REVIEW

# Splicing modulation therapy in the treatment of genetic diseases

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**Abstract:** Antisense-mediated splicing modulation is a tool that can be exploited in several ways to provide a potential therapy for rare genetic diseases. This approach is currently being tested in clinical trials for Duchenne muscular dystrophy and spinal muscular atrophy. The present review outlines the versatility of the approach to correct cryptic splicing, modulate alternative splicing, restore the open reading frame, and induce protein knockdown, providing examples of each. Finally, we outline a possible path forward toward the clinical application of this approach for a wide variety of inherited rare diseases.

**Keywords:** splicing, therapy, antisense oligonucleotides, cryptic splicing, alternative splicing

## Introduction

Genetic diseases are generally rare diseases caused by mutations in specific genes. Sometimes mutations in different genes can give rise to similar phenotypes, eg, there have been dozens of genes identified in patients suffering from muscular dystrophies.[1] However, mutations in a single gene can also give rise to multiple diseases with varying phenotypes. Probably the most notorious example is the *LMNA* gene, in which mutations are associated with multiple phenotypes, including Emery–Dreifuss muscular dystrophy, familial partial lipodystrophy, limb girdle muscular dystrophy, dilated cardiomyopathy, Charcot–Marie–Tooth disease, restrictive dermopathy, and Hutchinson–Gilford progeria syndrome (HGPS).[2] For a significant number of patients with, or suspected of having a genetic disease, the mutation in the causative gene has not yet been identified. However, with the rapid advances made by "next generation sequencing", it is anticipated that mutations will soon be identified for almost all patients with genetic disorders; the International Rare Disease Research Consortium (IRDiRC) has set itself an ambitious goal of having the means available to diagnose most rare genetic diseases by 2020 (http://www.irdirc.org).

With the availability of next generation sequencing, it is now possible to perform a more in-depth analysis of candidate genes. In the past, generally only exons and the donor and acceptor splice sites (ie, the first and last two base pairs of an intron, respectively) were analyzed. However, it is now feasible to analyze complete introns and there are multiple publications that report deep intronic mutations that activate cryptic splice sites and thus disrupt normal transcript processing.[3] In parallel, antisense-mediated splicing modulation has been developed from preclinical cell and animal models into the clinical trial phase for Duchenne muscular dystrophy (DMD) and spinal muscular atrophy (SMA).[4–8] This approach makes use of antisense oligonucleotides (ASOs, small

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pieces of chemically modified DNA or RNA) and would offer a potential treatment for genetic disorders caused by mutations that disrupt splicing, but can also be exploited in other ways to address genetic mutations.[9] ASOs need to be chemically modified to improve stability to nucleases and provide favorable pharmacokinetic and pharmacodynamic characteristics for in vivo use. The most commonly used ASO chemistries are the 2′-O-methyl RNA phosphorothioate chemistry, which is negatively charged, and the charge neutral phosphorodiamidate morpholino oligomer. For more background on ASO chemistry, we refer the reader to a recent book chapter.[10] This review will describe different ways antisense-mediated splicing modulation can be used to treat genetic diseases. Since there are already numerous reviews describing exon skipping for DMD and SMA,[11–14] we have chosen to discuss these neuromuscular applications only briefly and to focus on the potential of the exon skipping approach for other genetic diseases. For each approach we have chosen a few illustrative examples, focusing on those where data from animal models is available.

## Cryptic splicing

Cryptic splice sites are sequences in the pre-messenger RNA (pre-mRNA) that are not normally used as splice sites, but which are activated by mutations that either inactivate the canonical splice sites or create splice sites where one did not exist before.[15] The classical example of this phenomenon is in the β-globin gene, where mutations in the canonical splice sites cause activation of cryptic splice sites, leading to the inclusion of aberrant exons, resulting in nonfunctional transcripts, defective β-globin expression, and consequently β-thalassemia.[16] For the majority of patients, the disease is caused by mutations that activate cryptic splice sites.[17] In the early 1990s, ASOs were developed by Dominski and Kole using this paradigm (Figure 1A): by targeting the cryptic splice sites with 2′-O-methyl RNA ASOs to block the access of the spliceosome to these sites, their group was able to restore normal β-globin splicing in cells[18] and normal hemoglobin expression, to some extent, in a thalassemia mouse model.[19] The restoration of normal splicing by suppressing cryptic splicing, therefore, represents the very earliest manifestation of ASOs being used to modulate splicing for therapy. Even despite this early progress, clinical applications of this technology for the treatment of β-thalassemia have not materialized, likely as a result of the need to generate, validate, and trial specific ASOs for each type of mutation in patients, and the fact that ASO therapy only leads to a transient correction of splicing. As a result,

most gene therapy research in the field has concentrated on viral-mediated transduction of β-globin into hematopoietic stem cells as a means of achieving durable and long-term correction of β-globin expression, with one patient achieving independence from transfusions, the usual treatment for β-thalassemia.[20]

