**Rare Disease and
Orphan Drugs Journal**

Review

Check for updates

RNA antisense and silencing strategies using synthetic drugs for rare muscular and neuromuscular diseases

Daniel Scherman[1,2]

[1]Foundation for Rare Diseases, 96 rue Didot, Paris 75014, France.
[2]Université Paris Cité, Faculté de sciences pharmaceutiques et biologiques, Unité de Technologies Chimiques et Biologiques pour la Santé (UTCBS), CNRS UMR8258, Paris 75014, France.

Correspondence to: Prof. Daniel Scherman, Faculté de sciences pharmaceutiques et biologiques, Unité de Technologies Chimiques et Biologiques pour la Santé (UTCBS), CNRS UMR8258, Inserm U1267, 4, avenue de l'observatoire, Paris 75014, France. E-mail: daniel.scherman@parisdescartes.fr

Abstract

Rare diseases occur in their large majority from a genetic cause, which makes them good candidates for genetic RNA drugs. The basic concepts, principles, mechanisms of action and chemical optimizations of synthetic antisense oligonucleotides (ASO) and small interfering RNA (siRNA) are illustrated. These drugs act either by leading to RNA degradation, or as steric blockers of RNA translation, microRNA antagonists, splicing modulators or inducers of exon skipping. Chemical modifications and delivery techniques differ and are adapted to their distinct functions. The successes, potential, and challenges of synthetic RNA drugs are illustrated for several muscular and neuromuscular diseases: Duchenne muscular dystrophy, spinal muscular atrophy, transthyretin amyloidosis, Type 1 myotonic dystrophy, centronuclear myopathy, oculopharyngeal muscular dystrophy.

Keywords: Antisense oligonucleotide, neuromuscular disorders, rare disease, RNA drug, RNA interference, small interfering siRNA

INTRODUCTION

In the last century, Paul Ehrlich and Emil Fischer introduced the concepts of chemotherapy, magic bullet, and lock-key, in which a drug is defined as a small molecule that specifically binds to a biological target through a three-dimension spatial recognition pattern[1,2]. With the advancement of genetics and the sequencing of the human genome, the genetic defects being the cause of rare genetic diseases are being increasingly elucidated. This has led to the emergence of a new class of "genetic" drugs. In addition to binding to their target through a 3-dimensional hydrogen bonding recognition pattern, these drugs recognize a 1-dimensional linear genetic sequence. In such cases, drug design is based on genetic information, which opens the perspective of the fast and rational development of drugs against a considerable number of diseases characterized by a specific genetic defect.

Theoretically, any gene or mRNA can be targeted by a proper nucleotide sequence selected to be unique on a given genome or transcriptome. Thus, such a sequence recognition mechanism opens tremendous perspectives in medicinal chemistry. Instead of a painstaking customized search for specific spatial recognition of a targeted biological ligand by a chemical compound or a monoclonal antibody, genetic drugs are based on a robust universal platform that can be used for a very large number of applications. Protein targets that were until now considered "non-druggable" can be challenged on their genetic expression. In addition, the use of a common validated technology for all antisense oligonucleotides (ASO) or all small interfering RNA (siRNA) allows for shortening pharmaceutical development steps and reducing costs, which is of paramount importance for rare diseases. In the case of ultrarare diseases, personalized therapy can now be envisioned with these revolutionary platforms for an n-of-1 patient[3].

The basic concepts, principles, mechanisms of action, and chemical optimizations of ASOs and siRNAs are illustrated in the present review. These RNA-targeted drugs can cause RNA degradation or act as steric blockers. In the latter case, they can inhibit RNA translation, antagonize a miRNA, modulate splicing, or induce exon skipping. Their specific divergent functions correspond to adapted chemical and delivery optimizations. Thus, RNA drugs are able either to restore a therapeutic mRNA or, in most cases, suppress mRNA or block mRNA translation to correct "gain-of-function" dominant genetic disorders. But another important application is that of splice modulator, which can overcome a nonsense mutation and lead to the expression of a functional, although shortened, therapeutic protein.

Out of the scope of the present review are the cell and gene therapy approaches compensating for a genetic deficiency by replacing the wild-type correct gene. We will not detail either the more recent revolution of genome editing, in which a genetic defect is corrected "*in situ*" by tools such as clustered regularly interspaced short palindromic repeats (CRISPR) associated with the Cas9 (CRISPR-Cas9)[4]. Finally, the present review does not intend at an extensive description of the clinical trials realized so far, as this aspect has been extensively reviewed elsewhere[5-8].

REVIEW

Steric blocker ASOs

The first RNA drug was introduced in 1978 against Rous sarcoma viral replication[9,10]. It used a 13-mer synthetic antisense oligonucleotide (ASO). It was an oligodeoxynucleotide complementary to 13 nucleotides of the 3'- and 5'-reiterated terminal sequences of Rous sarcoma virus 35S RNA and annealing to it through Watson-Crick recognition. This creates an intracellular DNA/RNA heteroduplex which may either function as a translation steric blocker or lead to mRNA degradation. The ASOs acting as steric blockers of translation target the mRNA translation initiation site (start codon). This inhibits the binding of ribosomal subunits to the mRNA through steric hindrance, thus blocking protein synthesis [Figure 1A]. While this
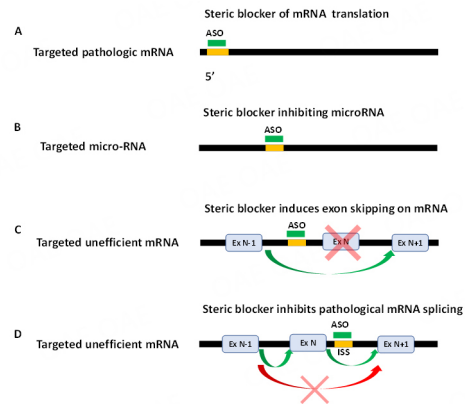
Figure 1. Non-RNase H-dependent ASO steric blocking effects. In yellow is indicated the complementary targeted sequence on the mRNA. ISS: Intronic splice silencer. A: mRNA Translation inhibition; B: Antagomir; C: Inducing skipping of a pathologic exon carrying stop or out-of-frame mutations; D: Splice modulator inhibiting a non-desired naturally occurring exon skipping.

antisense blocking effect on mRNA translation might be responsible for part of the observed decreased protein expression, it was later found that the loss of protein expression was also due to mRNA degradation induced by RNase H nuclease. This RNase specifically recognizes heteroduplexes and cleaves the associated mRNA. This nucleolytic mechanism of action drives most of the actual ASOs' clinical applications.

Antisense ASOs have the potential to induce numerous other steric blocking effects, which has led to major therapeutic successes. These are schematized in Figure 1.

MicroRNA (miRNAs) are a group of one to two thousand small non-coding RNA molecules containing 21 to 23 nucleotides. They play important biological functions as post-transcriptional regulators of gene expression. The miRNAs anneal to complementary sequences on mRNA molecules, leading to gene silencing by several mechanisms: mRNA cleavage mediated by the RNA-induced silencing complex RISC (see below the detailed section on RNA interference), mRNA destabilization by poly(A) tail shortening, or blocking of mRNA translation. As shown in Figure 1B, ASO steric blocking can antagonize these cellular "bandmasters", and thus display an antagomir action, which presents interesting therapeutic applications in neuronal and neuromuscular diseases[11].

An antisense blocker ASO can be used by sterically hindering a splice acceptor or splice enhancer site, thus promoting exon-skipping [Figure 1C]. This mechanism of action is of great interest for treating genetic diseases caused by stop or out-of-frame missense variants, and where the skipping of one or o several exons leads to a still functional or partially functional truncated form of the protein product. This strategy has led to clinically approved ASO RNA drugs for Duchenne Dystrophy. Exon skipping can also be of interest in cases where a pathologic alternative splicing of variant genes occurs, which leads to the inclusion of an additional exon, causing the expression of a non-functional protein. Finally, blocker ASOs can also treat cases where an intronic pathogenic variant results in aberrant inclusion of an intron segment into the mRNA transcripts, thus abolishing protein function.

Figure 1D displays the use of an ASO to inhibit a naturally occurring undesired splicing event leading to exon N skipping and translation of a non-functional truncated protein. By blocking this undesired exon skipping, therapeutic restoration of a complete functional protein is obtained. As will be described in Spinal muscular atrophy, this occurs in the case of spinal muscular atrophy (SMA), where a non-functional

truncated form of the SMN2 mRNA is produced. An ASO which sterically blocks the intronic splice silencer present on the SMN2 gene favors pre-mRNA maturation towards the complete form of the SMN2 mRNA, thus leading to the expression of a functional SMN protein. This approach has represented a historically major success as the first therapy for SMA [5,7,8, and more references in *Spinal muscular atrophy*].

ASO-induced mRNA degradation

RNA primers are required to initiate the synthesis of both the leading strand and Okazaki fragments on the lagging DNA strand during genome replication. Moreover, DNA replicases occasionally incorporate ribonucleotides into DNA. Finally, R-loops generated as a by-product of transcription when nascent mRNA molecules hybridize with the template DNA represent another example of naturally occurring RNA/DNA duplexes. Cells are ubiquitously equipped with ribonucleases H type 1 (called here RNase H), whose function is to remove RNA moieties from DNA, because such RNA/DNA duplexes might cause chromosomal instability and cell lethality during replication[12,13]. The RNases H nuclease belongs to the nucleotidyl transferase superfamily which relies on divalent cations to catalyze nucleophilic substitution reactions. These enzymatic reactions specifically hydrolyze either a single ribonucleotide or stretches of RNA in a diverse range of nucleic acids, such as RNA/DNA hybrids, R-loops, and double-stranded DNA with an embedded single ribonucleotide, *etc*.[14]. Other enzymes such as transposase, retroviral integrase, Holliday junction resolvase, and RISC nuclease Argonaute involved in the mechanism of RNA silencing belong to the same nucleotidyl transferase superfamily as RNase H.

As already mentioned, antisense oligodeoxynucleotides annealing to complementary mRNA sequence create an intracellular DNA/RNA heteroduplex to which RNase H binds, leading to cleavage and subsequent degradation of the targeted mRNA [Figure 2A]. After mRNA cleavage at the complementary site, the mRNA strand is released from the ASO, which thus becomes available for further association with another target mRNA.

RNase H-dependent ASOs are of interest for treating diseases caused by dominant-negative genetic variants. An important feature is that RNase H requires a non-modified deoxyribose moiety on the center of the complementary ASO sequence (the "seed" sequence) to maintain its catalytic efficiency. Thus, an efficient ASO must be either a pure deoxy oligomer bearing only unmodified ribose sugars, or a gapmer containing a stretch of about 10 natural ribose sugars with a variable number of modified sugars on the 3' and 5' ends.

mRNA degradation by RNA interference RNAi

The second strategy to suppress a targeted mRNA in a sequence-specific manner makes use of the RNA interference process (RNAi)[15-18]. As indicated above, interference is the natural microRNA-mediated process (mi-RNA) that is central to the post-transcriptional silencing regulation of many basic cellular and developmental programs. This process is schematized in Figure 3.

The basic steps of miRNA maturation involve the transcription by RNA polymerase II of primary miRNAs (pri-miRNAs). The miRNAs derive from regions of RNA transcripts that fold back on themselves to form short hairpins whose main characteristic is the presence of one or several mismatches in the self-complementary sequence. The primary transcript known as pri-miRNA is processed in the cell nucleus into ~70 nucleotides pre-miRNA by the microprocessor complex Drosha/DGCR8. The microprocessor complex subunit DGCR8 (DiGeorge syndrome critical region 8) contains an RNA-binding domain that stabilizes the primary miRNA for processing by the second microprocessor subunit Drosha, an RNase III enzyme. The pri-miRNA is cleaved by the Drosha/DGCR8 complex to a characteristic stem-loop structure known as a pre-miRNA. Pre-miRNAs have a hairpin structure with stems containing interspersed mismatches and are
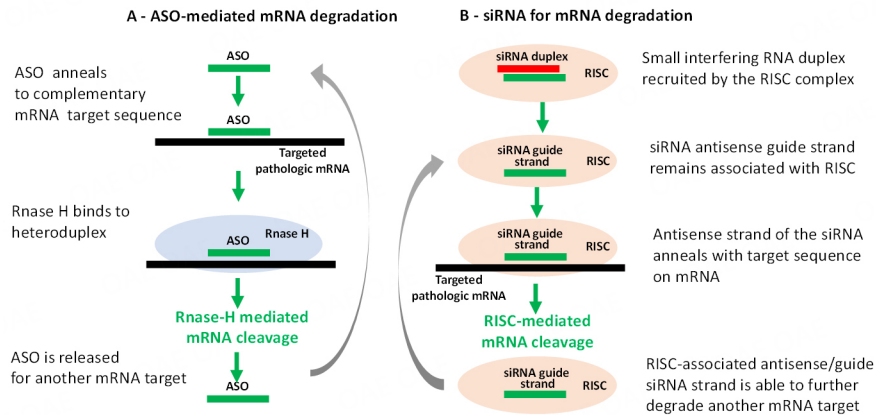
Figure 2. Comparison between the mechanism of action of genetically targeted mRNA degradation. A (left): ASO-mediated mRNA degradation through RNase H1 nucleolytic activity. ASO (green rod) binds to complementary sequences on mRNA. This heteroduplex induces RNase H binding and leads to mRNA cleavage. After mRNA cleavage, the mRNA fragments, ASO and RNase H are released from each other. Grey arrow: free ASO is recycled for binding to another mRNA on the target sequence, which induces a new cycle of RNase H binding and mRNA cleavage; B (right): siRNA-mediated mRNA degradation. Double-stranded homoduplex siRNA is recruited by the RISC complex. The passenger "sense" strand (red rod) is released from the complex. The guide strand (green rod) remains positioned in the RISC complex and stabilizes it on the targeted complementary mRNA sequence by Watson-Crick hybridization. This induces mRNA cleavage. The cleaved mRNA is dissociated, and the RISC/guide siRNA complex can associate and cleave another mRNA (grey arrow).

exported across the nuclear envelope to the cytosol by binding to exportin 5.

The next step occurs through a cytoplasmic process where the riboendonuclease Dicer mediates the cleavage of the pre-miRNA at the base of the stem (removing the terminal loop) to form the double-stranded mature miRNA duplex[19,20]. Then, the RNA Induced Silencing Complex (RISC) eliminates the miRNA sense passenger strand and keeps binding to the antisense strand. This antisense strand is also called the "guide" strand because it allows the specific binding of the RISC complex to the complementary Watson-Crick sequence on the targeted mRNA. The RISC complex is composed of the protein TRBP and the argonaute 2 nuclease (Ago).

Natural miRNA target sites tend to cluster in the mRNA 3' untranslated region, although other miRNA sites are being found in different mRNA regions. The binding of miRNA/RISC leads to the repression of mRNA translation and, in a certain proportion, to mRNA cleavage by Ago. The absence of mismatch induces complete mRNA cleavage, while the presence of mismatch favors translation repression.

For therapeutic use of the endogenous miRNA process, two strategies have been employed. In the first one, an expression cassette containing a short gene encoding a self-complementary mRNA is delivered to the cells. The resulting mRNA transcript forms a short hairpin (shRNA) [Figure 3 right side], which is processed by the Drocha/DGCR8 complex. The shRNAs have a completely base-paired 19-29 nt stem, ensuring the Dicer cleavage step to produce the final siRNA. The resulting siRNA duplex has symmetric 2 nucleotide 3' overhangs with a 19-21 base-paired region. It associates with the RISC ribonucleoprotein complex leading RNAi[21]. In most published cases, shRNA transgenes are delivered using either an AAV or a lentiviral gene delivery vector. The shRNA technique is widely used at the laboratory stage because it is available through standard expertise and ensures continuous intracellular siRNA production and efficient silencing.
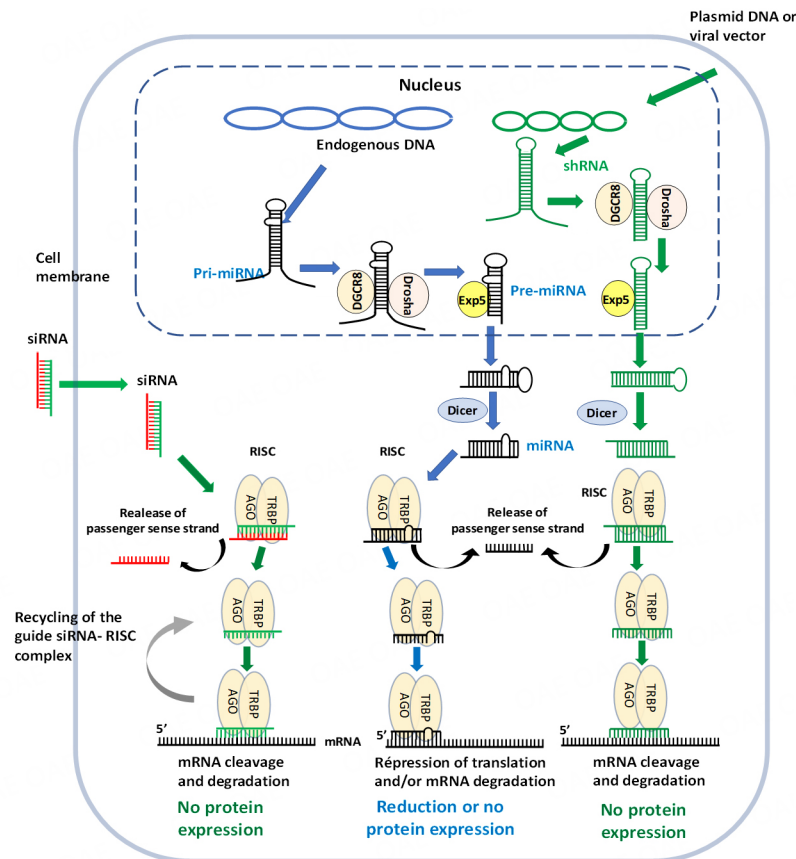
Figure 3. Mechanism of miRNA, siRNA, and shRNA action. The Center (blue arrows) represents the canonical miRNA maturation and mechanism of action pathway. Transcription leads to a pri-miRNA, which folds into a small hairpin RNA with one or several mismatches. The pri-miRNA is processed by Drosha and DGCR8 to a pre-miRNA. After association to exportin 5, the pre-miRNA is transported through the nuclear envelope by a process dependent upon the small GTPase ran-GTP. The pre-miRNA is finally processed by the Dicer endoribonuclease (a class III RNase), which deletes the hairpin loop. This generates an RNA duplex made of a passenger and a guide strand. After miRNA homoduplex association with the RISC complex composed of protein TRBP and the argonaute 2 nuclease, the passenger strand is eliminated. The RISC/guide strand complex binds to the target mRNA, in the 5' non-translated region, inducing a steric blocking of ribosome entry and translation. In some instances, miRNA binding can also lead to mRNA cleavage and degradation, but the mismatch generally make this less likely. Left side: siRNA mechanism. (green arrows), a double-strand siRNA with no mismatch is administered to the cells. All action takes place in the cytosol. After siRNA binding to the RISC complex composed of protein TRBP and the argonaute 2 nuclease, the passenger strand is eliminated. The RISC/guide strand complex binds to the target mRNA. The target sequence is generally chosen within the translated region. Because of the complete siRNA matching with the mRNA target sequence, argonaute 2 is now able to cleave the target mRNA with great efficiency. After mRNA cleavage and elimination, the RISC/guide siRNA complex can bind and cleave another mRNA (grey arrow). Right side: (green arrows): shRNA mechanism. A gene expression cassette coding a small hairpin RNA is administered either as a plasmid or by a viral gene delivery vector. The shRNA transcript is then processed as for the miRNA. However, no mismatch is introduced, which leads to fully efficient mRNA degradation and silencing.

