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Evaluation of 2'-deoxy-2'-fluoro antisense oligonucleotides for exon skipping in Duchenne muscular dystrophy

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Abstract

Duchenne muscular dystrophy (DMD) is a severe muscle wasting disorder typically caused by frame-shifting mutations in the DMD gene. Restoration of the reading frame would allow the production of a shorter but partly functional dystrophin protein as seen in Becker muscular dystrophy patients. This can be achieved with antisense oligonucleotides (AONs) that induce skipping of specific exons during pre-mRNA splicing. Different chemical modifications have been developed to improve AON properties. The 2'-deoxy-2'-fluoro (2F) RNA modification is attractive for exon skipping due to its ability to recruit ILF2/3 proteins to the 2F/pre-mRNA duplex, which resulted in enhanced exon skipping in spinal muscular atrophy models. In this study, we examined the effect of two different 2'-substituted AONs (2'-F phosphorothioate (2FPS) and 2'-O-Me phosphorothioate (2OMePS)) on exon skipping in DMD cell and animal models. In human cell cultures, 2FPS AONs showed higher exon skipping levels than their isosequential 2OMePS counterparts. Interestingly, in the mdx mouse model 2FPS was less efficient than 2OMePS and suggested safety issues as evidenced by increased spleen size and weight loss. Our results do not support a clinical application for 2FPS AON.

1. Introduction

Duchenne muscular dystrophy (DMD) is a severe X-linked muscle wasting disorder affecting 1 in 5,000 newborn boys. DMD is caused by out-of-frame or nonsense mutations in the DMD gene that lead to a truncated, non-functional dystrophin protein. Dystrophin is an important shock absorbing protein in muscle and without it, muscles are easily damaged. Restoration of the reading frame in DMD patients would in theory allow the production of a shorter, but partly functional dystrophin protein as seen in less severely affected Becker muscular dystrophy (BMD) patients. This can be achieved with antisense oligonucleotides (AONs) that target and induce skipping of specific exons during pre-mRNA splicing. Exon skipping AONs are thought to act by sterically hindering splicing factors in the recognition of the exon and/or splicing sites.

Over the years chemical modifications have been developed to improve AON characteristics, such as improved binding affinity to the target transcript, increased resistance against nuclease degradation and improved cellular uptake. Two different AON chemistries, phosphorodiamidate morpholino oligonucleotides and 2'-O-methyl phosphorothioate (2OMePS), are currently in clinical development for exon skipping in DMD.

The 2'-deoxy-2'-fluoro (2F) chemistry may also be an attractive chemistry for exon skipping AONs. Recently it has been shown that the duplex of 2F AON and its target pre-mRNA attracts interleukin enhancer binding factors 2 and 3 (ILF2/3 proteins) resulting in unanticipated exon skipping in a model of spinal muscular atrophy (SMA). Probably this is based on enhanced steric hindrance by the duplex/protein complex, which impedes binding of splicing factors to splice sites or exonic regions in the pre-mRNA transcript beyond the AON target sequence. As enhanced exon skipping is relevant for DMD therapeutics, we compared the efficiencies of isosequential 2'-F phosphorothioate (2FPS) and 2OMePS AONs targeting exonic regions within different human dystrophin exons, or in the 5' donor splice site of mouse dystrophin exon 23. In in vitro transfection experiments, 2FPS AONs outperformed their 2OMePS counterparts, while in vivo they appeared less effective.

2. Material and Methods

AONs

Sequences of 2FPS and 2OMePS AONs are provided in Supplementary Table 1. 2FPS AONs contained 2'-deoxy-2'-fluoro RNA with a phosphorothioate backbone (ChemGenes corporation, Wilmington, MA USA). 2OMePS AONs consisted of 2'-O-methyl RNA with a phosphorothioate backbone (Prosensa Therapeutics/BioMarin, Leiden, the Netherlands).

Cell culture

Human myoblasts

Primary human control myoblasts and patient myoblasts with a deletion of exon 45-52 were a kind gift from Vincent Mouly. Cells were grown in skeletal muscle cell growth medium (Promocell, C-23160) supplemented with an additional 15% fetal bovine serum (FBS) (Gibco-BRL, the Netherlands) and 50 µg/ml gentamicin (PAA Laboratories) in uncoated flasks until 70-80% confluence was reached. Cells were plated in a 6 wells plate coated with 0.5% gelatin (Sigma Aldrich Chemie B.V., the Netherlands), at a density of 10^4 cells per well, 48 hours prior to differentiation. Reaching 90% confluence, medium was switched to differentiation medium (Dulbecco’s medium, Dulbecco’s medium (without phenol red) supplemented with 2% FBS, 50µg/ml gentamicin, 2% glutamax and 1% glucose (all from Gibco-BRL, the Netherlands)) 2-4 days. Cells were allowed to differentiate for 4-5 days.

