Chapter 2

Antisense RNA Therapeutics: A Brief Overview

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Abstract

Nucleic acid therapeutics is a growing field aiming to treat human conditions that has gained special attention due to the successful development of mRNA vaccines against SARS-CoV-2. Another type of nucleic acid therapeutics is antisense oligonucleotides, versatile tools that can be used in multiple ways to target pre-mRNA and mRNA. While some years ago these molecules were just considered a useful research tool and a curiosity in the clinical market, this has rapidly changed. These molecules are promising strategies for personalized treatments for rare genetic diseases and they are in development for very common disorders too. In this chapter, we provide a brief description of the different mechanisms of action of these RNA therapeutic molecules, with clear examples at preclinical and clinical stages.

Key words RNA therapy, Antisense oligonucleotides, Clinical trials, Splicing, Personalized medicine

1 Introduction

Nucleic acid therapeutics is still a growing field. With the irruption of the mRNA vaccines against SARS-CoV-2 special attention has been given to this type of therapies but other types of nucleic acid therapeutics, coined antisense oligonucleotides (AONs), have been studied for many years. Although only a dozen therapeutic oligonucleotides have been formally approved for clinical use, there are many new such drugs in the pipeline for a plethora of (mainly rare) diseases. These AON molecules interact with different nucleic acids (mRNA, non-coding RNA, and DNA) thanks to sequence specific Watson–Crick base pairing. Their mechanism of action, that may be designed to bind specific targets, makes these drugs easy to design, less likely to cause side effects and, therefore, potential candidates to lead the next wave of precision medicine. In this chapter, we describe the most frequently used AON-based therapeutic strategies, their mechanisms of action (Fig. 1), and the results of several clinical trials, with special emphasis in eye and muscle diseases.

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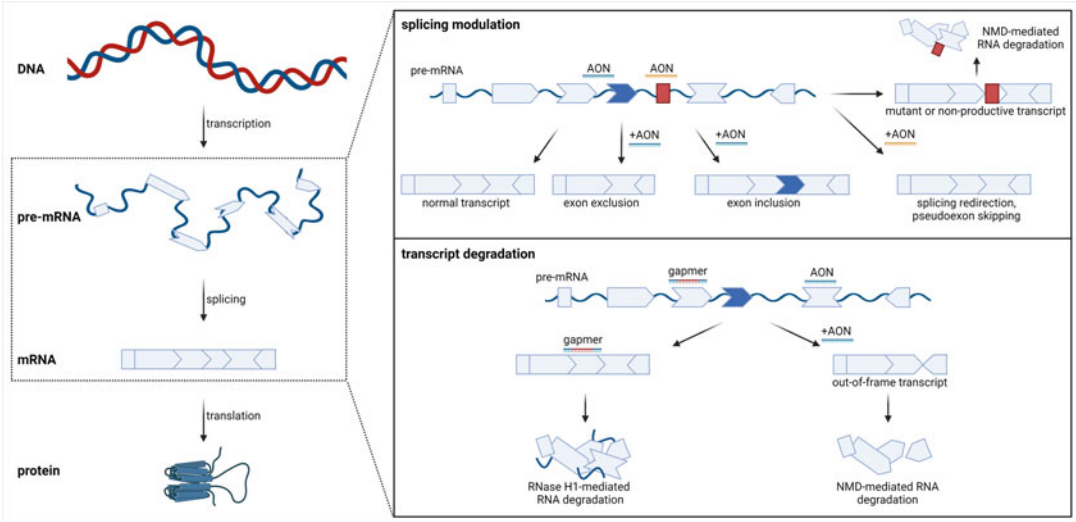

Fig. 1 Schematic representation of the multiple mechanisms of action of antisense oligonucleotide (AON) molecules. AONs can act at pre- and mRNA levels of the synthesis of a functional protein (left panel). They can be used to modulate splicing (upper right panel) or to degrade (pre-)mRNA (lower right panel). Splice-modulating AONs bind to pre-mRNA and promote the insertion or skipping of regular exons. In addition, they can redirect splicing when mutations in a gene lead to splicing defects (such as pseudoexon insertions). This splicing modulation causes the degradation of the transcript and a consequent reduction of protein levels. Alternatively, transcript degradation can also be achieved by using AONs binding to the pre-mRNA to disrupt the open reading frame and degrade transcripts via nonsense-mediated decay (NMD). Gapmers, in contrast, can bind to both pre-mRNA and mRNA and activate RNase-H1 RNA degradation. (Created with BioRender.com)

2 Mechanisms of Action

2.1 Splicing Modulation

The majority of existing therapeutic AONs are designed to alter the splicing pattern of specific pre-mRNAs [1]. This can be used to treat disorders caused by splicing alterations, which with the current widespread availability of better and cheaper sequencing options are being identified more easily and can be specifically targeted [2].