The second RNAi therapeutic strategy uses exogenous siRNAs, which are made of synthetic 20-25 base pairs. As shown in Figure 2B and Figure 3 left side, their mechanism of action also involves RISC. The siRNAs are administered as two-strand perfect matching Watson-Crick homoduplexes. One "antisense" strand, which is also called the "guide" strand, is complementary to a sequence on the targeted mRNA. In addition, both 3' ends possess two supplementary over-hanging non-hybridized nucleotides.

Such an siRNA homoduplex is recognized by the RISC nucleoprotein complex which dissociates the sense siRNA strand. As with miRNAs, the RISC complex is then guided by the antisense strand to bind to the complementary sequence on the targeted mRNA, leading to mRNA nucleolytic cleavage and degradation [

Figure 2B and Figure 3, left side]. Since the guide sense is designed for a perfect match with its complementary sequence on the targeted mRNA, the Ago-mediated mRNA cleavage is complete.

Once this has occurred, the RISC /guide siRNA complex can be used again to target and cleave another pathologic mRNA. This recycling process associated with optimized chemistry enabling high siRNA metabolic stability ensures a long duration of action[22]. Indeed, *in vivo* effects have been reported for the most recently developed siRNA drugs with a spectacular duration of action superior to 6 months after a single administration[23].

Comparison between ASO and RNAi properties

The differences between natural phosphodiester ASOs and siRNAs are summarized in Table 1.

The ASO and siRNA characteristics and specificities listed in Table I are responsible for marked differences in physicochemical and pharmacokinetic properties which dictate different required chemical optimization, depending on the nature of the drug and the targeted tissue. A single-stranded ASO is more flexible and accessible to an endonuclease and thus necessitates complete protection of each phosphodiester linkage against nucleases. By contrast, a double-helix siRNA shows more resistance in its internal phosphodiester linkages. A single-stranded ASO also displays hydrophobic moieties from its nucleic bases, which is not the case for double-helical siRNA. Hence, ASOs will bind to plasma proteins such as albumin, which leads to longer circulation time and enhanced biodistribution to tissues. On the contrary, natural siRNAs are rapidly eliminated by kidney filtration and need a delivery vector such as a lipid nanoparticle (LNP) formulation or a functional targeting moiety towards an extracellular receptor.

Other differences originate from the fact that only binding to the target mRNA is required to achieve a blocker ASO function, as for mRNA degradation, the catalytic activity of RNase H (for ASOs) and of Argonaute 2 (for siRNAs) must be maintained. Finally, it is generally considered that ASOs can block or cleave both pre-mRNA and mRNA either in the nucleus or cytosol, while siRNA ensures the degradation of the mature mRNA in the cytosol only. This represents an important point for diseases caused by nuclear aggregation of variant mRNA, such as Myotonic Dystrophy Type 1 (MD1) (see section *Type 1 myotonic dystrophy: different ASO modes of action*). This is, however, a disputed point, because several siRNAs and shRNAs have been shown to lead to nuclear foci degradation in MD1 cellular and animal models[24-26] thus suggesting that the RISC-induced cleavage and subsequent degradation could also occur in the nucleus.

Chemical optimization of steric blocker ASOs

After their initial discovery, the first enthusiastic attempts towards the clinical use of both ASOs and siRNAs were very disappointing, leading to a drastic decrease in the investment into these genetic pharmacology drugs. These initial approaches used natural oligonucleotides, which have poor pharmacokinetics because they undergo fast degradation by endo- and exonucleases. They are degraded in less than 20 minutes after intravenous administration. In addition, these first-generation ASOs and siRNAs were rapidly eliminated by kidney filtration. This decreases tissue biodistribution and might induce undesirable glomerular toxicity resulting from temporary elevated local concentrations of these biologically active agents. Moreover, natural oligonucleotides are small hydrophilic polyanionic compounds due to the phosphodiester linkages, which hinder cell penetration through the lipophilic plasma membrane. Finally, innate immune response to double-stranded RNA, especially through the TLR3 receptor[27-30], was found to represent a strong bottleneck for the development of siRNA drugs. All these considerations led to the conclusion that chemical modifications were required for the ultimate success of an ASO or siRNA drug.

Table 1. Comparison between natural phosphodiester ASOs and Double-stranded siRNA

ASO	siRNA
Single-stranded	Double-stranded
14-20 bases - linear deoxynucleotide	21-23 base pairs RNA
Flexible with ~ 1 nm width	Rigid duplex ~ 2 nm diameter
Single-stranded nature requires full backbone modification with phosphorothioate (PS) linkages to protect from nucleases	Double-stranded nature ensures relative protection against nucleases
Design must retain ribose sugars moieties in the seed center sequence to allow RNase H activity	Ribose sugars can be modified to a certain limit with respect to Argonaute efficiency
Sugar modifications are tolerated only on wings of a gapmer	Sugar modifications tolerated
Hydrophobic surfaces accessible for protein interactions allow binding to plasma proteins such as albumin and increase blood circulation and biodistribution to tissues.	Little exposed hydrophobic surface since aromatic bases are paired and buried in duplex. Hydrophilic surface causes rapid kidney clearance.

ASO: antisense oligonucleotides; siRNA: small interfering RNA.

Many comprehensive excellent reviews have been dedicated to the intensive chemistry efforts performed on ASO or siRNA derivatives, which have concerned their different components: backbone, sugar, and base)[31-33]. The state-of-the-art will be briefly described here. The most successful chemical modifications introduced so far on ASO and siRNA nucleotides are displayed in Figure 4. Because of their different properties and mode of action, different chemical modifications have been selected after more than 20 years of intensive research for each class of RNA drug.

The single-stranded nature of ASOs requires full phosphodiester backbone modification because of their high exposure to exo- and endonucleases. Figure 4B displays the most successful modified backbone linkage used so far, phosphorothioate (PS), in which a sulfur atom replaces one non-linking phosphate oxygen and which partially protects against nucleolytic activity. In addition, phosphorothioate modification has been shown to increase pharmacokinetics and cellular uptake[34-36]. Indeed, the first ASO drug introduced to the market was the full 21 nt full phosphorothioate oligodeoxynucleotide Vitravene, which has the sequence 5'-GCG TTT GCT CTT CTT CTT GCG-3' targeting the CMV protein IE2 mRNA[37]. Several other phosphodiester linkage modifications also confer nuclease resistance, for instance, methylphosphonate, phosphoramidate [Figure 4], and mesylphosphoramidate[38,39].

It should be noted that each PS substitution introduces a chiral phosphate with two stereoisomers, the Sp and Rp forms. The Sp diastereoisomers are more resistant to nucleases and should be preferred for a blocker ASO. In addition, the Rp diastereoisomers display a higher binding affinity and induce more efficient RNase H cleavage, which is more favorable for an mRNA degradation strategy. It has been shown that precise localization of Rp and Sp PS links could optimize the desired function and increase selectivity by decreasing off-target mismatch binding[29].

While phosphorothioate links confer improved relative nuclease resistance and metabolic stability, the protection is not complete. Moreover, PS links decrease ASO and siRNA binding affinity to the complementary mRNA. This can be corrected by introducing modifications to the ribose moiety. Modifications on the 2' carbon, such as 2'-fluoro, 2'-O-methyl or 2'-O-metoxyethyl are extensively used for both ASOs and siRNAs [Figure 4C]. Another way to increase nuclease resistance is by introducing constraints in the ribose ring, such as in the bicyclic locked nucleic acids (LNA) or in the constrained ethyl locked nucleic acids (cet-LNA).
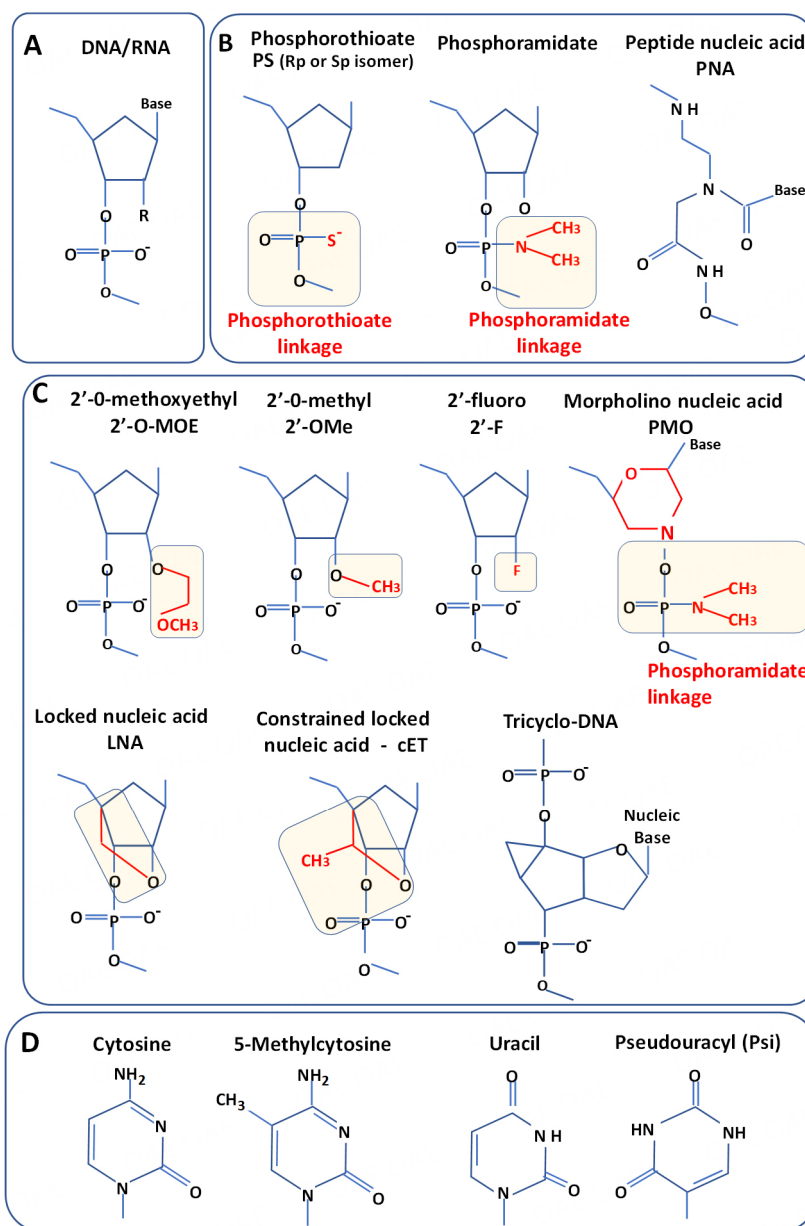
Figure 4. Most popular nucleotide analogs used in ASO and siRNA drugs. A: Natural DNA (R = H) and RNA (R = OH) units; B: linkage analogs. C: Ribose analogs; D: Base analogs. 5-methyl cytosine is the only one having been used in clinically approved drugs at the present time. Pseudouridine (Psi) is not used in RNA drugs but in mRNA vaccines, because it suppresses the innate immune response against double-stranded RNA stretch.

Other quite original structures, which are further modified from natural ribose or deoxyribose rings, have demonstrated their value in increasing metabolic stability while maintaining a high binding affinity toward mRNA. These are the morpholino, tricyclo-DNA, or PNA analogs [Figure 4C]. Morpholinos nucleic acids (commonly designated as PMOs) are methylenemorpholine rings linked through phosphorodiamidate groups instead of phosphates[40]. They display a strong affinity for mRNA leading to an efficient steric blocking effect[41]. Preclinical and clinical studies have shown that PMOs demonstrate improved efficacy, excellent kinetic behavior, biological stability, and a good safety profile[42]. Tricyclo-DNA oligomers display strong antisense and exon-skipping activity[43-44]. PNAs are peptide nucleic acids where the ribose-

phosphodiester backbone has been replaced by the peptide linkage analog N-(2-aminoethyl)-glycine units, which allows using convenient peptide synthesis technology[45]. However, it has been reported that PNAs had the disadvantage of rapid kidney elimination[46].

Finally, base modifications have been introduced, such as the base analog 5-methylcytosine, because this increases nuclease resistance while reducing innate immune response [Figure 4D]. However, the risk of genomic incorporation of these non-natural bases has hampered up to now their clinical use, except for 5-methyl cytosine, while pseudouridine (Psi) is not used in RNA drugs but in mRNA vaccines to alleviate the innate immune response against double-stranded RNA stretches.

More chemical refinements have been proposed to improve the pharmacokinetics and blocking properties of ASOs. For instance, it was found that a mixture of LNA with 2'O-methyl and 2'F nucleotide together with a PS backbone was most efficient in inhibiting miRNAs[47]. Such mixed oligomers are called "mixmers" and are schematized in Figure 5.

Other means to improve ASOs bioavailability to the desired tissue and cells imply nanoparticle encapsulation (see section *Type 1 myotonic dystrophy: different ASO modes of action*), or covalent coupling to a penetration enhancer or to a targeting moiety. Coupling morpholino nucleic acids to a peptide rich in alanine and the cationic amino-acid arginine has been reported to increase tissue delivery and the efficacy of exon skipping or exon restoration in models of Duchenne dystrophy and spinal muscular atrophy[48,49]. Linking ASO to a fatty acid chain showed promising results in a spinal muscular atrophy model[50]. The use of triantennary N-acetyl-galactosamine (GalNac) for targeting and high-performance delivery to liver hepatocytes via the asialoglycoprotein receptor (ASGPR) represents one of the most successful strategies, both for ASOs and siRNAs. It has led to impressive therapeutic achievements[51-54] (see below *Tri-GalNac siRNA Vutrisiran for transthyretin hereditary amyloidosis treatment*). Attempts to deliver ASOs through the intestine and blood-brain barrier have been reported[55-56].

Enhanced bioavailability and nuclease resistance are sufficient conditions for achieving the distinct ASOs therapeutic mechanisms of cation illustrated in Figure 1: steric block of mRNA translation, microRNA inhibition (antagomir effect), exon skipping, and exon restoration. Several FDA-approved drugs demonstrate the success of these chemical modifications for treating rare diseases, which will be further detailed below for muscular and neuromuscular diseases.

ASO chemical optimization for RNase H - induced mRNA cleavage

The necessity to maintain RNase H activity represents a major constraint that limits the use of many of the chemical modifications presented above. Phosphorothioate linkages are compatible with RNase H activity. Inversely, methylphosphonate substitution must be finely optimized. In a typical model study, duplexes formed with deoxy oligonucleotides or phosphorothioate analogs were allowing mRNA cleavage by RNase H, whereas a duplex formed with an oligonucleotide containing six methylphosphonate deoxynucleosides alternating with normal deoxynucleotides was not permissive to RNase H attack. The mRNA susceptibility to cleavage by RNase H increased in parallel to a reduction in the number of methylphosphonate linkages[38].

Sugar modifications such as morpholino or LNA are not tolerated by RNase H. Uniformly modified 2'-deoxy-2'-fluoro phosphorothioate oligonucleotides led to antisense molecules with strong binding affinity, high selectivity for the RNA target, and stability towards nucleases, but they did not support RNase H activity on mRNA. However, the incorporation of a mixture of these modifications into "chimeric"
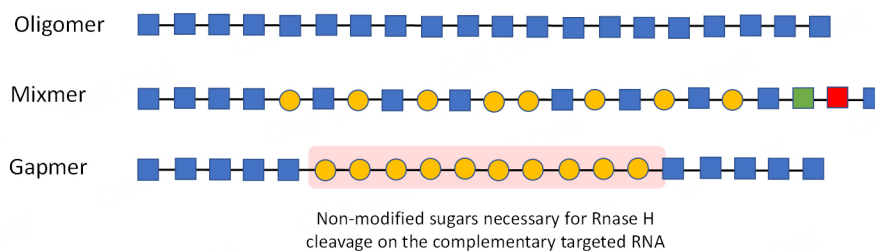
Figure 5. Different types of ASOs developed at the preclinical and clinical stages. Squares, circles, and assorted colors represent the diverse types of nucleotides displayed in Figure 4. The linkage between each nucleotide might also vary, for instance, the phosphorothioate Sp and Rp diastereoisomers. In a gapmer, the yellow circles represent non-modified nucleotides allowing cleavage by RNase H of the annealed complementary mRNA.

oligonucleotides has been shown to activate mammalian RNase H-mediated degradation[57]. Consequently, a sizable proportion of RNase H-dependent ASOs are "gapmers," in which a gap of 10 unmodified deoxyribose is flanked by 3' and 5' "wings" whose partial composition in chemically modified nucleotides leads to metabolic stability, enhanced binding to target, and cellular availability[Figure 5].