Even though in β-thalassemia, ASO-mediated correction is being side-lined in favor of other approaches, for other diseases targets that could be amenable to ASO-mediated cryptic splice site suppression continue to be identified. A recent example is Usher syndrome type 1C, characterized by congenital deafness, retinitis pigmentosa, and vestibular dysfunction (Figure 1B). This is caused by defects in the *USH1C* gene that encodes harmonin. Harmonin is an actin-binding scaffold protein that, together with myosin VIIa and cadherin 23, are required to ensure that the stereocilia in the inner ear cohere together.[21,22] The USH1C216G>A mutation, found in all Louisiana Acadian cases of Usher syndrome type 1C, creates a cryptic 5′ splice site within exon 3 of the gene, which is used preferentially over the canonical 5′ splice site, and the creation of a frameshift and premature truncation of the harmonin protein.[23] In a collaborative project, Lentz et al were able to show that an ASO targeted to the cryptic 5′ splice site was able to correct the splicing of *USH1C* transcript, restore harmonin expression, and rescue cochlear hair cells, vestibular function, and hearing for at least 6 months when given to an animal model of USH1C216G>A.[24]

## Alternative splicing

In humans, a myriad of proteins is responsible for most of the functions that form the basis of life. However, this array of different proteins is coded by a much (3–4-fold) smaller number of genes. This puzzled the field at first,[25] but can be explained by the occurrence of alternative splicing, a mechanism that allows the production of several proteins from the same gene by means of the exclusion, inclusion, or trimming of specific exons. In fact, 95% of human pre-mRNA is alternatively spliced.[26] Alternative splicing is responsible for generating proteins with very different, sometimes even antagonistic roles. Alternative splicing can also introduce stop codons that cause nonsense-mediated decay, and therefore, suppress the expression of the gene.[27] In healthy individuals, this flexibility contributes to the clockwork functioning of human biology. However, aberrant alternative splicing can also be the origin of disease, eg, a shift from an antiproliferative isoform to a proproliferative isoform can underlie tumor formation. From the therapeutic viewpoint, this provides researchers
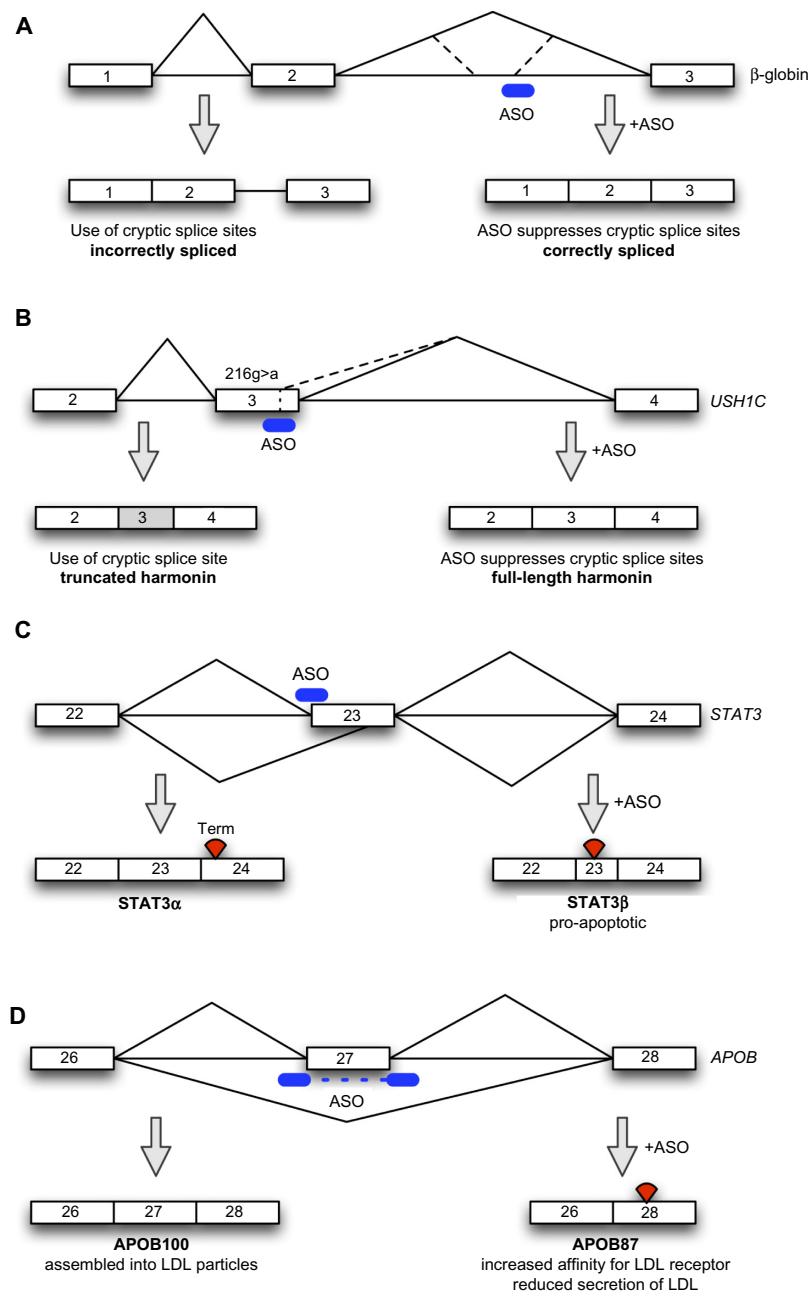