2.1.1 Exon Exclusion (Shortened Proteins)

In many genes, deleting an exon would result in the production of a non-functional protein, as their structure and function would be compromised. However, there are some cases in which internally "trimmed" proteins could be partially functional due to the existence of less-vital structures within a large protein. Mutations in the *DMD* gene disrupt the open reading frame (ORF) and the expression of the dystrophin protein, leading to Duchene muscular dystrophy (DMD). In contrast, in the much milder Becker muscular dystrophy mutations in the same gene that maintain the ORF produce an internally deleted but functional protein. This real-life example was rapidly seized as an opportunity to achieve the same

effect using therapeutic AONs, and several of the recently approved AON molecules target different *DMD* exons [3–9]. Because there are many different *DMD* mutations, the skipping specific exons would be therapeutic for different subsets of patients.

This concept has also been employed in the development of new AONs to skip in-frame exons carrying single-nucleotide changes generating premature stop codons in large genes. Mutations in *USH2A* cause either Usher syndrome (deafness combined with blindness) or isolated blindness in the form of retinitis pigmentosa. Exon 13 of *USH2A* is prone to carry truncating variants and by deleting it, a protein with residual function is potentially produced [10, 11]. This is also the case of a stop codon introduced by a variant in exon 36 of *CEP290*, which is naturally skipped at low levels in the retina and involved in retinal dystrophy. AONs designed to skip exon 36 restored the reading frame and produced a functional protein able to rescue the cellular phenotype in patient-derived cells [12]. Following the same strategy, AON molecules to skip different exons of *COL7A1* have been developed for dystrophic epidermolysis bullosa, a skin disease inherited in both dominant and recessive fashion [13–16].

2.1.2 Exon Inclusion

A seemingly opposite mechanism of action is at the core of nusinersen, an AON approved for the treatment of spinal muscular atrophy (SMA). In this case, mutations in the *SMN1* gene cause low or lack of SMN protein production. However, SMN protein can be produced by two nearly identical genes, *SMN1* and *SMN2*. The latter, however, contributes at very low levels due to the high rate of exon-7 skipping that disrupts the ORF. Nusinersen is used to alter the splicing of *SMN2* and include exon 7, and therefore produce sufficient amounts of SMN protein to ameliorate the patient's disease [17, 18].

2.1.3 Splicing Redirection

Variants close to the splice sites result often in either exon skipping or exon elongation. In the second scenario, mutations decrease the recognition of the original splice site and a cryptic splice site present in the intron is recognized; in the most extreme case, the entire intron is retained. Exonic variants may cause a synonymous or a predicted non-deleterious missense change at protein level, and in addition they can have a dramatic effect at RNA level by creating a novel splice site. In any case, these splicing defects are also amenable for AON intervention. For instance, a mutation in exon 3 of *USH1C* linked to deafness generates a novel splice donor site (SDS) upstream the regular SDS of the exon. This new SDS is preferentially used by the cells, leading to a disrupted reading frame. By using AONs to block the newly generated SDS, the normal transcript can be produced [19]. A similar approach has been used to target the exon elongation caused by near-exon

intronic variants in *ABCA4* linked to Stargardt macular degeneration. This study showed that by blocking the newly created SDS the normal splicing can be restored. However, this approach turned out to be not that successful when targeting exon elongations caused by variants in the splice acceptor site (SAS) [20].

Another elegant way to modulate splicing using AONs is by targeting the non-productive transcripts. These transcripts often are generated by (a) alternative splicing causing insertion or skipping of exons; (b) using alternative cryptic splice sites; and (c) retaining the introns. In any case, these splicing events lead to a disrupted ORF being the transcript degraded via nonsense-mediated decay (NMD). A very recent study has shown that 1246 potentially disease-associated genes present at least one of these non-productive transcripts. By targeting these splicing events to insert or skip an exon, exclude a retained intron, or redirect splicing when a cryptic splice site is used, the overall protein levels can be increased and this might be a promising therapeutic tool for haploinsufficiency cases [21].