Mouse myoblasts

Mouse myoblasts were grown in Dulbecco’s medium (without phenol red) supplemented with 10% FBS, 1% Penicillin/Streptomycin (P/S), 2% Glutamax and 1% glucose (all from Gibco-BRL, the Netherlands) in collagen coated flasks. Cells were seeded in collagen coated 6 wells plates with proliferation medium and grown until confluence. The cells were washed twice with Hank’s Balanced Salt Solution (HBSS) and differentiation medium, Dulbecco’s medium (without phenol red) supplemented with 2%
horse serum (HS), 1% P/S, 2% glutamax and 1% glucose (all from Gibco-BRL, the Netherlands) was added to induce differentiation. The cells were differentiated for 7-9 days before AON transfection.

**Primary myoblasts from mdx mice**

Primary myoblasts were isolated from the extensor digitorum longus (EDL) muscle of one mdx mouse by collagenase treatment followed by single fiber isolation. Fibers were grown in SC+ medium (Dulbecco’s medium (without phenol red) supplemented with 10% (HS), 30% fetal calf serum, 1% chicken embryonic extract (all from Gibco-BRL, the Netherlands) and 10µl/30ml final medium of fibroblast growth factor (Promega) on matrigel (GFR Matrigel BD Biosciences) coated plates. After 3 days myoblasts were separated from fibroblasts by pre-plating in proliferation medium (Dulbecco’s medium (without phenol red) supplemented with 10% FBS, 1% P/S, 2% glutamax and 1% glucose (all from Gibco-BRL, the Netherlands). Finally primary myoblast cells were plated in 12 wells plates at 70-80% confluence, the following day the cells were washed with PBS and differentiation medium (Dulbecco’s medium (without phenol red) supplemented with 2% HS, 1% P/S, 2% glutamax and 1% glucose (all from Gibco-BRL, the Netherlands)) was added.

**In vitro delivery**

**AON transfection of myotube cultures**

Primary human control, patients myotubes and mouse myotubes were transfected with either 100, 200 or 500 nM of AONs using 6 µl of Lipofectamin 2000 (according to manufacturer’s protocol) per well. After incubation for 3-4 hours at 37 ℃ and 5% CO₂, cells were washed twice with PBS and 2 ml of fresh differentiation medium was added. Forty-eight hours later, RNA was isolated.

**Gymnotic delivery**

Primary myoblasts from mdx mice were incubated with 2 or 4 µM of 23M or 23F AON for 96 hours at initiation of differentiation.

**In vivo delivery**

**Intramuscular injection of mdx mice**

Two mdx mice were IM injected in the gastrocnemius and triceps muscles with cardiotoxin 2 days prior to injection with 2.9 nmol of 23AON or 2FPS23AON contralateral for 2 consecutive days. One week after the last injection the mice were sacrificed, quadriceps (non-injected control), gastrocnemius and triceps muscles were isolated.

**Systemic treatment in mdx mice**

Five weeks old mdx mice (4-5 mice per group) were subcutaneously injected 4 times per week, with 50 mg/kg of 23M AON in 100 µl of saline or the molar equivalent (6.8 µmol) for 23F AON. Mdx mice were treated for 8 weeks and sacrificed 1 week after the last injection. All mice were weighed prior to injection and at the day of sacrifice. Blood samples were obtained from the tail vein for plasma pharmacokinetics analysis (PK) at the day of sacrifice. Gastrocnemius, quadriceps, tibialis anterior, triceps and diaphragm muscles, heart, liver and kidney were isolated to determine exon skipping levels and AON concentrations. The spleen was isolated and weighed.

**RNA isolation**

**RNA isolation and cDNA synthesis of myotube cultures**

Human and mouse cells were washed twice with PBS. RNA was isolated by adding 500 µl TriPure (Roche diagnostics, the Netherlands) to each well to lyse the cells. This was followed by chloroform extraction in a 1:5 ratio on ice for 5 minutes. The remaining cell debris was spun down by centrifugation (4°C, 15 minutes, 15,400 rcf) and the upper aqueous phase precipitated for 30 minutes on ice with equal volume of isopropanol. The RNA/isopropanol precipitate was centrifuged (4°C, 15 minutes, 15,400 rcf) and the pellet washed with 70% ethanol. The final RNA pellet was dissolved in 15 µl of RNase/ DNase free water. For complementary DNA (cDNA) synthesis, 11 µl of RNA was used in a 20 µl reaction with random hexamers and transcriptor reverse transcriptase (Roche Diagnostics, the Netherlands) for 30 minutes at 55°C and 5 minutes at 85°C to terminate the reaction according the manufacturer’s instructions.