**Figure 1** Schematic depiction of antisense-mediated splicing modulation approaches.
**Notes:** (**A**) Restoration of cryptic splicing for β-globin mutations. Due to a mutation in intron 3, a cryptic exon is included into the β-globin mRNA, abrogating the production of β-globin proteins. ASOs targeting the cryptic splice sites prevent the inclusion of the cryptic exon, restoring normal splicing and production of β-globin protein. (**B**) Restoration of cryptic splicing for USH1C. Due to a mutation in exon 3, a cryptic splice site within exon 3 is activated, leading to the exclusion of the last part of the exon from the USCH1C transcript and loss of harmonin protein production. Using ASOs targeting the cryptic splice site in exon 3 reactivates the original splice site and restores normal splicing and protein production. (**C**) Shifting of alternative splicing. The *STAT3* gene produces STAT3α and STAT3β proteins based on the use of a proximal or more distal acceptor splice site in exon 23 during pre-mRNA splicing. ASOs targeting the proximal acceptor splice site lead to a shift into the production of the proapoptotic STAT3β protein. (**D**) Isoform-specific knockdown. The *APOB* gene gives rise to APOB100 and APOB48 proteins. The latter derives from RNA editing in exon 26. Skipping of exon 27 gives rise to a prematurely truncated APOB protein with cholesterol-lowering properties, without affecting the APOB48 isoform.
**Abbreviations:** APOB, apolipoprotein B100; ASOs, antisense oligonucleotides; LDL, low-density lipoprotein; mRNA, messenger RNA; STAT3, signal transducer and activator of transcription 3; USH1C, *Usher syndrome type 1C* gene.

the opportunity to use ASOs as tools to knowingly shift alternative splicing to treat disease.

A first example involves lamin A, a nuclear envelope protein involved in the regulation of gene expression, nuclear stability, and chromatin structure that is encoded by the *LMNA* gene. A point mutation in this gene leads to a shift in alternative splicing,[28] and increased production of progerin, a truncated form of lamin A that is present in small amounts in normal aging, but which accumulates in high levels in HGPS. This leads to accelerated aging and shortened life

span due to growth impairment, lipodystrophy, dermal and bone abnormalities, and cardiovascular alterations.[29] This shift in the standard splicing of the *LMNA* gene favoring the truncated progerin protein represents an ideal example of how the usually firmly kept balance can be disturbed by the presence of this cryptic splice site: the new protein is only 50 amino acids shorter than the original one, but lacks a vital target sequence for protein cleavage,[30] and this in turn leads to the deleterious effects experienced by HGPS patients. A phosphorodiamidate morpholino oligomer ASO masking the progerin splice site has been successfully used in fibroblast cultures from HGPS patients to restore normal splicing and correct nuclear abnormalities and mislocalization of other nuclear envelope proteins.[31] Following these advances, in a recent study on a murine model that recapitulates the main clinical signs of HGPS, vivo-morpholino ASOs were able to reverse many of the phenotypical alterations seen in these mice.[32]