A large variety of gapmer geometries can be envisioned. A reduced gap size confers more precision in the cleavage zone and potentially contributes to allele specificity. Different flanking wings have been proposed to increase affinity and selectivity to the target RNA. However, care should be taken that the cleaved mRNA must dissociate to initiate another mRNA degradation. Thus, ASOs' affinity for the mRNA target must not be too high. For instance, with LNA-containing wings, an increased binding affinity has been reported, which leads to an optimal size of 12 to 15 nucleotides[58]. On the opposite, the clinically approved gapmer Inotersen for treating transthyretin amyloidosis is a fully phosphorothioate-modified ASO with five 2'-O-methoxyethyl ribonucleotides on each side, thus consisting of a 5-10-5 structure)[7]. In addition, a too strong affinity might induce off-target binding and RNase H cleavage of mismatched mRNAs (see section *Challenges faced by the synthetic ASO and siRNA technology*). Due to a high ASO concentration observed in the liver, off-target hepatic toxicity has been reported for 2'Fluoro or LNAs[59-61].

Chemical and delivery optimization of siRNAs
Contrary to ASOs which might be efficient as steric blockers with no concomitant nuclease activity, siRNAs efficacy strictly depends on argonaute 2 nucleolytic activity. As for RNase H-dependent ASOs, the modifications introduced for increasing metabolic stability are limited and must be finely optimized for the guide siRNA strand. More freedom for modifications is allowed on the passenger sense strand, which is not involved in the RISC-guided nuclease activity. An additional constraint to be considered is to limit the innate immune response induced by natural RNA duplexes, which has initially represented a major obstacle to the development of the technology.

A useful mapping of the functional domains of a typical siRNA gives guidance on where chemical diversity can be introduced [Figure 6A]. While most siRNA designs are based on a non-covalent two strands double helix [Figure 6A and 6B], corresponding to the presently clinically approved RNA drugs (see *Chemical and delivery optimization of siRNAs*), other concepts have been proposed and shown to possess promising properties at least at the preclinical level, such as di-siRNA composed of two double-helical siRNAs made of one double-sized passenger strand and two guide strands Figure 6C[62]. The geometry developed by the DIcerna company is that of a nicked hairpin [Figure 6D]. While the guide antisense strand is of 22 bases canonical length, the sense passenger strand is longer (36 bases), and it auto-hybridizes through a GC-rich sequence (GCAGCC hybridized to GGCUGC) to form a GAAA loop at its extremity. Hence, a nick of the
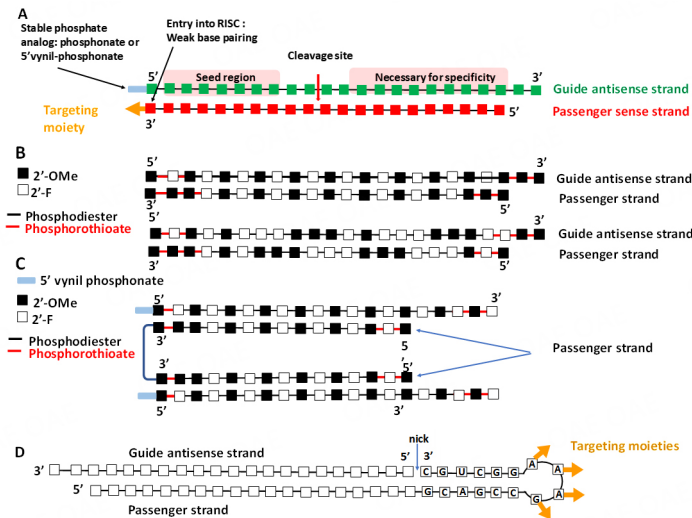
Figure 6. A: The siRNA functional regions. More modifications are tolerated on the passenger strand (red squares) which is not involved in argonaute 2-mediated mRNA cleavage. A targeting moiety can be covalently linked to the 3' strand (orange arrow). A stable phosphonate resistant to dephosphorylation enzymes can be included instead of phosphate (blue rod) at the 5'end of the guide siRNA (green square). Both are necessary and compatible for binding the guide strand to the RISC complex. Fewer modifications are allowed on the seed region of the guide siRNA strand (nucleotides 2 to 8). An optimized guide strand must contain a flexible 5' end (which can be obtained by lowering base pairing and facilitates capture by the RISC complex), a high affinity 'seed' region, which drives the initial base pairing between the guide strand and mRNA target, and a lower affinity 3'-region required for cleaved mRNA release. B: Frequently used siRNAs are made resistant to exonucleases by 2 to 3 terminal phosphorothioate linkages on both 3' and 5' terminals of each strand. Modified sugars 2'-OMe and 2'-fluoro have led to siRNAs highly resistant to nuclease, of improved bioavailability, and of high binding affinity to the RISC complex. This leads to an unprecedented duration of action which can reach up to 6 months or more after a single administration, due to the recycling mechanism displayed in Figures 4 and 5 inspired from [18,29]. C: Scheme of a di-siRNA [62,145]. D: In this presented geometry, the antisense strand is of 22 bases canonical length, while the sense passenger strand is much longer (36 bases), and it auto-hybridizes through a GC-rich sequence (GCAGCC hybridized to GGCUGC) to form a GAAA loop at its extremity.

hairpin structure is located between the sense and antisense strands[63,64]. Targeting moieties such as GalNac sugars have been linked to the GAAA loop, thus providing a high tetravalent sugar moiety for targeting liver hepatocytes.

The presence of a 5' phosphate on the siRNA guide strand is an essential factor for entry into the RISC complex and loading to the Ago2 nuclease. Stable phosphate analogs, such as phosphonate, have been introduced with a strong enhancing effect [Figure 6A][65]. A non-cleavable targeting moiety can be linked to the passenger strand. With a tri-antennary GalNac targeting head that binds to the hepatocyte asialoglycoprotein receptor, a dramatic increase in liver uptake and silencing efficiency has been observed[66-67], leading to several months silencing effect after a single dose. This represents one of the most exciting perspectives of siRNA therapeutics for liver-associated diseases[53] (see *Exon-skipping ASO for Duchenne muscular dystrophy*).

While 2'fluoro ribose modification is well tolerated by the RISC machinery, phosphodiester linkage substitution by phosphorothioate can only be introduced in a limited number, and this is outside the seed region and cleavage site. Similarly, sugar modifications such as 2'OMe or LNA are more tolerated on the passenger strand, and they are favored at the 5'end because they block passenger strand entry into the RISC complex and consequently favor RISC exclusive loading with the guide strand. Two typical popular siRNA geometries are displayed in Figure 6B. In the upper siRNA, 2'fluoro and 2'OMe are intercalated and face each other to obtain a canonical alpha helix geometry. In the second more widespread example, stretches of 3 consecutive 2'fluoro and 2'OMe nucleotides are present and frequently facing each other. Two to three

phosphorothioate linkages are introduced in the 5' and 3' ends of both strands, which ensures sufficient metabolic stability against exonucleases. Another backbone modification has been recently introduced in the form of a divalent siRNA, in which two passenger moieties are covalently linked. These di-siRNAs display a favorable distribution in the central nervous system and promising efficacy in neurodegenerative disease models in rodent and non-human primates[62].

While the maximum number of 2'fluoro must be controlled because of potential toxicity, other modifications have proven their utility, such as 5' carbon pyrimidines. Moreover, using a systematic iterative screening technology, it has been shown that optimizing the positioning of 2'-deoxy-2'-fluoro and 2'-O-methyl ribose across both strands enhanced metabolic stability. This could be obtained with a low 2'-deoxy-2'-fluoro content[68].

Numerous formulations have been proposed to improve siRNA pharmacokinetics, such as lipid nanoparticles (LNP), a detailed reviewing of which is out of the scope of the present review. Lipid nanoparticles have proven their efficacy in targeting the liver *in vivo*, leading to the clinically approved Patisiran siRNA drug in transthyretin amyloidosis[69,70]. While liver targeting is presently well mastered using either LNP or the GalNac technologies (see *Exon-skipping ASO for Duchenne muscular dystrophy*), challenges remain for other organs, particularly the brain. Nonetheless, progress is being made in terms of oral and ocular delivery[71-73] and intravenous (IV) delivery to inflammatory sites[74-76].

The following sections illustrate the above concepts by a selection of typical examples of ASOs and siRNAs approaches for treating muscular and neuromuscular disorders, and for which either marketing approval or very promising results have been obtained. Since any genetic disease caused by a dominant negative variant might benefit from an RNase H-dependent ASO or a siRNA approach, and since many others might benefit from anti-miRNA or splice modulation properties, this review can by no means be fully exhaustive concerning the ongoing preclinical studies.

Exon-skipping ASO for duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is caused by anomalies in the dystrophin gene located on the X chromosome (Xp21.2). Because the dystrophin gene is the largest known gene in the human genome, genetic variants and deletions occur at a higher frequency than in other genes, and DMD has one of the highest prevalence rates among rare diseases (about 6/100,000). Diagnosis is suspected based on the clinical picture, family history, and laboratory findings (serum creatine kinase being 100-200 times the normal level). Genetic testing is a critical tool for accurate DMD diagnosis (Orpha 98,896)[77].

DMD onset occurs in early childhood, and affected boys may show a delay in walking accompanied by speech and/or global developmental retardation. Autism and behavioral problems, such as ADHD (attention deficit hyperactivity disorder), anxiety, and obsessive-compulsive disorder, are common. Untreated DMD children rarely achieve the ability to run or jump. The condition progresses rapidly, and the child develops a waddling gait and a positive Gowers sign. Proximal muscles are affected first, then distal limb muscle. Climbing stairs becomes difficult and the child falls frequently. Loss of independent ambulation occurs between the ages of 6 and 13 years, the average being 9.5 years in non-steroid treated patients. Once ambulation is lost, joint contractures and scoliosis develop rapidly. Until recently, untreated patients might not survive over late teens to early twenties because of respiratory failure and/or cardiomyopathy, but life expectancy is increasing with adapted cardiac care and assisted ventilation[78].

DMD belongs to the larger group of rare genetic progressive muscular dystrophies called dystrophinopathies, which also include Becker muscular dystrophy (BMD) and a symptomatic form in female carriers. Dystrophinopathies present a spectrum of severity ranging from progressive skeletal and cardiac muscle wasting and weakness (DMD, BMD) to less severe muscle weakness or isolated cardiomyopathy affecting carrier females. At the mildest end of the spectrum exercise-induced muscle cramps and myoglobinuria may be the only feature, while at the severe end, there may be a complete loss of muscle function, cardiomyopathy, and respiratory failure. BMD presents a mild phenotype and a broad spectrum of clinical severity, with the onset of symptoms occurring from early childhood to as late as sixty. A very severe, rapidly progressive, X-linked dilated cardiomyopathy (code Orphanet 262) may also be caused by mutations in the dystrophin gene[77].

Muscle damage in DMD is caused by the complete absence of the cytoplasmic sarcolemmal protein dystrophin, which participates in a complex connecting muscle fiber cytoskeleton to the surrounding extracellular matrix through the cell membrane. Dystrophin protects myotubes' integrity during muscle contraction[79]. Dystrophin possesses a central rod domain of twenty-four spectrin-like repeats. Its primary muscular transcript measures about 2,100 kilobases. The mature mRNA, which is formed by the junction of seventy-nine exons, measures 14.0 kilobases and encodes a protein of 3,685 amino acid residues. Hence, intensive splicing is necessary for dystrophin biosynthesis[80].

Dystrophinopathies are allelic conditions caused by deletions, duplications, and mutations in the dystrophin gene. While DMD genetic variants are frameshift, BMD variants are in-frame. While severe DMD results from dystrophin complete absence, the moderate BMD form is only observed when one or several of the spectrin-liked repeats is missing. Deletions identified in DMD patients are shifting the translational open reading frame (ORF), thus resulting in a non-completed abnormal protein product of which the COOH terminal fragment is either non-functional or lost. On the opposite, BMD patients' deletions maintain the in-frame translational ORF for amino acids and predict a shorter protein of lower molecular weight. This indicates that the smaller protein product devoid of a certain number of internal spectrin-like domains remains semi-functional, resulting in the milder Becker clinical phenotype[81,82]. From this, it was predicted that, for some DMD patients, an in-frame skipping of the exon containing a nonsense mutation or deletion could have a therapeutic value. Figure 7 illustrates an exon skipping obtained by the binding of a steric blocker ASO targeting an intronic splice acceptor or donor site or an exonic splice enhancer site.

Given the fact that several thousands of variants have been reported for DMD patients, a search identified which in-frame exon skipping was favorable to treat a high proportion of patients. As shown in Figure 8, skipping exon 51 results in an in-frame skipping and leads to a partially functional protein in several deletion scenarios. Such partially internally shortened proteins would contain the following junctional exons, respectively: 50-53, 49-52, 49-52, 48-52, 47-52, 46-52, and 44-52. The deletions displayed in Figure 8 correspond to about 15 to 17% of the DMD population[83,84].

An elegant *in vivo* exon skipping proof of concept leading to the expression of a partially functional dystrophin molecule devoid of a certain number of spectrin-like domains was obtained by using an AAV viral vector delivering an antisense moiety born by a U7 or U1 small nuclear RNA[85,86]. The muscle force recovery was restored in these experiments. These very promising results prompted the development of synthetic steric blockers ASOs. It has led to the drug Eteplirsen (AVI-4658; Exondys 51), which has been approved by FDA but not by EMA, indicating that its efficacy is a matter of debate[87]. Eteplirsen is a thirty morpholino nucleotide oligomer delivered intravenously. Its sequence is CTCCAACATCAAGGAAGATGGCATTTCTAG. Other commercialized ASOs for DMD are skipping
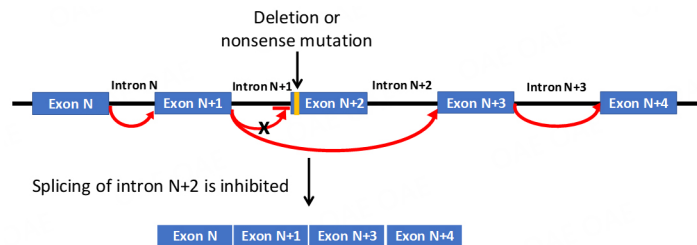
Figure 7. Therapeutic exon skipping. An ASO targeting and blocking an intronic splice acceptor or donor site or an exonic splice enhancer site enforces exon skipping. In the case of the picture, the ASO targets and binds to the acceptor site of intron N+1, resulting in the skipping of ExonN+2. The exons N+1 and N+3 must be in frame to ensure the expression of a protein devoid of only exon N+2, which might still be fully or partially functional.
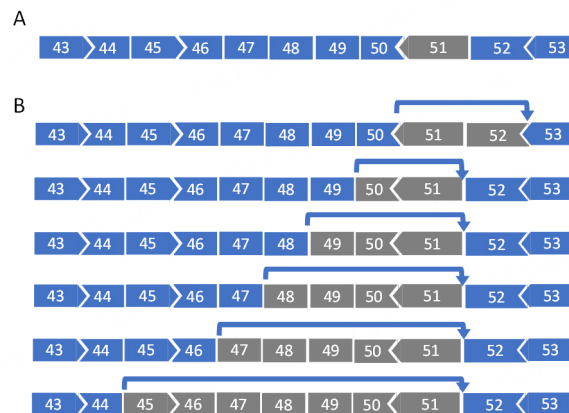
Figure 8. A: Exons 43 to 53 of the integral dystrophin gene. B: In grey, the group of exons whose concomitant deletion could, in theory, lead to a shortened functional or semi-functional dystrophin protein comprising only the protein encoded by the exons in blue, and allowing to skip mutated Exon 51.

exons 53 and 45, and at present times, there are 4 ASOs on the market for DMD, all being morpholinos. Other chemistries are in clinical development: 2'MOE-PS 20 mer ASO and peptide-conjugated ASO[88,89]. The clinical results obtained with Etiplisiren and other exon 51, exon 53, or exon 45 exclusion drugs (Golodirsen and Casimersen) have been reviewed recently[7,8].

Spinal muscular atrophy
Spinal muscular atrophy (SMA) is the second most common fatal, autosomal recessive disease of infants. It is a rare neuromuscular disease affecting 1/6000 children and is characterized by progressive general muscle waste. Spinal muscular atrophy is caused by mutations or deletions in the Survival Motoneuron 1 gene (SMN1)[90,91]. The gene for SMA was mapped to chromosome 5q13 within the telomeric region. The 20 kb gene encodes a 294 amino acids protein.

SMA occurs under different levels of severity. Less severe forms are classified as Type II, Type III, and Type IV, based on age of onset and ultimate motor disability. The most severe form is Type I (Werdnig-Hoffmann disease), which occurs in about 60% of the cases and is associated with quadriplegia, respiratory muscle paralysis, and mortality shortly after birth. Werdnig-Hoffmann patients are never able to sit by themselves and necessitate to be supported for their nutritional and ventilation function[92]. SMA patients who can sit but are unable to walk without help are classified as Type II. Milder type III SMA patients can be able to sit and walk, but they lose the walking capacity in adulthood[93]. Type IV patients have a normal

life expectancy but develop muscle weakness with time. Homozygous deletions or deleterious mutations in the SMN1 gene are present in all SMA patients.