**RNA isolation and cDNA synthesis of tissue**

Samples were homogenized in TriPure (Roche diagnostics, the Netherlands) solution using a MagNA Lyser (Roche Diagnostics, the Netherlands) and MagNA Lyser green beads (Roche Diagnostics, the Netherlands). Total RNA was isolated and purified according manufacturer’s instructions. For the cDNA synthesis, 400 ng of RNA was used in a 20 µl reaction with random hexamers and transcriptor reverse transcriptase (Roche Diagnostics, the Netherlands) for 45 minutes at 42°C and put on ice.
In vitro exon skip evaluation

Exon skipping was determined by nested RT-PCR. For RT-PCR analysis 3 µl of cDNA was incubated with 0.625 U AmpliTaq polymerase (Roche Diagnostics, the Netherlands), 10 pM of primers (in exon 43 and 48 for exon 45 skipping, for exon 53 skipping in exon 56 and 43 (patient) or exon 50 (control), and mouse exon 21 and 26 for mouse exon 23 skipping) 5 pmol of dNTPs and 1 times Supertaq PCR buffer (Sphaero-q, the Netherlands) and amplified for 20 cycles each consisting of 40 seconds at 94 °C, 40 seconds at 60 °C and 80 seconds at 72 °C. This PCR was followed by a nested PCR. For the nested PCR analysis 1.5 µl of the first PCR product was incubated with 1.25 U AmpliTaq polymerase (Roche Diagnostics, the Netherlands), 20 pmol of primers (human exon 44 and 46 for evaluating exon 45 skipping, human exon 55 and 44 (patient) or 51 (control for evaluating exon 53 skipping, and mouse exon 22 and 24 for exon 23 skipping) 10 pmol of dNTPs and 1 times Supertaq PCR buffer and amplified for 32 cycles each consisting of 40 seconds at 94 °C, 40 seconds at 60 °C and 60 seconds at 72 °C. PCR fragments were analyzed using 1.5 % agarose gel electrophoresis. Exon skip levels were semi-quantitatively determined as the percentages of the total (wild type and skipped) product with the Calipur LabChip GX (PerkinElmer, the Netherlands).

In vivo exon skip evaluation

Exon skip evaluation of intramuscular injected mice

Exon skipping was determined by nested RT-PCR and visualized on an agarose gel as described for the myotubes cultures detecting exon 45 skipping (hDMD) or exon 23 skipping (mdx mice).

Exon skip evaluation of systemically treated mdx mice

Exon skipping was determined by single RT-PCR. For RT-PCR analysis 1.5 µl of cDNA was incubated with 1.25 U taq polymerase (Roche Diagnostics, the Netherlands), 20 pmol of primers (reverse primer in exon 24, forward primer in exon 22) 10 pmol of dNTPs and 1 times Supertaq PCR buffer (Sphaero-q, the Netherlands) and amplified for 32 cycles each consisting of 30 seconds at 94 °C, 30 seconds at 60 °C and 30 seconds at 72 °C. PCR fragments were analysed by 2% agarose gel electrophoresis.

In vivo safety and quantification of AON levels

Plasma parameters

Blood was collected in lithium-heparin coated microvettces CB300 (Sarstedt B.V. the Netherlands). Glutamate pyruvate transaminase (GPT), alkaline phosphates (ALP) glutamic oxaloacetic transaminase (GOT) hemoglobin (HB), urea and creatine kinase (CK) were determined using Reflotron strips (Roche Diagnostics, the Netherlands) in the Reflotron Plus machine (Roche Diagnostics, the Netherlands).

Quantification of AON levels in tissue of mdx mice

For measuring the concentration of AONs in tissue samples a hybridization-ligation assay based on one previously published was used 24. Tissues were homogenized in 100 mM Tris-HCl pH 8.5, 200 mM NaCl, 0.2% SDS, 5 mM EDTA and 2 mg/ml protease K using zirconium beads (1.4 mm; OPS Diagnostics, Lebanon, NJ) in a MagNA Lyser (Roche Diagnostics, the Netherlands). Samples were diluted 600 and 6000 times (muscle) or 6000 and 60000 (liver and kidney) in pooled control mdx tissue in PBS.

Calibration curves of the analysed exon 23AONs prepared in 60 times pooled control mdx tissue in PBS were included. All analyses were performed in duplicate.