Signal transducer and activator of transcription 3 (STAT3) is involved in the activation of several oncogenic pathways. It is usually spliced to the isoform STAT3α, but an alternative acceptor site in exon 23 leads to the expression of isoform STAT3β, which lacks the C-terminal transactivation domain. Overexpression of STATβ can induce apoptosis and inhibit tumor growth. Zammarchi et al developed an ASO targeting a splicing enhancer site that was able to shift the balance of STAT3 expression to favor STAT3β expression (Figure 1C).[33] This approach has been tested in cell and animal models and has served a double purpose: on one hand it allowed a detailed study of the precise functions of STAT3β in its physiological environment. On the other hand, ASOs that shift splicing to the STAT3β isoform have antitumor applications.

## Knockdown

Exon-skipping ASOs can also be used to knockdown the function of an undesired gene, by inducing exon skipping and creating mRNA isoforms that encode nonfunctional proteins or trigger nonsense-mediated decay of the mRNA,[34] to inhibit the expression of the undesired gene; or encode alternative isoforms with desired physiological or therapeutic functions that could modify or antagonize the effect of an undesired gene. The use of exon-skipping ASOs for the first purpose is not generally widespread except in the special case of zebrafish genetics.[35] Other methods, such as RNA interference,[36] ASOs that trigger RNase H-mediated degradation of mRNAs,[37] or translation inhibition,[38] are more generally used if simple knockdown is desired. An example

of this is the knockdown of apolipoprotein B100 (APOB100) as a means of reducing low-density lipoprotein (LDL) secretion and cholesterol levels in familial hypercholesterolemia.[39] Both RNase H "gap-mer" ASOs and RNA interference have been used for this purpose, reducing LDL cholesterol in vivo.[40,41] These simple knockdown approaches, however, have the undesired effect of knocking down the APOB48 isoform, and therefore reduce chylomicron secretion.[42] This will interfere with fat transport from the intestine and malabsorption of fat-soluble vitamins such as vitamin K.[43]

Exon-skipping ASOs offer a distinct advantage over simple knockdown, as engineered alternative isoforms may possess their own therapeutic properties, inhibit the function of a normal isoform, and/or avoid side effects of knockdown of the normal isoform. Capitalizing on this advantage, exon-skipping exon 27 of the *APOB* gene using ASOs causes a reduction of LDL particle secretion and an increase in LDL affinity for the LDL receptor (Figure 1D).[44] These two effects combine to cause a powerful reduction of LDL cholesterol when the "APO-skip" ASOs are introduced into hypercholesterolemic mice transgenic for human *APOB*, validating the therapeutic concept.[45] Importantly, exon 27 skipping does not affect APOB48 expression, as the RNA editing necessary for APOB48 expression occurs in exon 26.[45] In this example, therefore, exon-skipping technology enables the reengineering of gene expression to produce a desired outcome with clear therapeutic advantages over other approaches to the same problem.