SMN2 gene is a paralog of SMN1 which results from the duplication of the 5q13 region gene and has been identified in a more centromeric position. The SMN2 gene is unique to Homo Sapiens and differs from SMN1 in less than 20 nucleotides, but a functionally crucial difference is the existence of thymine instead of cytosine in exon 7[94]. This modification inactivates a splicing enhancer and, on the opposite, creates a splicing silencer. Consequently, exon7 is skipped by the splicing machinery in about 90% of maturated SMN2 mRNA, leading to an unstable non-functional shorter SMN2 protein [Figure 9]. Still, about 10% of the maturated SMN2 transcripts contain Exon 7 and lead to a functional SMN2 protein with identical neuroprotective properties as those of SMN1.

The SMN2 gene is located in an unstable chromosomal region and is consequently present under a variable copy number in the population, leading to variable production of functional SMN2 protein. The broad spectrum of the severity of the SMA disease has been correlated with the number of SMN2 copies, which is coherent with the assumption that more SMN2 copies might, at least partially, compensate for the absence of SMN1 protein. As schematized in Figure 9, the presence of multiple copies of the SMN2 gene partially alleviates the disease symptoms by allowing more functional SMN2 protein to be produced, with Type I patients generally possessing 2 SMN2 copies, Type II having 3 SMN2 copies, and Type III having 3-4 SMN2 gene copies. Thus, the number of SMN2 copies is clearly inversely correlated to SMA severity, but other genes have also been proposed to be involved in SMA severity[95,96].

The observation that SMN2 was a strong disease modifier established the basis for the quest to increase the production of a complete and functional SMN2 protein by antagonizing the exon 7 skipping reaction. Using a systematic study of minigene mutants with different deletions at the 5' end of intron 7, a novel inhibitory element located immediately downstream of the 5' splice site in intron 7 was identified, which was called intronic splicing silencer N1 (ISS-N1)[97]. A pragmatic approach consisting of screening a large number of overlapping ASOs targeting introns 6 and 7, as well as exon 7, identified several sites on the 3 sequences whose steric blocking would inhibit undesired exon 7 skipping and confirmed that ISS-N1 was the most promising target sequence for enforcing full SMN2 protein expression[98]. Thus, ASOs targeting a sequence at approximately 10 nucleotides downstream of the 5'splice site were further developed, leading to the drug Nusinersen.

Nusinersen (Spinraza™) was made available in 2016 as the first treatment for spinal amyotrophy. Remarkably, Nursinersen's discovery rationale was based on the above-described thorough elucidation of the molecular mechanism of SMN1 and SMN2 mRNA maturation. Nusinersen increases child survival and is administered by repeated intrathecal delivery (i.e., in the spinal cord). Nusinersen is an 18-nucleotide oligomer whose 5'-3' sequence is: UCACUUUCAUAAUGCUGG. It is a highly modified ASO having a full phosphorothioate backbone and 2'O-Me modified ribose to increase metabolic resistance and bioavailability. In addition, all pyrimidines are 5-methylated: uracil replaced by thymine, and cytosine replaced by 5'-methyl cytidyl. The Nusinersen IUPAC formula is detailed in Table 2.

Nusinersen displays high efficacy for promoting exon 7 recovery into SMN2 protein. It was shown that increasing SMN exclusively in peripheral tissues completely rescued muscle necrosis in mild SMA mice models and robustly extended survival in severe SMA mice, with significant improvements in vulnerable tissues and motor function[99]. However, CNS effects upon IV injection could only be observed in neonates but not in adult mice. It is known that the blood-brain barrier is permeant in neonates and is subsequently

Table 2. Nusinersen and inotersen IUPAC formula

Nusinersen condensed IUPAC formula:
Thy-MeOEt(-2)Ribf-sP-m5Cyt-MeOEt(-2)Ribf-sP-Ade-MeOEt(-2)Ribf-sP-m5Cyt-MeOEt(-2)Ribf-sP-Thy-MeOEt(-2)Ribf-sP-Thy-MeOEt(-2)Ribf-sP-Thy-MeOEt(-2)Ribf-sP-m5Cyt-MeOEt(-2)Ribf-sP-Ade-MeOEt(-2)Ribf-sP-Thy-MeOEt(-2)Ribf-sP-Ade-MeOEt(-2)Ribf-sP-Ade-MeOEt(-2)Ribf-sP-Thy-MeOEt(-2)Ribf-sP-Gua-MeOEt(-2)Ribf-sP-m5Cyt-MeOEt(-2)Ribf-sP-Thy-MeOEt(-2)Ribf-sP-Gua-MeOEt(-2)Ribf-sP-Gua-MeOEt(-2)Ribf

Nusinersen detailed IUPAC formula:

O2'-(2-methoxyethyl)-5-methyl-P-thio-uridylyl-(3'- > 5')-O2'-(2-methoxyethyl)-5-methyl-P-thio-cytidylyl-(3'- > 5')-O2'-(2-methoxyethyl)-P-thio-adenylyl-(3'- > 5')-O2'-(2-methoxyethyl)-5-methyl-P-thio-cytidylyl-(3'- > 5')-O2'-(2-methoxyethyl)-5-methyl-P-thio-uridylyl-(3'- > 5')-O2'-(2-methoxyethyl)-5-methyl-P-thio-uridylyl-(3'- > 5')-O2'-(2-methoxyethyl)-5-methyl-P-thio-uridylyl-(3'- > 5')-O2'-(2-methoxyethyl)-5-methyl-P-thio-cytidylyl-(3'- > 5')-O2'-(2-methoxyethyl)-P-thio-adenylyl-(3'- > 5')-O2'-(2-methoxyethyl)-5-methyl-P-thio-uridylyl-(3'- > 5')-O2'-(2-methoxyethyl)-P-thio-adenylyl-(3'- > 5')-O2'-(2-methoxyethyl)-P-thio-adenylyl-(3'- > 5')-O2'-(2-methoxyethyl)-5-methyl-P-thio-uridylyl-(3'- > 5')-O2'-(2-methoxyethyl)-P-thio-guanylyl-(3'- > 5')-O2'-(2-methoxyethyl)-5-methyl-P-thio-cytidylyl-(3'- > 5')-O2'-(2-methoxyethyl)-5-methyl-P-thio-uridylyl-(3'->5')-O2'-(2-methoxyethyl)-P-thio-guanylyl-(3'- > 5')-O2'-(2-methoxyethyl)-guanosine

Inotersen compacted IUPAC formula:

Thy-MeOEt(-2)Ribf-sP-m5Cyt-MeOEt(-2)Ribf-sP-Thy-MeOEt(-2)Ribf-sP-Thy-MeOEt(-2)Ribf-sP-Gua-MeOEt(-2)Ribf-sP-dGuo-sP-dThd-sP-dThd-sP-dAdo-sP-m5Cyt-dRibf-sP-dAdo-sP-dThd-sP-dGuo-sP-dAdo-sP-dAdo-sP-Ade-MeOEt(-2)Ribf-sP-Thy-MeOEt(-2)Ribf-sP-m5Cyt-MeOEt(-2)Ribf-sP-m5Cyt-MeOEt(-2)Ribf-sP-m5Cyt-MeOEt(-2)Ribf

Figure 9. Summary of the genetic and molecular defects causing the several types of SMA severity and of treatment by a blocker ASO. A: In non-affected individuals, both SMN1 and SMN2 genes are functional wild-type. SMN1 pre-mRNA is transcribed into a complete mRNA corresponding to the 9 exons: 1,2a,2B,3,4,5, 6,7and 8. This leads to the production of a completely functional SMN1 protein, which does not occur in SMA patients. B: The SMN2 paralog gene contains several sequences which negatively impact the correct splicing and impair the incorporation of Exon 7 into the maturated SMN2 mRNA. The most important of these inhibitory sequences is the intronic splicing silencer N1 (ISS N1) located in 5' of Intron 7. Therefore, only a 10 to 20 % minority of SMN2 transcripts contain all necessary exonic sequences for a functional SMN2 protein. From 80 to 90% of SMN2 transcripts are translated in a shortened non-functional SMN2 protein devoid of Exon 7, which is unstable. The presence of multiple copies of the SMN2 gene results in a higher quantity of functional SMN2 being produced and partially alleviates the disease severity. Type I patients generally possess 2 SMN2 copies, Type II having 3 SMN2 copies, and Type III and IV having 4 SMN2 gene copies. **C:** In SMA patients, a mutation/deletion induces the loss of SMN1 protein production. An ASO designed as a steric blocker of ISS N1 is highly efficient in increasing the level of fully competent SMN2 protein, and has been clinically validated as the Nusinersen drug.

closing itself, concomitantly with the expression in capillary endothelium of tight junctions and Glut1 glucose transporter. This suggested that the PS- 2'O-Me ASOs Nusinersen did not cross the blood-brain barrier. However, direct Nusinersen administration into the cerebrospinal fluid or the brain parenchyma

allowed to achieve reasonable distribution through the brain and spinal cord, the latter being an absolute requisite for SMA.

Acutely conceived clinical development allowed us to prove the clinical benefit of intrathecal Nusinersen in Type I SMA in only a few years. The results obtained on patients were so spectacular that FDA approval was obtained within about 3 months, which represents one of the fastest delays ever observed. Recent clinical data show that the earlier the treatment is given after birth, the best are the clinical results. This opened the way to systematic SMA newborn screening in various countries' legislation, which was not the case before. More details on the clinical advances and ongoing development of Nusinersen have been published elsewhere[5,7,8].

Multiple other candidate ASOs are being tested as splice modifiers, such as an LNA/DNA mixmer[100]. Remarkably, quite a short time after the revolutionary Nusinersen commercialization, two other approaches led to approved drugs against SMA. Zolgensma is a self-complementary AAV-9 vector able to cross the blood-brain barrier[101] carrying the SMN1 cDNA sequence, which showed positive results by systemic IV administration[102] and has been FDA- and EMA-approved. Ridisplam is an orally administered splicing modifier of SMN2 which increases the level of functional SMN2 protein. The Ridisplam large domain of application covers all types of SMA, and it has been approved in the USA, Japan, and Europe as a treatment that does not necessitate hospital intervention.

RNase H-dependent ASO inotersen for the treatment of hereditary transthyretin amyloidosis
Hereditary transthyretin amyloidosis (hATTR) is a gain-of-function genetic rare disease. It represents a paramount example of clinical success for RNA drugs inducing mRNA degradation because both ASO and siRNA drugs have reached clinical use. This disease represents the first indication of a clinically approved siRNA. Moreover, two siRNA drugs with different delivery principles have been approved for hATTR in a brief period of time, and more are on the way.

The transthyretin protein (TTR) is secreted by the liver, choroid plexus, and retinal pigment epithelium. Its important function is to transport the thyroid hormone thyroxine (T4) and retinol to the liver. TTR is a 55 kDa homotetramer, which is formed by the first association of dimers, followed by dimer-dimer binding. Hereditary transthyretin amyloidosis is a group of several dominant negative diseases related to variants in the TTR gene. More than 140 different TTR variants have been identified. In these rare hereditary diseases, mutant TTR misfolding leads to the formation of amyloid aggregates which accumulate in various tissues and cause various pathologies: hereditary transthyretin amyloidosis (hATTR), familial amyloid polyneuropathy (FAP) and familial amyloid cardiomyopathy (FAC)[103-105]. Cardiomyopathy results from myocardial infiltration of abnormal amyloid protein. Moreover, wild-type transthyretin amyloidosis (wtATTR) has been observed, which occurs mainly at a late age and is caused by the aggregation of normal transthyretin[106]. It affects older age patients carrying the wild-type TTR gene. In addition to polyneuropathy and cardiomyopathy, other transthyretin amyloidosis symptoms involve nephropathy and ocular pathology.

Before the introduction of RNA drugs, hATTR treatment involved liver transplantation and, more recently, the small molecule drugs Diflunisal and Tafamidis, which stabilize TTR in a non-aggregating form. In recent years, however, a very efficient treatment for hATTR with polyneuropathy has been obtained through RNA drugs decreasing liver TTR mRNA. Either RNases H-dependent ASOs such as Inotersen (Tegsedi) or Ago-dependent siRNA Patisiran (Onpattro) and Vutrisiran have successfully reached the market.

The ASO Inotersen (Tegsedi) is a 20-mer gapmer similar to that represented in Figure 5. It leads to the degradation of TTR mRNA, with minimal off-target effect. It is fully PS-modified (phosphorothioate backbone), with 5 ribose on both 5' and 3'ends carrying 2'MOE (2'O-methoxyethyl) moieties [Figure 4C]. The 10 central nucleotides have a natural ribose. The nucleotidic sequence of Inotersen is UCUUGGTTACATGAAAUCCC, with the nucleotides UCUUG and AUCCC carrying a 2'OMOE. As for Nusinersen, all cytosines and uracil of Inotersen are 5'-methylated (uracil replaced by thymine and cytosine by 5-methyl cytosine). The Inotersen IUPAC formula is detailed in Table 2.

After subcutaneous (SC) injection, Inotersen leads to a robust decreasing effect on the level of both variant and wild-type transthyretin. It clearly inhibits TTR production and slows down the progression of the disease[107] but does not seem to induce any reverse effect on already-formed amyloid aggregates[7]. Reported Inotersen adverse events include thrombocytopenia. This could be linked to the presence of phosphorothioate linkages, which have been shown to be a potent platelet activator[108,109], glomerulonephritis, and hepatic toxicity[110].

siRNA patisiran for the treatment of transthyretin hereditary amyloidosis

Patisiran is a siRNA. The antisense guide strand formula is A-U-G-G-A-A-Um-A-C-U-C-U-U-G-G-U-Um-A-C-dT-dT). It is complexed with the complementary passenger sense strand (G-Um-A-A-Cm-Cm-A-A-G-A-G-Um-A-Um-Um-Cm-Cm-A-Um-dT-dT (A, adenosine; C, cytidine; G, guanosine; U, uridine; Cm, 2'-O-methylcytidine; Um, 2'-O-methyluridine; dT, thymidine). Thus, Patisiran bears eleven 2'O-methylated pyrimidines, which increases its lipophilicity. All inter-ribose linkages are phosphodiesters[111].

To protect this phosphodiester siRNA from rapid degradation and kidney filtration, the siRNA active component of Patisiran is formulated into a lipid nanoparticle (LNP). In addition to this protecting effect, LNP also facilitates Patisiran delivery to the liver. It has long been known that some colloidal delivery systems, such as nanoparticles or liposomes, accumulate rapidly into the liver and spleen upon IV administration, at a ratio superior to 90%[74,112-115]. Nanoparticles or liposomes containing cationic and/or ionizable lipids thus represent a very favorable system for high-yield encapsulation of negatively charged siRNA and for preferential liver uptake, as previously demonstrated for DNA.

The LNP formulation which has been selected for Patisiran includes buffer components (disodium hydrogen phosphate, heptahydrate potassium dihydrogen phosphate, anhydrous sodium chloride), as well as the lipid DLin-MC3-DMA [(6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31¬ tetraen-19-yl-4-(dimethylamino) butanoate], an amine-containing ionizable lipid with a pKa of 6,4. In addition, the formulation comprises a neutral lipid distearoylphosphatidylcholine, cholesterol, and the PEGylated lipid DMG-PEG 2,000 [Figure 10A][111]. Each 1 mL Patisiran also contains 6.2 mg cholesterol USP, 13.0 mg DLin-MC3-DMA, 3.3 mg DSPC, and 1.6 mg α-(3'-{[1,2-di(myristyloxy)propanoxy] carbonylamino}propyl)-ω-methoxy, polyoxyethylene (PEG2000 C-DMG0). In such a typical LNP formulation, cholesterol is added to provide rigidity to the 40-100 nm nanoparticles, and the neutral lipid distearoylphosphatidylcholine attenuates the charge repulsion between the DLin-MC3-DMA cationic heads. In addition, the PEGylated lipid PEG2000 C-DMG0 ensures colloidal stability through the highly hydrated polyethyleneglycol chains forming a protecting shield around the nanoparticle.

The mechanism by which the LNPs are targeted to the liver hepatocytes is schematized in Figures 10B and 10C. It is caused by the natural binding of the plasma protein ApoE to the PEGylated LNP. Since liver hepatocytes express a high concentration of the ApoE receptor, LNPs decorated with ApoE bind to the hepatocyte surface [Figure 10B]. The resulting ApoE clustering induces internalization by a clathrin-
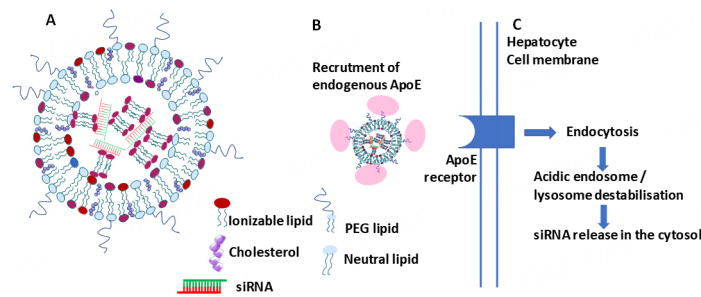
Figure 10. A: RNAi encapsulated in LNPs are protected from the actions of nucleases and targeted to the liver. LNPs are specifically targeted to the liver because they bind apolipoproteins E in the blood circulation and then bind to the ApoE-LDL receptor on the surface of the hepatocytes. B: LNP decorated with Apo E bind to Apo E receptor on the hepatocyte plasma membrane. C: DLin-MC3-DMA lipids at the LNP surface are neutral at pH 7. Inside the endosome, DLin-MC3-DMA becomes cationic, which favors either fusion with the endosome membrane and siRNA release or endosome breakage caused by the "proton pump" osmotic effect.

dependent endocytosis mechanism. Inside the endosome, it is postulated that the ionizable DLin-MC3-DMA lipid becomes cationic because of endosomal/lysosomal acidification. This induces endosome breakage, for which two different mechanisms have been proposed.

1) In the first one, the ionizable lipid captures and titrates the H+ ions taken up into the endosomes by the endolysosomal proton pump ATPase. This induces the sustained uptake of a large number of protons and chloride anions which diffuse into the endosomes to equilibrate the proton cationic charges. This "proton sponge" effect induces endosome swelling and breakage.