2.1.4 Deep-Intronic Variants

For many years intronic variants have been ignored. This is mainly because they lay in the non-informative regions of our genome, the introns. However, the implementation of novel and more robust sequencing tools has contributed to solve the missing heritability in several diseases by discovering deep-intronic mutations with a detrimental effect on pre-mRNA splicing. The study of these variants is complex but with the help of novel bioinformatic (e.g. SpliceAI [22]) and molecular tools (mini/midi/maxi-genes [23]), it is possible to predict the effect at pre-mRNA level. These variants often result in the insertion of a pseudoexon, a piece of the intron that it is recognized as an exon and leads to a disruption of the ORF and consequently the generation of a premature stop codon. Pseudoexon exclusion can be achieved by using AONs. In the *ABCA4* gene, AONs have shown splicing correction for most of the 35 intronic variants identified as cause of Stargardt disease [24–30].

2.2 Transcript Degradation

Antisense technology can be extremely useful to degrade transcripts and cause gene silencing (knockdown). From the therapeutic perspective, this might be a potential tool to treat autosomal dominant diseases caused by dominant-negative mutations. In this case, by degrading specifically the mutant allele, the correct protein can perform its function properly.

2.2.1 RNase H1-Activating Antisense Oligonucleotides (Gapmers)

These antisense molecules are characterized for being able to actively reduce the levels of the mRNAs in the nucleus and cytoplasm [31], therefore they are very useful to downregulate gene expression. These RNase H1-activating AONs or gapmers are chimeric molecules linked using a phosphorothioate (PS) backbone that usually present a conformation 5-10-5, where the two arms

consist of five modified RNA nucleotides (2′-*O*-methoxyethyl (2′MOE), 2′-*O*-methyl (2′OMe) or locked nucleic acid (LNA)) flanking ten DNA nucleotides [32]. The first-ever AON approved by the FDA was fomivirsen, a first-generation RNase H1-activating AON [33–36] (*see* Subheading 3). However, this is the only RNase H1-activating AON that does not have the chimeric RNA/DNA structure. So far, four molecules using this mechanism of action have received FDA and/or EMA approval to treat different disease conditions [32].

Gapmers can be used to downregulate genes in allele-independent and allele-specific manner. Below, we review some examples of each case.

Allele-independent mRNA degradation is often used to target genes or pathways that are overexpressed in certain disease conditions or can worsen the disease progression. Thus, reducing the levels of particular genes can be very beneficial. This is the case for two of the approved AON drugs: mipomersen and volanesorsen. These molecules target the mRNA of the apolipoprotein B-100 in familial hypercholesterolemia or apolipoprotein C3 in familial chylomicronaemia syndrome, hypertriglyceridemia and familial partial lipodystrophy, respectively, to lower the levels of specific lipids increased in these diseases [37–41].

In contrast, allele-specific mRNA degradation aims to target only the mutant allele. This way, specific mutations that cause a dominant-negative effect can be targeted. This is the case of inotersen, a gapmer designed to target the mRNA encoding the transthyretin (TTR) protein in autosomal dominant hereditary transthyretin amyloidosis [42, 43]. A single-nucleotide change in the gene produces misfolding of the TTR protein. As TTR protein needs to tetramerize in order to conduct its function, the addition of mutant monomers into the tetramer affects the overall function. Systemic amyloid depositions are formed, leading to progressive polyneuropathy of the sensory and motor systems with multiorgan dysfunction in late-disease stages. The therapeutic gapmer targets the mutant allele to reduce the amount of tetramers containing the mutant protein, and therefore prevent the aforementioned depositions [42, 43]. Another recent example is the use of gapmers to specifically degrade the mutant allele introduced by a mutation in the *COCH* gene, which causes autosomal dominant hearing impairment [44]. In this study, two strategies were used to degrade the mutant transcript: directly targeting the mutation or other single-nucleotide polymorphisms (SNPs) in *cis* with the mutation that are part of the mutant haplotype.