## Reading frame correction

The coding sequence for proteins is dispersed over exons, and the location where introns interrupt the code can be in three phases in relation to the coding sequence: either between complete codons (phase 0 introns), interrupting after the first nucleotide of the codon (phase 1 introns), or interrupting after the second nucleotide of the codon (phase 2 introns). As this will apply to both the beginning and the end of each exon, each exon will have two jigsaw-like ends that will require a complementary exon before and after to maintain the open reading frame (ORF).[46] Therefore, a symmetrical exon can be deleted without interrupting the ORF, but others, if deleted, would cause a disruption in the ORF (Figure 2). For most genes, the deletion of an exon would result in a nonfunctional protein regardless of whether the ORF is disrupted by the deletion, since the majority of proteins do not have redundant domains. However, there are some exceptions where deletions that maintain the ORF allow the production of internally deleted but partially functional proteins. This concept is best
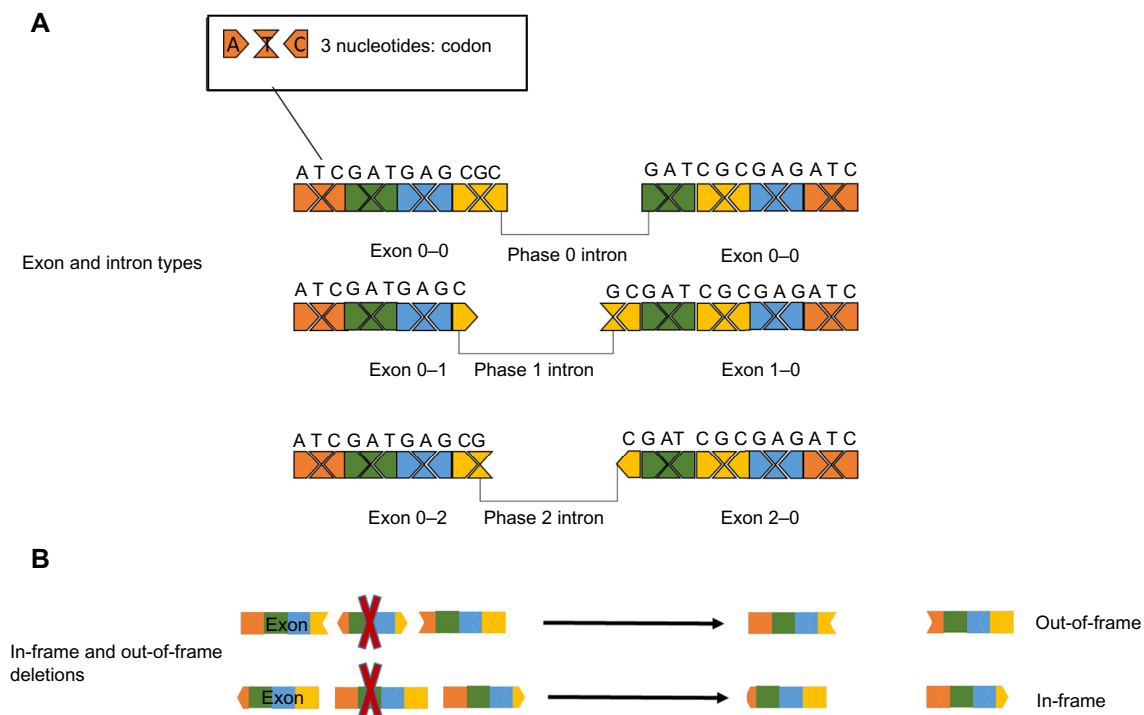
**A**



**B**

**Figure 2** Effect of exon deletions on the open reading frame.

**Notes:** (**A**) Different ways in which codons in exons may be split by introns: in phase 0 introns, the codon ends at the end of the exon, while in phase 1 and phase 2 introns the codon is split over the 2 exons, with the interruption after the first or the second nucleotide of the codon, respectively. (**B**) As each exon is separated from the next and previous one by the flanking introns, there are nine different types of exons due to the different combinations (0–0, 0–1, 0–2, 1–0, 1–1, 1–2, 2–0, 2–1, 2–2); if one of these exons is deleted, the open reading frame could be either disrupted or maintained, depending on these combinations: the deletion of "symmetrical" exons (0–0, 1–1, 2–2) would have no effect on the reading frame (in-frame), while others will disrupt it (out-of-frame).

illustrated in the neuromuscular disorder DMD, an early onset, severely progressive muscle-wasting disorder.[47] In the majority of DMD patients, deletions or duplications of one or more exons cause a disruption of the ORF and the deficit of its encoded protein, dystrophin.[48] In a stark contrast to DMD, another disorder in which one or more exons are also deleted from the *DMD* gene, Becker muscular dystrophy, has a much milder progression. The difference is that in Becker patients, the deletions do not disrupt the ORF and a shorter, but partially functional dystrophin protein is produced.[48] The existence of Becker muscular dystrophy is the conceptual basis for the model of exon skipping: ASOs are used to "mask" specific exons to the splicing machinery to restore the ORF and partial dystrophin function for DMD patients.