2) According to the alternative proposed mechanism, the ionizable DLin-MC3-DMA lipid at the LNP surface becomes cationic in the acidic endosomal compartment, leading to the fusion of the positively charged LNP surface with the negatively charged endosome membrane.

Both postulated mechanisms result in siRNA release into the cytosol and further interaction with the RISC complex.

Patisiran was granted orphan drug status, fast track designation, priority review, and breakthrough therapy designation due to its novel mechanism and the rarity of the condition it is treating. It was approved for medical use in USA ad EU in August 2018. However, with the development of other transthyretin amyloidosis treatments, such as Inotersen and Vutrisiran which is accumulated into the liver through a triGalNac N-acetylglycosamine targeting moiety, there is now intense competition between these different RNA drugs.

Tri-GalNac siRNA Vutrisiran for transthyretin hereditary amyloidosis treatment
Vutrisiran (AMVUTTRA™) is a subcutaneously administered TTR-specific siRNA developed for hATTR and wtATTR treatment. Vutrisiran was FDA approved in 2022 for the treatment of hATTR amyloidosis with polyneuropathy in adults, following fast-track designation in 2020 given the very promising efficacy of this RNA drug.

The Vutrisiran sequence was designed to bind to a conserved sequence on all TTR mRNA variants. Because Vutrisiran is administered as a naked siRNA, i.e., not associated with a lipid nanoparticle delivery system, the drug must be metabolically protected and targeted to hepatocytes. Nuclease resistance has been achieved by introducing much more modifications in Vutrisiran than in Patisiran. Indeed, while Patisiran contains

only eleven 2'O-Me nucleotides and only phosphodiester linkages, Vutrisiran is a heavily modified siRNA with 6 phosphorothioate linkages at the end of each strand, 35 nucleotides carrying a 2'OMe ribose and 9 nucleotides modified with a 2'F ribose. Thus, the two Vutrisiran strands (one of 21 nt and one of 23 nt) are fully modified[116,117]. Optimization of the number and respective positions of these modifications has been performed in view of reducing toxicity, particularly hepatotoxicity[19,46,54,116,117].

The most innovative feature of Vutrisiran is the functionalization of the sense passenger strand with a very efficient hepatocyte targeting moiety, the tri-N-acetyl-galactosamine (tri GalNac). The triGalNac head binds with high avidity to the ASGPR, a lectin that is present at a remarkably high density on the hepatocyte surface (500,000 ASGPR per cell). Binding to the ASGPR actively promotes the cellular uptake of the ligand upon clustering and aggregation of several receptors in coated pits. The ASGPR turnover is very fast since only about 5%-10% of ASGPR are permanently accessible at the cell plasma membrane. After endocytosis, ASGPR recycling occurs in ~15 min concomitantly with the release of the bound ligand in the acidified endosomal compartment, in a process analog to that of the transferrin receptor cycle. Hence, high ASGPR density on hepatocyte surface and fast turnover points Tri GalNac as an ideal targeting head candidate for any liver-targeted RNA drug[118]. The fact that more than 80% of an IV injected compound expressing galactose or galactosamine is taken up by the liver has been evidenced by SPECT or luminescent imaging techniques using lactosylated albumin[119,120].

As shown in Figure 11, the tri-GalNac is covalently linked to the 3' end of the siRNA sense strand, so as not to interfere with the binding of the complementary antisense to RISC. The improved metabolic stability of Vutrisiran and the very potent targeting efficacy of its tri-Gal-Nac lead to an exceptional intrinsic efficacy of Vutrisiran. This is demonstrated by the therapeutic efficacy of very low doses and the exceptionally long duration of action because a quarterly regimen is sufficient to achieve a similar therapeutic effect as Patisiran[121-125]. Both polyneuropathy, cardiomyopathy, and wt-TTR are envisioned as Vutrisiran indications.

As compared to the LNP formulation used for Patisiran, GalNac-siRNA conjugate allows simpler GMP preparation and storage conditions, and a more convenient administration protocol (SC versus IV). In addition, slow diffusion to capillaries through the extracellular conjunctive tissue creates a "depot" effect which, together with increased metabolic stability, allows less frequent administration. Remarkably, the last generation of GalNac siRNAs carrying a phosphate triglycan-end group allows a regimen as distant as twice a year for multiple indications requiring silencing of a liver-expressed protein. A one-year duration after a single dose has been reported for Cemdisiran, which has strong potential in the treatment of complement-mediated diseases such as paroxysmal nocturnal hemoglobinuria (PNH). This is a historically never achieved performance. Thus, the GalNac targeting technology represents actually the most promising approach for siRNAs, as illustrated by the fast development of Givosiran (FDA approved in 2019 for adults with acute hepatic porphyria), Lumasiran (FDA approved in 2020 for hyperoxaluria type 1), and Nedosiran (in phase III for primary hyperoxaluria[116,123]. Similarly, the GalNac targeting technology is also being pursued for ASO liver targeting[126,127].

Type 1 myotonic dystrophy: different ASO modes of action
Myotonic dystrophy (also called dystrophic myotonia) is a multisystemic disease with a frequency of 1/8000 worldwide. The most severe form (DM1) includes symptoms such as myotonia, muscle weakness, cardiac arrhythmias, cognitive dysfunction, and cataract. The genetic cause of DM1 comes from the repeated expansion of a CTG triplet motif in the DM Protein Kinase (DMPK) gene. Normal DMPK gene contains 5 to 37 repeats, while variant DM1 DMPK gene might contain up to thousands of CTG repeats, and the
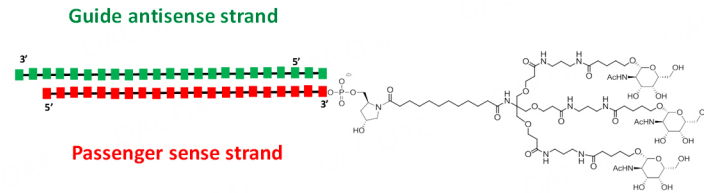
Figure 11. Tri-GalNac chemical group addressing RNAi to the hepatocyte asialoglycoprotein receptor ASGPR. The tri-GalNac is covalently linked to the 3' end of the siRNA sense strand, so as not to interfere with capture by RISC of the complementary antisense strand necessary for mRNA cleavage.

number of repeats correlates with disease severity[128].

Variant DMPK mRNA form hairpin structures made from CUG repeats and assembling in well-characterized nuclear foci. The molecular mechanism of DM1 pathology results from the toxicity of these nuclear foci, which bind and sequester proteins of the family muscleblind-like (MBNL), thus interfering with their natural splicing functions[129,130]. The mis-splicing reactions due to insufficient availability of the MBNL protein in the nucleus induce a fetal-like pattern in adult DM1 cells, which concerns a multiplicity of proteins, such as muscle chlorine channel, insulin receptor, cardiac troponin T, bypass integrator 1, skeletal rapid troponin T, dystrophin (DMD) and cardiac sodium channel SCN5A. This spliceopathy leads to myotonia and other clinical symptoms[131].

ASO and siRNA strategies have been intensively studied on DM1 cellular models such as patient fibroblasts and mouse or drosophila models containing up to 1000 CTG triplets in DMPK gene, and in clinical trials with a gapmer ASO containing constrained ethyl (cEt) locked nucleic acid (LNA)[132,133]. A recent clinical trial was initiated in 2022 with DYNE-101, a muscle membrane antigen-binding fragment antibody (Fab) conjugated to an ASO to enable targeted muscle tissue delivery[134,135].

The multiple strategies displayed in Figure 12 illustrate that DM1 represents a model disease case where multiple RNA drug interventions can be envisioned, and this either separately or in combination. Since MBNL proteins are sequestered by poly CTG DMPK nuclear foci, the use of ASO or siRNA carrying a poly ACG triplet might lead to DMPK nuclear foci cleavage and degradation. Alternatively, an ASO can also function as a steric blocker of MBNL binding, thus releasing the necessary amount of MBNL for restoring adult phenotype splicing pattern [Figure 12A and B][130,131,136]. A second strategy is to target the miRNA (miR)-23b, which has been shown to negatively control MBNL expression. Thus, the administration of a blocker ASO complementary to (miR)23b would up-regulate MBNL expression and potentially compensate for the sequestration of MBNL by variant DMPK mRNA [Figure 12C and D][137]. Finally, since MBNL sequestering and lack of availability induces mis-splicing towards the fetal phenotype of various proteins, splice correctors ASOs, similar to the one described in Figures 2 and 9 for SMA, represent an appealing strategy to restore the expression of the adult form of the critical mis-spliced proteins [Figure 12E and F]. Such a downstream approach has been successfully reported using a morpholino ASO to restore the muscle chloride channel. This was achieved in electroporated muscle fibers[138] or using ultrasound-enhanced delivery of morpholino with bubble liposomes[139].

Allele-specific silencing for centronuclear myopathy

In gain-of-function dominant-negative disorders, an ideal strategy would be to specifically target for ASO or siRNA degradation the variant sequence on the pre-RNA and mRNA. However, this would require a personalized ASO or siRNA drug for each variant. This might not be feasible for several pharmacological
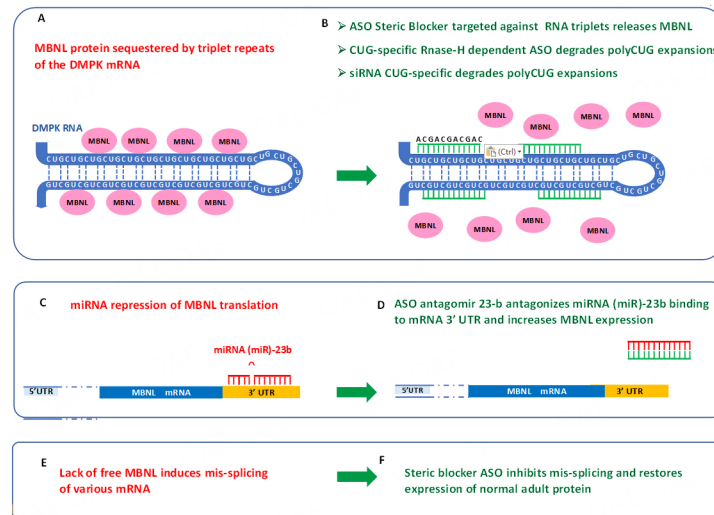
Figure 12. Different ASO- and siRNA-based strategies against Myotonic Dystrophy Type 1. A: MBNL proteins are sequestered by nuclear foci formed by the mRNA from the poly CTG DMPK variant. B: Poly ACG ASO or siRNA lead to DMPK mRNA cleavage and nuclear foci degradation. Alternatively, they can also function as a steric blocker of MBNL binding to the foci, thus releasing the necessary amount of MBNL to restore the adult phenotype splicing pattern. C, D: Since the miRNA (miR)-23b has been shown to repress MBNL expression, the administration of antagomir-23b antagonizes the miRNA (miR)23b which increases MBNL expression, thus compensating for the sequestration of MBNL by variant DMPK mRNA. E: The lack of free MBNL induces mis-splicing toward the fetal phenotype of various proteins. F: Splice correctors ASOs can restore the adult form of the mis-spliced proteins.

reasons including toxicity linked to the off-target effect of some of such specific ASO or siRNA, and cost considerations. In addition, this strategy is not usable for genetic diseases linked to gene duplication or to triplet repeat expansion such as Huntington's disease (CAG repeat expansion) or spinocerebellar ataxia subtypes (CAG or CTG repeats), in which the available variant sequences that could be targeted are present on both the wild-type and the mutated gene.

An ASO or siRNA targeting a non-variant sequence on the pathological gene is the most natural way to provide an RNA drug that could ideally treat all patients, independently of their specific variant genotype. However, this leads to complete repression of both the wild-type and dominant-negative variant alleles.

The above strategies cannot be applied if at least a minimal expression of the wild-type protein is required, and then allele-specific silencing must be sought[140]. Allele-specific silencing can be achieved by targeting one or several single nucleotide polymorphisms (SNP) associated with a variant allele in the patient population. This approach is only feasible if common specific SNPs can be identified in a high percentage of the various mutated alleles in the diseased population. This strategy has been followed in Huntington's disease (HD) and spinocerebellar ataxia[141,142].

Epidemiological studies on the HD patient population and targetable SNPs suggest that 80%-85% of HD patients could be treated with panels of 2 to 5 SNP heterozygosities, meaning that only the expanded variant allele possesses these 2 to 5 SNPs in these individuals[141,143,144]. Exciting recent preclinical results obtained with a brain-targeted di-siRNA have been reported with fully chemically modified, therapeutically translatable siRNAs targeting SNP heterozygosities specific to Huntingtin variants. It allowed a 50-fold discriminative power on the huntingtin variant genetic allele in a cell-based assay on human neurons derived from human Huntington chorea patient iPSCs. This optimized si-RNA was obtained by repeated targeted screening and chemical optimization. Selective silencing of the mutant huntingtin HTT allele

(> 85%) was detected throughout the brain in an HD mouse model with this technology[62,145].

Autosomal dominant centronuclear myopathy (AD-CNM, ORPHA 169 189) is a rare congenital myopathy characterized by numerous centrally placed nuclei on muscle biopsy. Clinical features are those of congenital myopathy: hypotonia, distal/proximal muscle weakness, rib cage deformities sometimes associated with respiratory insufficiency, ptosis, ophthalmoparesis, and weakness of the muscles of facial expression with dysmorphic facial features.

AD-CNM results from heterozygous mutations in the DNM2 gene, which encodes dynamin 2, and to date, 37 mutations (mainly missense) have been identified[146,147]. Dominant DNM2 mutations also cause rare cases of Charcot-Marie-Tooth peripheral neuropathy (CMT)[148] and hereditary spastic paraplegia[149]. The DNM2 protein belongs to the superfamily of large guanosine triphosphatases (GTPases) and is involved in endocytosis and intracellular vesicle trafficking through its role in the deformation of biological membranes, particularly in muscle cell T-tubule biogenesis[146,150]. The role of DNM2 as a regulator of actin and microtubule cytoskeletons has also been reported. A potential AD-CNM pathophysiological mechanism is the formation of abnormally stable polymers by mutant Dynamin 2[151].

A single copy of the wild-type allele in heterozygous knockout mice expressing 50% Dnm2 displays a healthy wild-type phenotype[152]. Moreover, homozygous DNM2 mutation reported in 3 consanguineous patients leads to a lethal congenital syndrome associating akinesia, joint contractures, hypotonia, and skeletal abnormalities, together with brain and retinal hemorrhages[153]. These and other data point to the necessity of an allele-specific siRNA drug targeting only the dynamin 2 variant, in order to maintain 50% of dynamin 2 production.

The proof of concept of silencing the variant mRNA without affecting the wild-type transcript has been obtained in a mouse model with an shRNA gene delivered by an AAV virus[146]. Complete rescue of the muscle phenotype was maintained for at least 1 year after a single injection of the shRNA AAV, leading to a maintained reduction of the variantDnm2 transcript. This study also suggested a new potential pathophysiological mechanism linked to mutant protein accumulation with age, which does not occur in wild-type animals, and which can be prevented by the shRNA treatment.

Further progress was obtained by screening a library of siRNAs targeted to specific heterozygous SNPs associated with the DNM2 dominant-negative variant gene in the AD-CNM patient population. About 75% of patients could be covered by four different siRNAs targeting the four SNPs most frequently associated in a heterozygote manner with the AD-CNM patient population.

One allele-specific siRNA strategy is now in a clinical trial for the treatment of pachyonychia congenital, a rare skin disorder linked to a dominant negative mutation in keratin 6a[154,155]. More considerations on the allele-specific siRNA technology have been developed recently[156].

Combined RNA drug silencing and replacement gene therapy for dominant-negative oculopharyngeal muscular dystrophy

For the gain-of-function disorders related to triplet expansion which have been discussed in the preceding section, an alternative approach has been proposed which does not require the presence of specific SNP on the heterozygote variant allele. The strategy is based on the simultaneous knockdown of both variant and wild-type endogenous mRNAs, together with the administration of a gene encoding the wild-type protein. The replacement gene sequence must be codon-optimized using the genetic code degeneration to carry

differences with the endogenous human gene, thus allowing it to be discriminated and untargeted by the therapeutic silencing ASO, siRNA, or shRNA. Additionally, codon optimization might also contribute to ensuring a high expression level of the wild-type protein.

Oculopharyngeal muscular dystrophy (OPMD) is a rare muscle disease characterized by an onset of weakness in the pharyngeal and eyelid muscles. Inherited in an autosomal dominant mode, the disease is found on all continents, with several clusters identified in Quebec and Israel. The clinical diagnosis is confirmed by a genetic test which in most cases shows an 11-18 expansion of GCA or GCG triplets in the gene of Poly(A) Binding Protein Nuclear 1 (PABPN1) on chromosome 14. The disease is caused by the extension of the polyalanine tract in the PABPN1 protein leading to the gain-of-function formation of intranuclear inclusions or aggregates in the muscle of OPMD patients, which are the hallmark of the disease[157]. Although PABPN1 is ubiquitously expressed and l contributes to control gene expression in all tissues, playing key roles in post-transcriptional processing of RNA, the PABPN1 variant pathological phenotype is restricted to a limited set of skeletal muscles affected in OPMD. The exact pathophysiological process leading to the localized pathology and the precise role of intranuclear aggregates are still unclear[158].

In a gene therapy approach, two AAV vectors were co-delivered, the first expressing a cassette including a triple shRNA under the control of RNA polymerase III promoter and the second expressing human codon-optimized PABPN1 under the control of a skeletal and cardiac muscle-specific promoter. This treatment significantly reduced the number of myonuclei containing PABPN1-positive insoluble intranuclear inclusions, showed significant improvements in several histopathological features (muscle regeneration, fibrosis, and muscle force), and almost completely normalized the transcriptome muscles of A17 mice to that of wild-type mice[159].

