entire heart except the septum.[178] The safety of this approach for epicardial catheter electrophysiological ablations has progressively improved but a major issue for ncRNA therapy may be restricted

delivery to the epicardial surface and removal of agent by the pericardial lymphatic system.[164,179]

Conclusions and future perspectives

Small nucleic acid therapies offer an exciting new therapeutic modality for CVD and an opportunity to tackle disease pathways that are not targeted by current small molecule approaches. Despite this perceived potential, there are several challenges that need to be overcome before wider clinical application of ncRNA therapies. A major issue that remains is improvements in the carriers and RNA modifications that would allow efficient cellular uptake and intracellular stability of the agents; lipid-based nanocarriers currently appear the most promising approach, at least until efficient CM-targeting systems are developed.

An additional problem relates to toxicity following the undesired activation of the innate immune system. As part of the antiviral defence mechanism, cells recognize both single- and double-stranded RNA via pathogen-associated molecular pattern receptors.[180] The predominant pathway that recognizes small RNAs starts with the detection of RNA structures by Toll-like receptors 3, 7, and 8 in endosomes.[181] This can result in the production of pro-inflammatory cytokines in dendritic cells and macrophages, or the stimulation of a Type I interferon response.[182] In addition, double-stranded molecules are recognized by intracytoplasmic receptors, such as retinoic acid-inducible gene I (RIG1) and melanoma differentiation-associated protein 5 (MDA5).[183] Further improvements in the design of ncRNA therapeutics should aim to reduce immune recognition. Already available evidence indicates that innate immune system activation by siRNAs is sequence specific[184] and that immunogenicity can be markedly decreased by the incorporation of 2′-OMe or 2′-F modifications in the ribose ring of nucleotides.[185–187]

Potential off-target effects of ncRNA therapies also need to be borne in mind. For example, siRNAs can induce off-target effects due to their miRNA-like activity, by which other cellular genes can also be down-regulated.[188–191] Analogous problems can occur with miRNA mimics, in which the passenger strand, which is usually chemically modified, can accumulate in transfected cells and exert effects on other cellular mRNAs.[192] This can be even more relevant considering that these off-target effects can be cell-type specific and thus difficult to predict or reproduce in cell culture.[193]

Notwithstanding these hurdles, the class of ncRNA therapies may truly portend a paradigm shift in our approaches to the management of many CVD.

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