From early proof of concept experiments to the latest clinical trials, the developments in the use of ASOs as a potential therapeutic agent in DMD have been fast: restoration of dystrophin has been achieved following intramuscular[49,50] and systemic[4,5,51] injections of ASOs targeting *DMD* exon 51, and these and several other ASOs targeting other exons are progressing through clinical trials.[52]

SMA, the most common genetic cause of death in infants under the age of 2 years, is caused by deletions and

mutations of *SMN1*, and consequently low levels of SMN protein, a ubiquitously expressed protein involved in RNA processing.[53] Although a duplicate of *SMN1*, *SMN2* also exists within the genome and also encodes SMN; it is usually inefficiently spliced due to minor differences in and around exon 7. As a result, only 20% of *SMN2* mRNA transcripts include exon 7, which is necessary for the stability of SMN protein.[54] In healthy individuals, this is irrelevant, but in SMA patients, increasing the amount of exon 7 inclusion from *SMN2* transcripts could allow the generation of sufficient amounts of SMN protein to ameliorate their disease. In fact, several studies using ASOs have already demonstrated in vitro and in vivo that this is possible,[6,55–58] and clinical trials are underway.

## Toward clinical application

From the above, it is clear that antisense-mediated splicing modulation may offer a potential treatment for rare inherited diseases, and as such, can be seen as a platform approach. For DMD and SMA, the approach has made it to the clinical trial phase, whereas other genetic diseases are still in preclinical development. While each of these diseases will have their own quirks and challenges (eg, delivery to the tar-

get tissue and dosing regimens will vary between diseases), there will be common challenges and hurdles as well, since they all use the exon-skipping platform. Furthermore, there is no reason why people embarking on developing exon skipping for their disease of choice should have to repeat mistakes others have made in the past. Therefore, networking between scientists involved in this endeavor is warranted. The European Cooperation of Science and Technology (COST) provides funding for networking to overcome challenges such as these, and has funded the Action "Networking towards clinical application of anti-sense-mediated exon skipping for rare diseases" (BM1207, see http://www.cost.eu and http://www.exonskipping.eu).[59] Within this Action, regular workshops are organized to discuss common challenges, such as delivery to the target tissue, ASO chemistry and safety, and to align preclinical work. Furthermore, the Action focuses on the development of new regulatory models and has recently had a workshop on translational and regulatory challenges with representatives from patient organizations, academia, and regulatory agencies,[60] following a larger workshop organized by the Translational Research in Europe – Assessment and Treatment of Neuromuscular Diseases (TREAT-NMD) network and hosted by the European Medicines Agency that focused on the particulars of exon skipping for DMD.[61]

One of the key factors for clinical development will be the harmonization of outcome measures. While the regulators will provide market approval only when clinically relevant benefit has been convincingly shown, biochemical outcome measures can provide additional information, eg, by confirming mechanism of action of ASOs. Lessons could be learned from the case of DMD: when the first clinical trials using ASOs were outlined, methods to measure dystrophin restoration relied heavily on standard clinical diagnostic techniques and academic protocols, and each research center used their own version of the methods.[4,5,49,51,52] The most commonly used method was immunofluorescent staining, but the large size and low expression level of the protein made accurate quantification technically challenging, especially when the amount of restored dystrophin is relatively small and it needs to be distinguished from preexisting dystrophin-positive revertant fibers and residual trace dystrophin expression.[52,62] To overcome some of those difficulties, refinements developed, in particular using better software analysis.[63,64] A group of laboratories from academia and industry have embarked on an effort to provide a data-driven reproducible methodology for dystrophin quantification that could be used in future trials. This group has revised methodologies,

validated them among their laboratories, and proposed a standard operating procedure.[52,65]

## Conclusion

ASO-mediated splicing modulation holds potential for treating rare genetic diseases as outlined in this review, and as corroborated by the advances made for DMD and SMA. International networks on exon skipping aim to help scientists developing this approach for other diseases benefit from the experience gained from the DMD field, to jointly overcome challenges and further this approach toward clinical application for as many rare genetic diseases as possible.

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## Disclosure

Dr Khoo discloses being co-inventor on UK Patent GB1117880.3. Dr Aartsma-Rus discloses being employed by the Leiden University Medical Center (LUMC) which has patents on exon skipping. Dr Aartsma-Rus is co-inventor on some of these patents and as such is entitled to a share of potential royalties. Dr Arechavala-Gomeza has nothing to declare.

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