Subsequent work managed to condense the two AAV vectors into one and reported reversion of already established insoluble aggregates and partial muscle rescue from atrophy, which are both crucially important since, in most cases, OPMD patients already have an established disease when diagnosed. Also reported were the prevention of the formation of muscle fibrosis and stabilization of muscle strength to healthy muscle levels[160]. Recent progress towards a clinical trial described a unique single bifunctional construct under the control of a muscle-specific promoter for the co-expression of both the codon-optimized PABPN1 protein and two siRNAs against PABPN1 modeled into microRNA (miRNA) backbones. A single intramuscular injection of the AAV9 vector in a murine model resulted in the inhibition of mutant PABPN1 and in PABPN1 replacement, leading to restoration at a normal level of muscle strength and other muscle parameters[161].

Challenges faced by the synthetic ASO and siRNA technology

RNA drugs face several challenges and bottlenecks. The first one concerns pharmacodynamic/ pharmacokinetic (PK/PD) properties. Except for the liver, delivery of RNA drugs to other organs and tissues is still not ideally performed. Lack of proper delivery of sufficient amount into muscles of the Eteplirsen morpholino ASO might be responsible for its suboptimal performance[7,87], and CNS delivery of RNA drugs still necessitates intrathecal, intraventricular or intracerebral administration, which is clinically demanding and associated with infection risk. For muscular and neuromuscular indications, a novel muscle-targeting platform obtained by conjugation of siRNAs with anti-CD71 Fab' fragment has been reported[134,162]. This conjugate led to one-month durable gene-silencing in the heart and skeletal muscle after IV administration in normal mice, and to significant gene-silencing when injected intramuscularly. In a mouse model of peripheral artery disease, the intramuscular administration of an anti-myostatin siRNA resulted in significant silencing of myostatin in muscle and led to the recovery of the running performance.

This technology is now in a clinical trial for a Type 1 myotonic dystrophy RNA drug[135].

Another PK/PD challenge paradoxically comes from the tremendous efficacy of the last generation of RNA drugs. The remarkable duration of the siRNA silencing effect after a single dose results from the siRNA metabolic stability and increased affinity to RISC, which have been brought by the optimized chemistries described in Figures 4 and 5. In addition, grafting functional groups such as 5'vynil phosphonate or methyl phosphonate facilitates guide strand interaction with the RISC complex [Figure 6]. In this way, the guide strand can remain stably bound to RISC and undergo sustained long-term recycling [Figure 3, left side]. The fact that a single administration can lead to 6 to 12 months of activity raises the necessity to find ways to terminate the treatment on demand, and to do this fast enough in case of severe adverse events. The stopping drug effect can be obtained by promoting guide strand dissociation from RISC. Such a reversal system has been described in the liver. It uses short synthetic high-affinity oligonucleotides complementary to the siRNA guide strand that can compete with RISC binding to the guide strand[163]. The authors reported that 9-mers with five locked nucleic acids (LNAs) have the highest potency across several targets to displace the guide strand from RISC and to stop the interference reaction. This 9-mer is targeted to the liver through a tri-GalNac moiety.

Concern must also be considered about the risk of potential toxicity effects of ASOs / siRNA. The first type of toxicity effects might result from direct interactions between the ASOs / siRNA and cellular proteins via a hybridization-independent mechanism. This potential toxicity has to be studied in a similar way than for any classical drug.

The hybridization of ASOs, siRNA/RISC complex, or of free single-strand siRNA to non-intended RNAs sharing some sequence homology with the targeted mRNA is called "off-target effect". For instance, ASO hepatotoxicity in mice has been described to be partly mediated via the RNase H-dependent degradation of off-target RNAs[164,165]. Some strategies are being developed in order to alleviate the ASO off-target effects. Optimization of the ASO nucleotide length by extending its size from a 14-mer to an 18-mer has been shown to reduce the number of off-target candidates, presumably by decreasing the number of matching with non-intended mRNAs[166].

siRNA might suppress the expression of unintended mRNAs with partially complementary sequences by a similar mechanism to that of miRNA-mediated RNA silencing. This siRNA-mediated off-target effect occurs mainly from similarities in the siRNA seed region (nucleotides 2-8). An in-depth analysis of which base pairing was responsible for the off-target effect involving a machine learning technique and using a random sampling procedure led to the conclusion that nucleotides 2-5 were mostly responsible for siRNA off-target effects on RNAi[167]. Enhanced stabilization chemistry has been recently proposed to substantially reduce siRNA seed-mediated binding to off-target transcripts while maintaining on-target activity[68].

Another cause of off-target silencing is improper strand selection by RISC. Passenger-strand silencing can be avoided by selecting siRNA sequences with high thermodynamic asymmetry or by chemically modifying the sense strand, as described above[168,169].

Abundant siRNAs or shRNA can overload the endogenous RNAi pathway leading to toxicity, which has been mostly reported in hepatocytes. Precise dosing must be carefully optimized to avoid this saturation of the RISC system and limit hepatotoxicity of short- and long-term clinical gene silencing by both approaches[169-171].

Other challenges for the future of synthetic ASO and siRNA drugs originate from the rapid advance of alternative techniques such as viral gene therapy for delivering a shRNA, which leads to the same effect as ASO and siRNA in terms of steric blocker for exon skipping or translation inhibition, and for inducing mRNA cleavage/degradation. Indeed, at the present time, most proofs of concept for RNA drugs have been obtained at the laboratory scale by specifically designed AAV or lentivirus vectors. However, this gene therapy approach is less flexible than synthetic ASOs and siRNA because no conditional expression system has been clinically approved at the present time, which renders hazardous the control of gene therapy treatment termination. Moreover, synthetic compounds might be preferable in terms of production costs, and finally, synthetic RNA drugs can be administered repeatedly without initiating any immune response, whereas distinct viral serotypes must be used for multiple-dose administration.

Genome editing is a rapidly evolving technology. Targeted DNA double-strand breaks (DSBs) using CRISPR-Cas9 have revolutionized genetic intervention by enabling efficient and accurate genome editing in a broad range of eukaryotic systems. Multiple applications are actively investigated, such as targeted knockout of dominant negative pathological genes or viral genomes (either integrated or episomal).

In 2021, a clinical trial involving the in vivo use of CRISPR-Cas9 in humans was disclosed[4]. The in vivo gene-editing therapeutic agent NTLA-2001 consists of lipid nanoparticles LNP containing a single guide RNA (sgRNA) that targets the human TTR gene. A human-codon-optimized mRNA sequence of Streptococcus pyogenes Cas9 protein was added to the LNP formulation. Preclinical results of IV NTLA-2001 were impressive, with a durable 95% drop in TTR concentration in monkeys. The genome-editing drug was then administered to patients with hereditary ATTR amyloidosis with polyneuropathy. The treatment was associated with only mild adverse events and led to a decrease in serum TTR protein concentration resulting from the targeted knockout of the TTR gene.

Multiple other genome editing preclinical studies are promising, for instance, in myotonic dystrophy targeting the DMPK gene [see Figure 12][171]. Since LNPs can efficiently deliver CRISPR-Cas9 to the liver, many ASO and siRNA indications might eventually also be treated by genome-editing technology. However, more time is necessary to assess the safety of this approach in the long term, especially concerning off-target effects, genotoxicity, and germ-line modification, since CRISPR-Cas9 makes cuts in the genomic DNA in contrary to ASOs and siRNA, which target mRNA. Also, genome editing represents an irreversible therapy through a one-shot knockout of the targeted gene. Thus, the pros and cons of this technology must be weighted for each specific indication in comparison to reversible synthetic RNA drugs.

CONCLUSION AND PERSPECTIVES

The examples detailed here illustrate the wide and versatile capacities of antisense therapies in muscular and neuromuscular disorders. A large additional number of these diseases could potentially benefit from RNA drugs. Without being exhaustive, one can mention several other diseases where specific silencing has shown benefit in preclinical models. In facioscapulohumeral muscular dystrophy (FSHD), the knockdown of the FSHD region gene 1 (FRG1) was achieved using miRNAs delivered using an AAV vector system[172]. The AAV2/9-mediated delivery of an shRNA targeting the Pmp22 mRNA and injected in the sciatic nerve prevented the development of pathological features in a rat model of Charcot-Marie-Tooth disease 1 A[173]. An allele-specific RNA interference using an AAV9 has been described in a Charcot-Marie-Tooth disease type 2D mouse model. RNAi sequences targeting the dominant mutant of glycyl-tRNA synthetase (GARS) mRNA, but not wild-type, were optimized and then packaged into AAV9 for in vivo delivery. This prevented neuropathy in mice treated at birth. However, delaying the treatment until after disease onset drastically reduced the benefit of gene therapy, and the therapeutic effect decreased with the delay in

treatment, which points to the value of early diagnosis[174]. In most cases, the proof of concept was obtained using shRNA delivered by an AAV vector because this is easily obtained at the laboratory level and because several AAV serotypes display suitable organ penetration and accessibility[100,175]. An AAV shRNA proof of concept then opens the way to the search for synthetic genetic RNA drugs.

Genetic pharmacology with synthetic RNA drugs, which is sometimes presented as belonging to the broader gene therapy field, has made remarkable progress after 20 years of relentless and intensive efforts to improve RNA drug performances. Two to three logs enhancement of their potency has led to recent extraordinary successes, such as being able to silence a pathological gene by a twice, or even once-a-year subcutaneous administration of a small amount of compound, which represents a historical performance never reached before by a chemical compound.

The chemical RNA drugs technology platform, whose chemistry is still continuously improving, is very versatile and can be applied to a large variety of therapeutic applications once the first proof of concept has been obtained, including for targeting previously non-druggable proteins. Of particular value for rare diseases is the possibility to tackle dominant negative gain-of-function genetic diseases, among them those linked to triplet expansion. Trinucleotide repeat diseases lead to either toxic RNA or toxic protein product, and they represent a widening class of rare diseases that is not restricted to the ones illustrated or mentioned in the present review: Type 1 myotonic dystrophy, centronuclear myopathy, Huntington chorea, and spinocerebellar ataxia[141-145], but also cover other expanding disease families[176,177].

The mRNA vaccine technology can be rapidly adapted to any new pathogen by administering pathogen-specific mRNA sequences through a unique delivery technology platform and could thus be rapidly applied to the COVID-19 pandemic spread. Similarly, RNA drug improvements are of general and extendable value, such as modifications in the phosphodiester linkage, sugars, and nucleic bases. However, it must be stressed that the number and respective position of the linkage, ribose, and base modifications need a tailor-made optimization that might be optimized for specific antisense sequences[68,145].

Tri-GalNac targeting has now demonstrated its extraordinary efficacy in delivering RNA drugs to the liver, and the majority of actual clinical trials concerning RNA drugs make a profit from this technology, not only for rare metabolic or neuromuscular disorders such as TTR, or blood disorders such as hemophilia but also for diseases concerning a much larger share of the population, such as hypercholesterolemia by targeting the PCSK9 gene[178], hypertension, diabetes or chronic hepatitis.

In parallel to this extension of RNA drugs application to highly prevalent diseases, the technology shows the potential to treat an increasing number of severely debilitating or life-threatening ultrarare diseases, which can affect worldly only 1 or a very limited number of patients. They are now referred to as "N-of-1" treatments. How this ultrarare diseases population could benefit from antisense RNA drugs is now raising increasing interest from scientists, GMP producers, and regulatory authorities[179]. Since there might be little or no commercial value for these unmet medical needs, patient advocacy groups, charities, and foundations are at the frontline of this challenge[180].

In 2018, the ASO Milasen was developed to treat a single 6-year-old patient with neuronal ceroid lipofuscinosis 7, a neurodegenerative lysosomal storage disorder originating from excessive accumulation of pigment lipofuscin in the body's tissues. The clinical trial result was published in 2019[181]. Remarkably, the molecular diagnosis of this fatal condition led to the rational design, testing, and manufacture of the splice-modulating antisense ASO tailored to this unique patient. Proof-of-concept experiments in cell lines from

the patient served as the basis for launching the N-of-1 clinical trial of Milasen within 1 year after first contact with the patient.

More N-of-1 ASOs drugs have been developed through accelerated regulatory pathways for specific forms of ataxia-telangiectasia and amyotrophic lateral sclerosis (ALS)[3]. It is worth mentioning that gene editing is also progressing to treat n-of-1 diseases, such as in the case of a rare mutation of Duchenne muscular dystrophy[182]. Treating N-of-1 diseases by personalized therapy, which was unthinkable a few years ago, is now becoming a reality with the availability of well mastered technological platforms which drastically reduce development duration and costs. This personalized medicine perspective for N-of-1 patients might represent the hallmark of the ongoing genetic drug revolution. However, this will necessitate solving the main obstacle to the generalization of RNA drug use, which consists in identifying efficient delivery methods to all tissues.

DECLARATIONS
Authors' contributions
The author contributed solely to the article.

Availability of data and materials
Not applicable.

Financial support and sponsorship

Conflicts of interest
All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Copyright

REFERENCES
1.   Kaufmann SH. Paul Ehrlich: founder of chemotherapy. *Nat Rev Drug Discov* 2008;7:373. DOI
2.   Drews J. Paul ehrlich: magister mundi. *Nat Rev Drug Discov* 2004;3:797-801. DOI
3.   Synofzik M, van Roon-Mom WMC, Marckmann G, et al. Preparing n-of-1 antisense oligonucleotide treatments for rare neurological diseases in europe: genetic, regulatory, and ethical perspectives. *Nucleic Acid Ther* 2022;32:83-94. DOI PubMed PMC
4.   Gillmore JD, Gane E, Taubel J, et al. CRISPR-Cas9 *in vivo* gene editing for transthyretin amyloidosis. *N Engl J Med* 2021;385:493-502. DOI
5.   Bennett CF, Krainer AR, Cleveland DW. Antisense oligonucleotide therapies for neurodegenerative diseases. *Annu Rev Neurosci* 2019;42:385-406. DOI PubMed PMC
6.   Hu B, Weng Y, Xia XH, Liang XJ, Huang Y. Clinical advances of siRNA therapeutics. *J Gene Med* 2019;21:e3097. DOI PubMed
7.   Holm A, Hansen SN, Klitgaard H, Kauppinen S. Clinical advances of RNA therapeutics for treatment of neurological and neuromuscular diseases. *RNA Biol* 2022;19:594-608. DOI PubMed PMC
8.   Brunet de Courssou JB, Durr A, Adams D, Corvol JC, Mariani LL. Antisense therapies in neurological diseases. *Brain* 2022;145:816-31. DOI PubMed

9.   Zamecnik PC, Stephenson ML. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc Natl Acad Sci USA* 1978;75:280-4. DOI PubMed PMC

10.   Stephenson ML, Zamecnik PC. Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. *Proc Natl Acad Sci USA* 1978;75:285-8. DOI PubMed PMC

11.   Sun P, Liu DZ, Jickling GC, Sharp FR, Yin KJ. MicroRNA-based therapeutics in central nervous system injuries. *J Cereb Blood Flow Metab* 2018;38:1125-48. DOI PubMed PMC

12.   Gan W, Guan Z, Liu J, et al. R-loop-mediated genomic instability is caused by impairment of replication fork progression. *Genes Dev* 2011;25:2041-56. DOI PubMed PMC

13.   Pang J, Guo Q, Lu Z. The catalytic mechanism, metal dependence, substrate specificity, and biodiversity of ribonuclease H. *Front Microbiol* 2022;13:1034811. DOI PubMed PMC

14.   Nowotny M, Gaidamakov SA, Crouch RJ, Yang W. Crystal structures of RNase H bound to an RNA/DNA hybrid: substrate specificity and metal-dependent catalysis. *Cell* 2005;121:1005-16. DOI

15.   Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* 1998;391:806-11. DOI PubMed

16.   Montgomery MK, Xu S, Fire A. RNA as a target of double-stranded RNA-mediated genetic interference in Caenorhabditis elegans. *Proc Natl Acad Sci U S A* 1998;95:15502-7. DOI PubMed PMC

17.   Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001; 411: 494-498. DOI PubMed

18.   Bumcrot D, Manoharan M, Koteliansky V, Sah DW. RNAi therapeutics: a potential new class of pharmaceutical drugs. *Nat Chem Biol* 2006;2:711-9. DOI PubMed PMC

19.   Lisa J. Scherer LJ, Rossi JJ. Principles of RNAi trigger expression for gene therapy. Advanced Textbook on Gene Transfer, Gene Therapy and Genetic Pharmacology. 2014; pp53-72; Editor Scherman Daniel. Imperial College Press. ISBN: 9781786346872. DOI

20.   Snead NM, Rossi JJ. Biogenesis and function of endogenous and exogenous siRNAs. *Wiley Interdiscip Rev RNA* 2010;1:117-31. DOI PubMed

21.   Scherer L, Rossi JJ. RNAi applications in mammalian cells. *Biotechniques* 2004;36:557-61. DOI PubMed

22.   Foster DJ, Brown CR, Shaikh S, et al. Advanced siRNA designs further improve *in vivo* performance of GalNAc-siRNA conjugates. *Mol Ther* 2018;26:708-17. DOI PubMed PMC

23.   Gosselin NH, Schuck VJA, Barriere O, et al. Translational population-pharmacodynamic modeling of a novel long-acting siRNA therapy, inclisiran, for the treatment of hypercholesterolemia. *Clin Pharmacol Ther* 2023;113:328-38. DOI PubMed

24.   Langlois MA, Boniface C, Wang G, et al. Cytoplasmic and nuclear retained DMPK mRNAs are targets for RNA interference in myotonic dystrophy cells. *J Biol Chem* 2005;280:16949-54. DOI

25.   Sobczak K, Wheeler TM, Wang W, Thornton CA. RNA interference targeting CUG repeats in a mouse model of myotonic dystrophy. *Mol Ther* 2013;21:380-7. DOI PubMed PMC