2.2.2 Disrupting Reading Frame

Splice-switching AONs can also be used to induce transcript degradation. Skipping regular exons can also be used to knockdown the function of an undesired gene, by creating mRNA isoforms that

encode non-functional proteins or trigger degradation of the mRNA by NMD [45]. For instance, exon skipping of hepatic APOB100 was able to sustainably reduce LDL cholesterol levels in mice [46], downregulation of *MAPT* gene has been proposed as a possible treatment for tauopathies [47], and skipping exon 2 of *ALK5* may modulate the TGF-β signaling cascade, reducing the components related to the overproduction of extracellular matrix in hypertrophic scar [48].

3 Therapeutic Potential

While years ago oligonucleotides were considered a useful research tool and just a curiosity in the clinical market, this has rapidly changed into approved therapeutic strategies for several diseases and promising personalized treatments for many other (rare inherited) diseases. In this section, we will focus on the development of AON-based therapeutic strategies for two particular tissues: muscle and retina.

3.1 Examples of Clinical Trials for Muscle Diseases

The use of AONs to treat neuromuscular disorders has been at the forefront of the clinical development of AON-based therapies and more than half of the AONs currently in the market target either Duchenne muscular dystrophy (DMD) or spinal muscular atrophy (SMA). As previously described, AONs targeting the *DMD* gene aim to skip specific exons to restore the reading frame. This gene has 79 exons and patients present a large variety of mutations, mostly deletions and duplications, that require the design of specific AONs to treat a small subset to patients. The first such drug, eteplirsen, targeted exon 51 of *DMD*. Skipping this exon could potentially be therapeutic for 13% of DMD patients [3, 6]. Since then, golodirsen, viltolarsen, and casimersen have been approved, all applicable to decreasing percentages of patients [9, 49, 50].

All DMD exon-skipping drugs currently in the market are phosphorodiamidate morpholino oligomers (PMO). In contrast, the development of the first AON drug in clinical trials for this disorder, drisapersen (a 2′OMe/PS oligonucleotide) [51] as well as that of many others targeting *DMD* with the same chemistry were halted due to side effects [52]. Despite the apparent success of PMO chemistries to reach the market, these drugs are yet not very efficient, and their clinical outcomes are still poor. This is the main reason why they are yet to be approved in Europe, while in the USA and Japan have been given "accelerated approval" based on dystrophin protein expression as a surrogate endpoint, which is very low and there is debate about its clinical relevance [53]. Currently, several efforts are driven toward increasing the delivery efficacy of these drugs to the target tissue [32, 54]. Several next generation AONs targeting the same exon as eteplirsen (exon 51)

have been or are being developed. This is the case of the stereopure suvodirsen, which was halted after poor results in a phase I clinical trial (NCT03907072) or the peptide-conjugated PMO currently in Phase I/II clinical trials (MOMENTUM, NCT04004065).

While AONs for DMD do not offer yet the clinical benefits that were hoped to achieve at initial stages, the journey to their development has provided very valuable lessons to stakeholders interested in developing these drugs, particularly in the context of orphan drugs [55]. A drug that benefited from some of the previous knowledge was nusinersen, a 2′MOE/PS AON targeting another neuromuscular disorder (SMA). Nusinersen was approved only months after eteplirsen and has been quickly approved worldwide due to the robust clinical data derived from the clinical trials [18, 56]. As described before, this AON is based on an exon inclusion approach to restore the expression of SMN protein in motoneurons. In this case, the target tissue is treated directly by intrathecal infusion, circumventing any delivery hurdles that may have hampered the efficacy of AONs targeting muscle or other organs when delivered systemically. Indeed, nusinersen's delivery approach, chosen chemistry and formulation has been replicated in several *n*-of-1 clinical trials of other AONs targeting motoneurons, such as milasen and jacifusen (NCT04768972) [57] (*see* Subheading 4).

3.2 Examples of Clinical Trials for Eye Diseases

The eye is one of the most promising organs for therapeutic development. Among other characteristics, it is contained, easily accessible, and immune-privileged [58]. In fact, the first-ever FDA-approved AON (fomiversen) was a first-class oligonucleotide to treat human cytomegalovirus retinitis, an eye condition in immunocompromised patients [33–36]. Furthermore, a growing group of genes and mutations causing retinal diseases have been targeted at preclinical level using AONs. This includes pseudoexon exclusion for *CEP290* [59–63], *OPA1* [64], *CHM* [65] *USH2A* [66], and *ABCA4* [24–29]; splicing modulation for *USH2A* [10] and *CEP290* [12]; or transcript degradation for *NR2E3* [67] and *RHO* [68]. Three of these molecules are currently in different clinical trial phases detailed below.