26.   Bisset DR, Stepniak-Konieczna EA, Zavaljevski M, et al. Therapeutic impact of systemic AAV-mediated RNA interference in a mouse model of myotonic dystrophy. *Hum Mol Genet* 2015;24:4971-83. DOI PubMed PMC

27.   Gantier MP, Williams BR. The response of mammalian cells to double-stranded RNA. *Cytokine Growth Factor Rev* 2007;18:363-71. DOI PubMed PMC

28.   Gantier MP, Sadler AJ, Williams BR. Fine-tuning of the innate immune response by microRNAs. *Immunol Cell Biol* 2007;85:458-62. DOI PubMed

29.   Kleinman ME, Yamada K, Takeda A, et al. Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. *Nature* 2008;452:591-7. DOI PubMed PMC

30.   Cho WG, Albuquerque RJ, Kleinman ME, et al. Small interfering RNA-induced TLR3 activation inhibits blood and lymphatic vessel growth. *Proc Natl Acad Sci U S A* 2009;106:7137-42. DOI PubMed PMC

31.   Bramsen JB, Pakula MM, Hansen TB, et al. A screen of chemical modifications identifies position-specific modification by UNA to most potently reduce siRNA off-target effects. *Nucleic Acids Res* 2010;38:5761-73. DOI PubMed PMC

32.   Khvorova A, Watts JK. The chemical evolution of oligonucleotide therapies of clinical utility. *Nat Biotechnol* 2017;35:238-48. DOI PubMed PMC

33.   Crooke ST, Liang XH, Baker BF, Crooke RM. Antisense technology: a review. *J Biol Chem* 2021;296:100416. DOI PubMed PMC

34.   Koller E, Vincent TM, Chappell A, De S, Manoharan M, Bennett CF. Mechanisms of single-stranded phosphorothioate modified antisense oligonucleotide accumulation in hepatocytes. *Nucleic Acids Res* 2011;39:4795-807. DOI PubMed PMC

35.   Eckstein F. Phosphorothioates, essential components of therapeutic oligonucleotides. *Nucleic Acid Ther* 2014;24:374-87. DOI PubMed

36.   Geary RS, Norris D, Yu R, Bennett CF. Pharmacokinetics, biodistribution and cell uptake of antisense oligonucleotides. *Adv Drug Deliv Rev* 2015;87:46-51. DOI PubMed

37.   Crooke ST. Vitravene-another piece in the mosaic. *Antisense Nucleic Acid Drug Dev* 1998;8:vii-viii. DOI PubMed

38.   Furdon PJ, Dominski Z, Kole R. RNase H cleavage of RNA hybridized to oligonucleotides containing methylphosphonate, phosphorothioate and phosphodiester bonds. *Nucleic Acids Res* 1989;17:9193-204. DOI PubMed PMC

39.   Zhang L, Liang XH, De Hoyos CL, et al. The combination of mesyl-phosphoramidate inter-nucleotide linkages and 2'-O-methyl in selected positions in the antisense oligonucleotide enhances the performance of RNaseH1 active PS-ASOs. *Nucleic Acid Ther*

2022;32:401-11. DOI PubMed PMC

40.   Summerton J, Weller D. Morpholino antisense oligomers: design, preparation, and properties. *Antisense Nucleic Acid Drug Dev* 1997;7:187-95. DOI PubMed

41.   Moulton JD. Using morpholinos to control gene expression. *Curr Protoc Nucleic Acid Chem* 2017;68:4.30.1-4.30.29. DOI PubMed PMC

42.   Amantana A, Iversen PL. Pharmacokinetics and biodistribution of phosphorodiamidate morpholino antisense oligomers. *Curr Opin Pharmacol* 2005;5:550-5. DOI PubMed

43.   Renneberg D, Bouliong E, Reber U, Schümperli D, Leumann CJ. Antisense properties of tricyclo-DNA. *Nucleic Acids Res* 2002;30:2751-7. DOI PubMed PMC

44.   Goyenvalle A, Leumann C, Garcia L. Therapeutic potential of tricyclo-DNA antisense oligonucleotides. *J Neuromuscul Dis* 2016;3:157-67. DOI PubMed PMC

45.   Nielsen PE, Egholm M, Berg RH, Buchardt O. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science* 1991;254:1497-500. DOI PubMed

46.   McMahon BM, Mays D, Lipsky J, Stewart JA, Fauq A, Richelson E. Pharmacokinetics and tissue distribution of a peptide nucleic acid after intravenous administration. *Antisense Nucleic Acid Drug Dev* 2002;12:65-70. DOI PubMed

47.   Lennox KA, Behlke MA. A direct comparison of anti-microRNA oligonucleotide potency. *Pharm Res* 2010;27:1788-99. DOI PubMed

48.   Jearawiriyapaisarn N, Moulton HM, Buckley B, et al. Sustained dystrophin expression induced by peptide-conjugated morpholino oligomers in the muscles of mdx mice. *Mol Ther* 2008;16:1624-9. DOI PubMed PMC

49.   Hammond SM, Hazell G, Shabanpoor F, et al. Systemic peptide-mediated oligonucleotide therapy improves long-term survival in spinal muscular atrophy. *Proc Natl Acad Sci U S A* 2016;113:10962-7. DOI PubMed PMC

50.   Tajik-Ahmadabad B, Polyzos A, Separovic F, Shabanpoor F. Amphiphilic lipopeptide significantly enhances uptake of charge-neutral splice switching morpholino oligonucleotide in spinal muscular atrophy patient-derived fibroblasts. *Int J Pharm* 2017;532:21-8. DOI PubMed

51.   Hangeland JJ, Flesher JE, Deamond SF, Lee YC, Ts'O PO, Frost JJ. Tissue distribution and metabolism of the [32P]-labeled oligodeoxynucleoside methylphosphonate-neoglycopeptide conjugate, [YEE(ah-GalNAc)3]-SMCC-AET-pUmpT7, in the mouse. *Antisense Nucleic Acid Drug Dev* 1997;7:141-9. DOI PubMed

52.   Maier MA, Yannopoulos CG, Mohamed N, et al. Synthesis of antisense oligonucleotides conjugated to a multivalent carbohydrate cluster for cellular targeting. *Bioconjug Chem* 2003;14:18-29. DOI

53.   Debacker AJ, Voutila J, Catley M, Blakey D, Habib N. Delivery of oligonucleotides to the liver with GalNAc: from research to registered therapeutic Drug. *Mol Ther* 2020;28:1759-71. DOI PubMed PMC

54.   Schlegel MK, Janas MM, Jiang Y, et al. From bench to bedside: improving the clinical safety of GalNAc-siRNA conjugates using seed-pairing destabilization. *Nucleic Acids Res* 2022;50:6656-70. DOI PubMed PMC

55.   Gennemark P, Walter K, Clemmensen N, et al. An oral antisense oligonucleotide for PCSK9 inhibition. *Sci Transl Med* 2021;13:eabe9117. DOI

56.   Angeli E, Nguyen TT, Janin A, Bousquet G. How to make anticancer drugs cross the blood-brain barrier to treat brain metastases. *Int J Mol Sci* 2019;21:22. DOI PubMed PMC

57.   Kawasaki AM, Casper MD, Freier SM, et al. Uniformly modified 2'-deoxy-2'-fluoro phosphorothioate oligonucleotides as nuclease-resistant antisense compounds with high affinity and specificity for RNA targets. *J Med Chem* 1993;36:831-41. DOI PubMed

58.   Seth PP, Siwkowski A, Allerson CR, et al. Short antisense oligonucleotides with novel 2'-4' conformationaly restricted nucleoside analogues show improved potency without increased toxicity in animals. *J Med Chem* 2009;52:10-3. DOI PubMed

59.   Shen W, De Hoyos CL, Sun H, et al. Acute hepatotoxicity of 2' fluoro-modified 5-10-5 gapmer phosphorothioate oligonucleotides in mice correlates with intracellular protein binding and the loss of DBHS proteins. *Nucleic Acids Res* 2018;46:2204-17. DOI PubMed PMC

60.   Burel SA, Hart CE, Cauntay P, et al. Hepatotoxicity of high affinity gapmer antisense oligonucleotides is mediated by RNase H1 dependent promiscuous reduction of very long pre-mRNA transcripts. *Nucleic Acids Res* 2016;44:2093-109. DOI PubMed PMC

61.   Kasuya T, Hori S, Watanabe A, et al. Ribonuclease H1-dependent hepatotoxicity caused by locked nucleic acid-modified gapmer antisense oligonucleotides. *Sci Rep* 2016;6:30377. DOI PubMed PMC

62.   Alterman JF, Godinho BMDC, Hassler MR, et al. A divalent siRNA chemical scaffold for potent and sustained modulation of gene expression throughout the central nervous system. *Nat Biotechnol* 2019;37:884-94. DOI PubMed PMC

63.   . Dicerna. Methods and compositions for the specific inhibition of transthyretin (TTR) by double-stranded RNA. US Pat; 2019. 1-240.

64.   Hu B, Zhong L, Weng Y, et al. Therapeutic siRNA: state of the art. *Signal Transduct Target Ther* 2020;5:101. DOI PubMed PMC

65.   Elkayam E, Parmar R, Brown CR, et al. siRNA carrying an (E)-vinylphosphonate moiety at the 5´ end of the guide strand augments gene silencing by enhanced binding to human Argonaute-2. *Nucleic Acids Res* 2017;45:3528-36. DOI PubMed PMC

66.   Brown CR, Gupta S, Qin J, et al. Investigating the pharmacodynamic durability of GalNAc-siRNA conjugates. *Nucleic Acids Res* 2020;48:11827-44. DOI PubMed PMC

67.   Kel'in AV, Zlatev I, Harp J, et al. Structural basis of duplex thermodynamic stability and enhanced nuclease resistance of 5'-C-Methyl Pyrimidine-Modified Oligonucleotides. *J Org Chem* 2016;81:2261-79. DOI PubMed

68.   Schlegel MK, Janas MM, Jiang Y, et al. From bench to bedside: improving the clinical safety of GalNAc-siRNA conjugates using seed-pairing destabilization. *Nucleic Acids Res* 2022;50:6656-70. DOI PubMed PMC

69.   Quan D, Obici L, Berk JL, et al. Impact of baseline polyneuropathy severity on patisiran treatment outcomes in the APOLLO trial. *Amyloid* 2023;30:49-58. DOI

70.   Ranasinghe P, Addison ML, Dear JW, Webb DJ. Small interfering RNA: discovery, pharmacology and clinical development-an introductory review. *Br J Pharmacol* 2022. DOI PubMed

71.   Wei Y, Li X, Lin J, et al. Oral Delivery of siRNA Using fluorinated, small-sized nanocapsules toward anti-inflammation treatment. *Adv Mater* 2023;35:e2206821. DOI PubMed

72.   Busignies V, Arruda DC, Charrueau C, et al. Compression of Vectors for Small Interfering RNAs Delivery: Toward Oral Administration of siRNA Lipoplexes in Tablet Forms. *Mol Pharm* 2020;17:1159-69. DOI

73.   Fattal E, Bochot A. Ocular delivery of nucleic acids: antisense oligonucleotides, aptamers and siRNA. *Adv Drug Deliv Rev* 2006;58:1203-23. DOI PubMed

74.   Scherman D, Rousseau A, Bigey P, Escriou V. Genetic pharmacology: progresses in siRNA delivery and therapeutic applications. *Gene Ther* 2017;24:151-6. DOI PubMed

75.   Khoury M, Escriou V, Courties G, et al. Efficient suppression of murine arthritis by combined anticytokine small interfering RNA lipoplexes. *Arthritis Rheum* 2008;58:2356-67. DOI

76.   Courties G, Baron M, Presumey J, et al. Cytosolic phospholipase A2α gene silencing in the myeloid lineage alters development of Th1 responses and reduces disease severity in collagen-induced arthritis. *Arthritis Rheum* 2011;63:681-90. DOI

77.   Available from: Orphanet: https://www.orpha.net/consor/cgi-bin/Disease.php?lng=EN [Last accessed on 29 May 2023].

78.   Angelini G, Mura G, Messina G. Therapeutic approaches to preserve the musculature in duchenne muscular dystrophy: the importance of the secondary therapies. *Exp Cell Res* 2022;410:112968. DOI PubMed

79.   Koenig M, Monaco AP, Kunkel LM. The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell* 1988;53:219-28. DOI PubMed

80.   Tennyson CN, Klamut HJ, Worton RG. The human dystrophin gene requires 16 hours to be transcribed and is cotranscriptionally spliced. *Nat Genet* 1995;9:184-90. DOI PubMed

81.   Monaco AP, Bertelson CJ, Liechti-Gallati S, Moser H, Kunkel LM. An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. *Genomics* 1988;2:90-5. DOI PubMed

82.   Aartsma-Rus A, Van Deutekom JC, Fokkema IF, Van Ommen GJ, Den Dunnen JT. Entries in the Leiden Duchenne muscular dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule. *Muscle Nerve* 2006;34:135-44. DOI PubMed

83.   Aartsma-Rus A, Fokkema I, Verschuuren J, et al. Theoretic applicability of antisense-mediated exon skipping for Duchenne muscular dystrophy mutations. *Hum Mutat* 2009;30:293-9. DOI

84.   Neri M, Rossi R, Trabanelli C, et al. The genetic landscape of dystrophin mutations in Italy: a nationwide study. *Front Genet* 2020;11:131. DOI PubMed PMC

85.   Goyenvalle A, Vulin A, Fougerousse F, et al. Rescue of dystrophic muscle through U7 snRNA-mediated exon skipping. *Science* 2004;306:1796-9. DOI

86.   Denti MA, Rosa A, D'Antona G, et al. Chimeric adeno-associated virus/antisense U1 small nuclear RNA effectively rescues dystrophin synthesis and muscle function by local treatment of mdx mice. *Hum Gene Ther* 2006;17:565-74. DOI PubMed

87.   Stein CA. Eteplirsen Approved for duchenne muscular dystrophy: the FDA faces a difficult choice. *Mol Ther* 2016;24:1884-5. DOI PubMed PMC

88.   Flanigan KM, Voit T, Rosales XQ, et al. Pharmacokinetics and safety of single doses of drisapersen in non-ambulant subjects with Duchenne muscular dystrophy: results of a double-blind randomized clinical trial. *Neuromuscul Disord* 2014;24:16-24. DOI PubMed PMC

89.   Deng J, Zhang J, Shi K, Liu Z. Drug development progress in duchenne muscular dystrophy. *Front Pharmacol* 2022;13:950651. DOI PubMed PMC

90.   Melki J, Lefebvre S, Burglen L, et al. De novo and inherited deletions of the 5q13 region in spinal muscular atrophies. *Science* 1994;264:1474-7. DOI

91.   Lefebvre S, Bürglen L, Reboullet S, et al. Identification and characterization of a spinal muscular atrophy-determining gene. *Cell* 1995;80:155-65. DOI

92.   Finkel RS, McDermott MP, Kaufmann P, et al. Observational study of spinal muscular atrophy type I and implications for clinical trials. *Neurology* 2014;83:810-7. DOI PubMed PMC

93.   Kaufmann P, McDermott MP, Darras BT, et al; Muscle Study Group (MSG); Pediatric Neuromuscular Clinical Research Network for Spinal Muscular Atrophy (PNCR). Prospective cohort study of spinal muscular atrophy types 2 and 3. *Neurology* 2012;79:1889-97. DOI PubMed PMC

94.   Rochette CF, Gilbert N, Simard LR. SMN gene duplication and the emergence of the SMN2 gene occurred in distinct hominids: SMN2 is unique to Homo sapiens. *Hum Genet* 2001;108:255-66. DOI PubMed

95.   Burlet P, Bürglen L, Clermont O, et al. Large scale deletions of the 5q13 region are specific to Werdnig-Hoffmann disease. *J Med Genet* 1996;33:281-3. DOI PubMed PMC

96.   Wirth B, Brichta L, Schrank B, et al. Mildly affected patients with spinal muscular atrophy are partially protected by an increased

SMN2 copy number. *Hum Genet* 2006;119:422-8. DOI

97. Singh NK, Singh NN, Androphy EJ, Singh RN. Splicing of a critical exon of human Survival Motor Neuron is regulated by a unique silencer element located in the last intron. *Mol Cell Biol* 2006;26:1333-46. DOI PubMed PMC

98. Hua Y, Vickers TA, Okunola HL, Bennett CF, Krainer AR. Antisense masking of an hnRNP A1/A2 intronic splicing silencer corrects SMN2 splicing in transgenic mice. *Am J Hum Genet* 2008;82:834-48. DOI PubMed PMC

99. Hua Y, Liu YH, Sahashi K, Rigo F, Bennett CF, Krainer AR. Motor neuron cell-nonautonomous rescue of spinal muscular atrophy phenotypes in mild and severe transgenic mouse models. *Genes Dev* 2015;29:288-97. DOI PubMed PMC

100. Touznik A, Maruyama R, Hosoki K, Echigoya Y, Yokota T. LNA/DNA mixmer-based antisense oligonucleotides correct alternative splicing of the SMN2 gene and restore SMN protein expression in type 1 SMA fibroblasts. *Sci Rep* 2017;7:3672. DOI PubMed PMC

101. Benkhelifa-Ziyyat S, Besse A, Roda M, et al. Intramuscular scAAV9-SMN injection mediates widespread gene delivery to the spinal cord and decreases disease severity in SMA mice. *Mol Ther* 2013;21:282-90. DOI PubMed PMC

102. Mendell JR, Al-Zaidy SA, Rodino-Klapac LR, et al. Current clinical applications of *in vivo* gene therapy with AAVs. *Mol Ther* 2021;29:464-88. DOI PubMed PMC

103. ANDRADE C. A peculiar form of peripheral neuropathy: familiar atypical generalized amyloidosis with special involvement of the peripheral nerves. *Brain* 1952;75:408-27. DOI

104. Jacobson DR, Pastore RD, Yaghoubian R, et al. Variant-sequence transthyretin (isoleucine 122) in late-onset cardiac amyloidosis in black Americans. *N Engl J Med* 1997;336:466-73. DOI

105. Dyck PJB, Coelho T, Waddington Cruz M, et al. Neuropathy symptom and change: Inotersen treatment of hereditary transthyretin amyloidosis. *Muscle Nerve* 2020;62:509-15. DOI PubMed PMC

106. Westermark P, Sletten K, Johansson B, Cornwell GG 3rd. Fibril in senile systemic amyloidosis is derived from normal transthyretin. *Proc Natl Acad Sci U S A* 1990;87:2843-5. DOI PubMed PMC

107. Benson MD, Waddington-Cruz M, Berk JL, et al. Inotersen treatment for patients with hereditary transthyretin amyloidosis. *N Engl J Med* 2018;379:22-31. DOI PubMed

108. Sewing S, Roth AB, Winter M, et al. Assessing single-stranded oligonucleotide drug-induced effects in vitro reveals key risk factors for thrombocytopenia. *PLoS One* 2017;12:e0187574. DOI PubMed PMC

109. Flierl U, Nero TL, Lim B, et al. Phosphorothioate backbone modifications of nucleotide-based drugs are potent platelet activators. *J Exp Med* 2015;212:129-37. DOI PubMed PMC

110. Severi D, Palumbo G, Spina E, et al. A case of severe increase of liver enzymes in a ATTRv patient after one year of inotersen treatment. *Neurol Sci* 2023;44:1419-22. DOI PubMed PMC

111. Alnylam pharmaceuticals highlights of US prescribing information. Available from:https://www.alnylam.com/ [Last accessed on 29 May 2023].

112. Marimani MD, Ely A, Buff MC, et al. Inhibition of replication of hepatitis B virus in transgenic mice following administration of hepatotropic lipoplexes containing guanidinopropyl-modified siRNAs. *J Control Release* 2015;209:198-206. DOI

113. Kulkarni JA, Witzigmann D, Chen S, Cullis PR, van der Meel R. Lipid nanoparticle technology for clinical translation of sirna therapeutics. *Acc Chem Res* 2019;52:2435-44. DOI PubMed

114. van der Meel R, Chen S, Zaifman J, et al. Modular lipid nanoparticle platform technology for siRNA and lipophilic prodrug delivery. *Small* 2021;17:e2103025. DOI

115. Böttger R, Pauli G, Chao PH, et al. Lipid-based nanoparticle technologies for liver targeting. *Adv Drug Deliv Rev* 2020;154-155:79-101. DOI PubMed

116. Zhang MM, Bahal R, Rasmussen TP, Manautou JE, Zhong XB. The growth of siRNA-based therapeutics: updated clinical studies. *Biochem Pharmacol* 2021;189:114432. DOI PubMed PMC

117. Weng YH, Xiao HH, Zhang JC, Liang XJ, Huang YY. RNAi therapeutic and its innovative biotechnological evolution. *Biotechnol Adv* 2019;37:801-25. DOI

118. Springer AD, Dowdy SF. GalNAc-siRNA conjugates: leading the way for delivery of RNAi therapeutics. *Nucleic Acid Ther* 2018;28:109-18. DOI PubMed PMC

119. Chaumet-Riffaud P, Martinez-Duncker I, Marty AL, et al. Synthesis and application of lactosylated, 99mTc chelating albumin for measurement of liver function. *Bioconjug Chem* 2010;21:589-96. DOI PubMed

120. Salmon H, Gahoual R, Houzé P, et al. Europium labeled lactosylated albumin as a model workflow for the development of biotherapeutics. *Nanomedicine* 2019;18:21-30. DOI

121. Habtemariam BA, Karsten V, Attarwala H, et al. Single-dose pharmacokinetics and pharmacodynamics of transthyretin targeting N-acetylgalactosamine-small interfering ribonucleic acid conjugate, vutrisiran, in healthy subjects. *Clin Pharmacol Ther* 2021;109:372-82. DOI PubMed

122. Adams D, Tournev IL, Taylor MS, et al; HELIOS-A Collaborators. Efficacy and safety of vutrisiran for patients with hereditary transthyretin-mediated amyloidosis with polyneuropathy: a randomized clinical trial. *Amyloid* 2023;30:1-9. DOI

123. Ando Y, Adams D, Benson MD, et al. Guidelines and new directions in the therapy and monitoring of ATTRv amyloidosis. *Amyloid* 2022;29:143-55. DOI

124. Echaniz-Laguna A, Cauquil C, Labeyrie C, Adams D. Treating hereditary transthyretin amyloidosis: Present & future challenges. *Rev Neurol* 2023;179:30-4. DOI PubMed

125. Aimo A, Castiglione V, Rapezzi C, et al. RNA-targeting and gene editing therapies for transthyretin amyloidosis. *Nat Rev Cardiol* 2022;19:655-67. DOI

126. Tanowitz M, Hettrick L, Revenko A, Kinberger GA, Prakash TP, Seth PP. Asialoglycoprotein receptor 1 mediates productive uptake of N-acetylgalactosamine-conjugated and unconjugated phosphorothioate antisense oligonucleotides into liver hepatocytes. *Nucleic Acids Res* 2017;45:12388-400. DOI PubMed PMC

127. Kim Y, Jo M, Schmidt J, et al. Enhanced potency of GalNAc-conjugated antisense oligonucleotides in hepatocellular cancer models. *Mol Ther* 2019;27:1547-57. DOI PubMed PMC

128. Brook JD, McCurrach ME, Harley HG, et al. Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. *Cell* 1992;69:385. DOI PubMed

129. Konieczny P, Stepniak-Konieczna E, Sobczak K. MBNL proteins and their target RNAs, interaction and splicing regulation. *Nucleic Acids Res* 2014;42:10873-87. DOI PubMed PMC

130. Goodwin M, Mohan A, Batra R, et al. MBNL Sequestration by toxic RNAs and RNA misprocessing in the myotonic dystrophy brain. *Cell Rep* 2015;12:1159-68. DOI PubMed PMC

131. Overby SJ, Cerro-Herreros E, Llamusi B, Artero R. RNA-mediated therapies in myotonic dystrophy. *Drug Discov Today* 2018;23:2013-22. DOI PubMed

132. Lee JE, Bennett CF, Cooper TA. RNase H-mediated degradation of toxic RNA in myotonic dystrophy type 1. *Proc Natl Acad Sci USA* 2012;109:4221-6. DOI PubMed PMC

133. A safety and tolerability study of multiple doses of ISIS-DMPKRx in adults with myotonic dystrophy Type 1. Available from: https://clinicaltrials.gov/ct2/show/NCT02312011 [Last accessed on 29 May 2023].

134. Sugo T, Terada M, Oikawa T, et al. Development of antibody-siRNA conjugate targeted to cardiac and skeletal muscles. *J Control Release* 2016;237:1-13. DOI

135. Safety, tolerability, pharmacodynamic, efficacy, and pharmacokinetic study of dyne-101 in participants with myotonic dystrophy Type 1 (ACHIEVE). Available from: https://www.clinicaltrials.gov/ct2/show/NCT05481879 [Last accessed on 29 May 2023].

136. Nguyen Q, Yokota T. Degradation of Toxic RNA in myotonic dystrophy using gapmer antisense oligonucleotides. In: Yokota T, Maruyama R, editors. Gapmers. New York: Springer US; 2020. pp. 99-109. DOI

137. Cerro-Herreros E, González-Martínez I, Moreno-Cervera N, et al. Therapeutic potential of antagomiR-23b for treating myotonic dystrophy. *Mol Ther Nucleic Acids* 2020;21:837-49. DOI PubMed PMC

138. Wheeler TM, Lueck JD, Swanson MS, Dirksen RT, Thornton CA. Correction of ClC-1 splicing eliminates chloride channelopathy and myotonia in mouse models of myotonic dystrophy. *J Clin Invest* 2007;117:3952-7. DOI PubMed PMC

139. Negishi Y, Endo-takahashi Y, Ishiura S. Exon skipping by ultrasound-enhanced delivery of morpholino with bubble liposomes for myotonic dystrophy model mice. In: Yokota T, Maruyama R, editors. Exon Skipping and Inclusion Therapies. New York: Springer; 2018. pp. 481-7. DOI

140. Xia X, Zhou H, Huang Y, Xu Z. Allele-specific RNAi selectively silences mutant SOD1 and achieves significant therapeutic benefit in vivo. *Neurobiol Dis* 2006;23:578-86. DOI PubMed

141. Lombardi MS, Jaspers L, Spronkmans C, et al. A majority of Huntington's disease patients may be treatable by individualized allele-specific RNA interference. *Exp Neurol* 2009;217:312-9. DOI

142. Hauser S, Helm J, Kraft M, Korneck M, Hübener-Schmid J, Schöls L. Allele-specific targeting of mutant ataxin-3 by antisense oligonucleotides in SCA3-iPSC-derived neurons. *Mol Ther Nucleic Acids* 2022;27:99-108. DOI PubMed PMC

143. Pfister EL, Kennington L, Straubhaar J, et al. Five siRNAs targeting three SNPs may provide therapy for three-quarters of Huntington's disease patients. *Curr Biol* 2009;19:774-8. DOI PubMed PMC

144. Kay C, Collins JA, Caron NS, et al. A comprehensive haplotype-targeting strategy for allele-specific HTT suppression in huntington disease. *Am J Hum Genet* 2019;105:1112-25. DOI PubMed PMC

145. Conroy F, Miller R, Alterman JF, et al. Chemical engineering of therapeutic siRNAs for allele-specific gene silencing in Huntington's disease models. *Nat Commun* 2022;13:5802. DOI PubMed PMC

146. Trochet D, Prudhon B, Mekzine L, et al. Benefits of therapy by dynamin-2-mutant-specific silencing are maintained with time in a mouse model of dominant centronuclear myopathy. *Mol Ther Nucleic Acids* 2022;27:1179-90. DOI PubMed PMC

147. Dudhal S, Mekzine L, Prudhon B, et al. Development of versatile allele-specific siRNAs able to silence all the dominant dynamin 2 mutations. *Mol Ther Nucleic Acids* 2022;29:733-48. DOI PubMed PMC

148. Züchner S, Noureddine M, Kennerson M, et al. Mutations in the pleckstrin homology domain of dynamin 2 cause dominant intermediate charcot-marie-tooth disease. *Nat Genet* 2005;37:289-94. DOI

149. Sambuughin N, Goldfarb LG, Sivtseva TM, et al. Adult-onset autosomal dominant spastic paraplegia linked to a GTPase-effector domain mutation of dynamin 2. *BMC Neurol* 2015;15:223. DOI PubMed PMC

150. Fujise K, Noguchi S, Takeda T. Centronuclear myopathy caused by defective membrane remodelling of dynamin 2 and BIN1 variants. *Int J Mol Sci* 2022;23:6274. DOI PubMed PMC

151. Wang L, Barylko B, Byers C, Ross JA, Jameson DM, Albanesi JP. Dynamin 2 mutants linked to centronuclear myopathies form abnormally stable polymers. *J Biol Chem* 2010;285:22753-7. DOI PubMed PMC

152. Cowling BS, Chevremont T, Prokic I, et al. Reducing dynamin 2 expression rescues X-linked centronuclear myopathy. *J Clin Invest* 2014;124:1350-63. DOI PubMed PMC

153. Koutsopoulos OS, Kretz C, Weller CM, et al. Dynamin 2 homozygous mutation in humans with a lethal congenital syndrome. *Eur J*

Hum Genet 2013;21:637-42. DOI PubMed PMC

154. Study of TD101, a Small interfering RNA (siRNA) designed for treatment of pachyonychia congenita. Available from: https://clinicaltrials.gov/ct2/show/NCT00716014 [Last accessed on 29 May 2023].

155. Leachman SA, Hickerson RP, Schwartz ME, et al. First-in-human mutation-targeted siRNA phase Ib trial of an inherited skin disorder. *Mol Ther* 2010;18:442-6. DOI PubMed PMC

156. Trochet D, Prudhon B, Vassilopoulos S, Bitoun M. Therapy for dominant inherited diseases by allele-specific RNA interference: successes and pitfalls. *Curr Gene Ther* 2015;15:503-10. DOI PubMed

157. Roth F, Dhiab J, Boulinguiez A, et al. Assessment of PABPN1 nuclear inclusions on a large cohort of patients and in a human xenograft model of oculopharyngeal muscular dystrophy. *Acta Neuropathol* 2022;144:1157-70. DOI PubMed PMC

158. Banerjee A, Apponi LH, Pavlath GK, Corbett AH. PABPN1: molecular function and muscle disease. *FEBS J* 2013;280:4230-50. DOI PubMed PMC

159. Malerba A, Klein P, Bachtarzi H, et al. PABPN1 gene therapy for oculopharyngeal muscular dystrophy. *Nat Commun* 2017;8:14848. DOI PubMed PMC

160. Malerba A, Klein P, Lu-Nguyen N, et al. Established PABPN1 intranuclear inclusions in OPMD muscle can be efficiently reversed by AAV-mediated knockdown and replacement of mutant expanded PABPN1. *Hum Mol Genet* 2019;28:3301-8. DOI PubMed PMC

161. Strings-Ufombah V, Malerba A, Kao SC, et al. BB-301: a silence and replace AAV-based vector for the treatment of oculopharyngeal muscular dystrophy. *Mol Ther Nucleic Acids* 2021;24:67-78. DOI PubMed PMC

162. Cao W, Lia R, Pei X, et al. Antibody–siRNA conjugates (ARC): emerging siRNA drug formulation. *Medicine in Drug Discovery* 2022;15:100128. DOI

163. Zlatev I, Castoreno A, Brown CR, et al. Reversal of siRNA-mediated gene silencing in vivo. *Nat Biotechnol* 2018;36:509-11. DOI

164. Burel SA, Hart CE, Cauntay P, et al. Hepatotoxicity of high affinity gapmer antisense oligonucleotides is mediated by RNase H1 dependent promiscuous reduction of very long pre-mRNA transcripts. *Nucleic Acids Res* 2016;44:2093-109. DOI PubMed PMC

165. Kasuya T, Hori S, Watanabe A, et al. Ribonuclease H1-dependent hepatotoxicity caused by locked nucleic acid-modified gapmer antisense oligonucleotides. *Sci Rep* 2016;6:30377. DOI PubMed PMC

166. Yasuhara H, Yoshida T, Sasaki K, Obika S, Inoue T. Reduction of off-target effects of gapmer antisense oligonucleotides by oligonucleotide extension. *Mol Diagn Ther* 2022;26:117-27. DOI PubMed PMC

167. Kobayashi Y, Tian S, Ui-Tei K. The siRNA off-target effect is determined by base-pairing stabilities of two different regions with opposite effects. *Genes* 2022;13:319. DOI PubMed PMC

168. Kanasty RL, Whitehead KA, Vegas AJ, Anderson DG. Action and reaction: the biological response to siRNA and its delivery vehicles. *Mol Ther* 2012;20:513-24. DOI PubMed PMC

169. Alagia A, Eritja R. siRNA and RNAi optimization. *Wiley Interdiscip Rev RNA* 2016;7:316-29. DOI PubMed

170. Grimm D, Wang L, Lee JS, et al. Argonaute proteins are key determinants of RNAi efficacy, toxicity, and persistence in the adult mouse liver. *J Clin Invest* 2010;120:3106-19. DOI PubMed PMC

171. Cardinali B, Provenzano C, Izzo M, et al. Time-controlled and muscle-specific CRISPR/Cas9-mediated deletion of CTG-repeat expansion in the DMPK gene. *Mol Ther Nucleic Acids* 2022;27:184-99. DOI PubMed PMC

172. Wallace LM, Garwick-Coppens SE, Tupler R, Harper SQ. RNA interference improves myopathic phenotypes in mice over-expressing FSHD region gene 1 (FRG1). *Mol Ther* 2011;19:2048-54. DOI PubMed PMC

173. Gautier B, Hajjar H, Soares S, et al. AAV2/9-mediated silencing of PMP22 prevents the development of pathological features in a rat model of Charcot-Marie-Tooth disease 1 A. *Nat Commun* 2021;12:2356. DOI PubMed PMC

174. Morelli KH, Griffin LB, Pyne NK, et al. Allele-specific RNA interference prevents neuropathy in charcot-marie-tooth disease type 2D mouse models. *J Clin Invest* 2019;129:5568-83. DOI PubMed PMC

175. Muraine L, Bensalah M, Dhiab J, et al. Transduction efficiency of adeno-associated virus serotypes after local injection in mouse and human skeletal muscle. *Hum Gene Ther* 2020;31:233-40. DOI PubMed PMC

176. Boivin M, Charlet-Berguerand N. Trinucleotide CGG repeat diseases: an expanding field of polyglycine proteins? *Front Genet* 2022;13:843014. DOI PubMed PMC

177. Glineburg MR, Todd PK, Charlet-Berguerand N, Sellier C. Repeat-associated non-AUG (RAN) translation and other molecular mechanisms in fragile X tremor ataxia syndrome. *Brain Res* 2018;1693:43-54. DOI PubMed PMC

178. German CA, Shapiro MD. Small interfering RNA therapeutic inclisiran: a new approach to targeting PCSK9. *BioDrugs* 2020;34:1-9. DOI PubMed

179. Lemaitre MM. Individualized antisense oligonucleotide therapies: how to approach the challenge of manufacturing these oligos from a chemistry, manufacturing, and control-regulatory standpoint. *Nucleic Acid Ther* 2022;32:101-10. DOI PubMed

180. Crooke ST. Meeting the needs of patients with ultrarare diseases. *Trends Mol Med* 2022;28:87-96. DOI PubMed

181. Kim J, Hu C, Moufawad El Achkar C, et al. Patient-customized oligonucleotide therapy for a rare genetic disease. *N Engl J Med* 2019;381:1644-52. DOI PubMed PMC

182. Treatment of a single patient with CRD-TMH-001. Available from: https://clinicaltrials.gov/ct2/show/NCT05514249 [Last accessed on 29 May 2023].