The most advanced molecule in a clinical setting is sepofarsen (QR-110). This is a 17-mer 2′OMe/PS oligonucleotide aiming to correct the inclusion of a pseudoexon caused by a deep-intronic mutation in *CEP290*-associated autosomal recessive Leber congenital amaurosis [69]. In the phase 1/2 clinical trial (NCT03140969), all patients were injected with an initial loading dose of either 320 or 160 μg followed by a maintenance dose every 3 months (160 or 80 μg) [70, 71]. Interim results showed that sepofarsen was well tolerated and safe with no serious adverse events [70, 71]. Although the final results of the trial have not yet been published, the improvement that most patients showed led to the design and approval of a phase 2/3 clinical trial (Illuminate,

NCT03913143). This is a multi-center, double-masked, randomized, controlled, multiple-dose study to evaluate efficacy, safety, tolerability, and systemic exposure in patients older than 8 years carrying the specific mutation in at least one of the two alleles. Two different doses and a sham-procedure group will be assessed, for a total period of 2 years. In addition, two other clinical trials for the same molecule are ongoing. One is the extension of the phase 1/2 clinical trial to continue treating the patients of the first trial by administering sepofarsen every 3 months in both the already intervened and the contralateral eye (NCT03913130). The second is a multi-center, open-label, dose-escalation, and double-masked randomized controlled trial to evaluate safety and tolerability in children below age of 8 years old (Brighten, NCT04855045).

A multi-center phase 1/2 clinical trial to assess safety and tolerability of QR-421a (Stellar, NCT03780257) is currently ongoing. This 21-mer 2′MOE/PS oligonucleotide aims to skip the frequently mutated exon 13 of *USH2A* [10] causing autosomal recessive Usher syndrome or isolated retinitis pigmentosa. Preliminary results, presented in a press release seem to indicate that QR-421a is well tolerated with no serious adverse events. Furthermore, after treatment with this molecule, improvements in several measures of vision were detected. With these encouraging results, two preliminary phase 2/3 clinical trials have been designed in order to study different patient populations based on the best corrected visual acuity. Both trials will be double-masked, randomized, controlled, 24-month, and multiple-dose study (Sirius and Celeste).

The third molecule in a clinical setting is QR-1123, a gapmer designed to degrade the mutant allele (known as P23H) in the *RHO* gene [68], which has a dominant-negative effect leading to autosomal dominant retinitis pigmentosa. Thus, the hypothesis is that by degrading the allele carrying the mutation, the other allele will be able to produce a functional protein. This molecule is in an early stage of a multi-center open-label, double-masked, randomized, phase 1/2 trial (NCT04123626).

Other molecules for eye-related genetic diseases in late stages of preclinical development are QR-504a for *TCF4*-associated Fuchs endothelial corneal dystrophy and QR-411 for pseudoexon exclusion in *USH2A*-associated Usher syndrome or isolated retinitis pigmentosa.

As well as to target specific mutations, AONs have also been explored for multifactorial eye conditions. This is the case of primary open angle glaucoma, in which *TGF-β2* was targeted with a 14-mer 3 + 3 LNA-modified gapmer in a phase I clinical trial. Results showed that the molecule was tolerated, safe and potentially clinically efficacious [72]. Besides this, other type of antisense molecules (small interference RNA, siRNA) have been clinically tested for glaucoma [73], dry eye syndrome [74], diabetic macular edema [75], and age-related macular degeneration [73, 76, 77].

4 Future of AON Trials and Personalized Medicine: $n = 1$ Trials?

In 2019, an AON molecule (milasen) to treat a single patient pushed the bounds of personalized medicine and raised many regulatory and ethical questions never explored before for genetic treatments [57, 78].

Milasen was customized exclusively for Mila, a child suffering from a form of Batten disease (neuronal ceroid lipofuscinosis 7) caused by the insertion of an SVA (SINE–VNTR–Alu) retrotransposon, with a detrimental effect on splicing, in the intron 6 of the *MFSD8* gene [57]. Using a 22-mer 2′MOE/PS AON it was possible to redirect splicing avoiding the insertion of the SVA in the final mRNA transcript. Besides the exclusivity of this treatment, another extraordinary achievement was that it took only 13 months to go from the clinical diagnosis to the first dosing: Mila had a clinical diagnosis in mid-November of 2016, the genetic defect was identified in May 2017, approval to proceed was received in January 2018 and first patient dosing occur in the same month.

The AON delivery regime via intrathecal bolus injection was highly similar to the one of nusinersen, the AON used for SMA [17, 18, 56]. The treatment did not show any safety concerns and the frequency and duration of the seizures was reduced. Unfortunately, despite the treatment had some effect, Mila passed away early 2021. Nevertheless, this study is the hallmark of personalized medicine, and although not all diseases are amenable for this type of therapies, it has highlighted this as a possible approach and managed to re-evaluate the speed and type of safety studies and regulatory requirements. In a similar development, a drug was designed and provided to patient suffering from amyotrophic lateral sclerosis (ALS) with mutations *FUS* gene, following the same delivery route as nusinersen and milasen. Unfortunately, this patient, Jaci Hermstad, also died recently. However, the drug originally developed for this single patient, ION363 or jacifusen, is currently being tested in a phase III trial for patients with the same disease (NCT04768972). Thus, AON technology can be considered as a platform for individualized treatments which may, sometimes, be extended to other patients.

5 Hurdles

A drawback when compared to small molecule drugs is the relatively large size of AON molecules which limits their delivery into the cells where they exert their action. Therefore, their distribution is limited, their naked uptake is poor, and it is highly determined by the chemistry of their backbones [54]. Often, these AON molecules are not even able to reach their target organ. To circumvent

this, most of these AONs rely on their conjugation or formulation with different delivery systems to be able to reach and access their intracellular targets [32]. In addition, when delivered systemically, these molecules can barely reach the central nervous system due to the blood retina and brain barriers. However, as described before, local delivery of naked modified AONs to these specific organs have shown to be efficient and safe in several clinical trials [17, 18, 56, 70, 71, 79].

Another drawback is the high exposure of certain organs upon systemic delivery of AONs. For instance, after intravenous injection of AONs a significant proportion is taken by the liver and kidney. This limits the biodistribution to other tissues and derivate on toxic effects in these organs. However, many of the liver and kidney injuries were found when using high and not clinically relevant doses of AONs [32]. In that sense, novel delivery methods or conjugates are required to be able to target the organs of interest and bypass the high clearance by the liver and kidneys.

Finding proper models to assess the sequence-dependent efficacy and safety of AONs is still a pending issue. Their safety assessment is often performed in rodents, non-human primates, and human plasma. However, these studies only provide sequence- and chemical modification-specific effects. The generation of humanized models have provided very good results, however, generating a humanized animal model for every mutation to be targeted is not feasible nor ethical. It is also possible to generate almost any human cell from patient-derived cells reprogrammed to a pluripotent stage. While these models can provide good readouts at RNA, protein, or even functional levels the entire context will still be missing. Currently, significant efforts are being made in the generation of organ-on-chips. This technology allows the combination of multiple tissues or even organs to study the interaction between them and test therapeutic interventions [80, 81]. In addition, this technology enables other type of measurements that in the near future might be very valuable to perform drug screenings and evaluate the efficacy and safety of many molecules, including AONs [80–83].

Finally, clear guidelines and novel clinical trial designs are needed to explore the full therapeutic potential of AONs when investigated as treatments for rare diseases. The case of milasen has proven that this is possible and new of such trials are being planned.

6 Conclusions

The therapeutic potential of AONs has been, for many years, subject of speculation and theoretical discussion and, while these molecules were widely applied in a research laboratory setting, their

clinical application was anecdotal and limited to rare diseases. However, this landscape has recently changed completely thanks to several factors. On one hand, many of such drugs have been approved, being splice-switching AON and siRNA drugs at the forefront of this wave. Secondly, several breakthroughs in the delivery formulation of these drugs have increased the uptake of AONs targeting the liver and this has open wide open the field to consider these as reliable treatment options for several disorders where the liver is the target tissue. Thirdly, much more attention has been given to antisense technology due to the *n*-of-1 case of milasen. Lastly, RNA-therapies have gained extraordinary popularity due to vaccines against SAR-CoV-2 based on mRNA technology, highlighting the development of drugs based on nucleic acids. All of this will contribute to make these drugs a main resource in the therapeutic toolbox of the twenty-first century.

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