Statistical analyses

A Student's T-Test was used to determine significant differences in exon skipping levels, AON levels, plasma protein levels and spleen/bodyweight ratios. Mixed model linear regression analysis was used to determine significant differences in bodyweight over time. Results were deemed significantly different when $P <0.05$. 
Chapter 6

3. Results

In vitro evaluation

To test whether 2FPS AONs are capable of inducing dystrophin exon skipping, human control myotube cultures were transfected with 100-500 nM of several 2FPS AONs and their isosequential 2OMePS counterparts. These AONs have different activity profiles and target exon 45 or exon 53 (Supplementary table 1, fig. 1). RNA was isolated after 48 hours and exon skipping levels were determined semi-quantitatively by lab-on-a-chip analysis after nested RT-PCR amplification. We observed highest exon 45 skipping levels for each of the 2FPS AONs, with 3 out of 4 of the 2FPS AONs having exon skipping levels over 90% at all concentrations tested (fig. 1A). For exon 53 skipping, although percentages were more variable, all 2FPS AONs induced relatively higher exon skipping levels than their 2OMePS AON counterparts (fig. 1B). This effect was also confirmed in DMD patient-derived Δ45-52 myotube cultures, in which skipping of exon 53 is frame-restoring and potentially therapeutic (fig. 1C).

We also evaluated the potential of 2FPS AONs targeting mouse dystrophin exon 23 in mouse control myotube cultures. Upon the use of a transfection reagent we observed a slight increase in exon 23 skipping with 2FPS AON (23F) compared to the 2OMePS AON (23M) at 500 nM. However, no differences between 23F and 23M were observed at 200nM (fig. 1D). Finally, we also tested the activity of 2FPS AON in primary myoblasts derived from extensor digitorum longus (EDL) muscles of an mdx mouse, a mouse model for DMD. In this case, we did not use a transfection reagent (‘gymnotic delivery’). Primary myoblasts were incubated with 2 or 4 µM of 23M or 23F AON at initiation of differentiation into myotubes. After 96 hours RNA was isolated and analyzed by nested RT-PCR. Exon 23 skipping was confirmed for both AONs at comparable levels (fig. 1E).

In vivo evaluation

Two mdx mice were intramuscularly (IM) injected with 2.9 nmol of 23M or 23F AON for 2 consecutive days in gastrocnemius and triceps muscles. One week after the last injection the mice were sacrificed and the injected muscles harvested for RNA isolation. Exon skipping was determined by nested RT-PCR and visualized on an agarose gel. Surprisingly, again no exon skipping could be detected in 23F AON treated mice in any of the muscles analyzed, while exon skipping was detectable in the case of 23M AON treated mice for each muscle analyzed (fig. 2A). As anticipated, dystrophin restoration was observed by Western blot for 23M AON treated mice, but not for 23F AON or saline treated mice (Supplementary figure 2).

Assessment of the AON concentrations in different organs revealed lower levels of 23F AON in skeletal and cardiac muscle, liver and kidney compared to 23M AON treated animals (fig. 2C,D). The calculated target tissue muscle/kidney and muscle/liver ratios were higher for 23F AON suggesting that while uptake in muscle is lower for 23F than 23M AON, it is even further reduced in kidney and liver (fig. 2E,F).

Blood and plasma were determined for markers of liver and kidney damage and function as part of the safety profiling of the AONs. Glutamic oxaloacetic pyruvate transaminase (GOT) and glutamate pyruvic transaminase (GPT) are both enzymes that leak into the bloodstream upon liver and muscle damage. No significant differences were observed for GOT, while significantly lower GPT levels were found for mice treated with either AON compared to saline treated mice. Alkaline phosphatase (ALP, a marker for hepatobiliary function), urea (a marker for kidney function), and haemoglobin levels showed no significant differences between the 3 groups and were in the normal range for mdx mice. Large variations in individual levels of creatine kinase (CK), an enzyme that leaks into the bloodstream upon muscle damage, prevented comparisons between the groups (fig. 3A-D).
Figure 1. Exon skip evaluation of human and mouse myotube cultures. RT-PCR analysis of human and mouse myotubes transfected with 100-500 nM of 2OMePS or isosequential 2FPS AON (n=4). A) Exon 45 AONs in control myotubes. B) Exon 53 AONs in control myotubes. C) Exon 53 AONs in DMD patient-derived (Δ45-52) myotubes. D) Mouse exon 23 AONs in mouse myotubes. E) Exon 23 skipping in primary mouse myoblasts without the use of a transfection reagent (gyratory delivery). Bars represent means ± SD.
Figure 2. Exon skipping and pharmacokinetic analysis of 23F and 23M-treated mdx mice. A) RT-PCR analysis of muscles from two mdx mice, IM injected with 2.9 nmol of 23M or 23F contralaterally for 2 consecutive days. B) RT-PCR analysis of skeletal and heart muscles isolated from mdx mice (4-5 mice/group) subcutaneously treated 4 times per week with 50 mg/kg of 23M, an equimolar amount of 23F or saline for 8 weeks. C) AON concentrations in skeletal muscles and heart assessed with a hybridization ligation assay. D) AON concentrations in liver and kidney as assessed with a hybridization ligation assay. E) Ratios of AON levels in muscle compared to kidney. F) Ratios of AON levels in muscle compared to liver. M= 23M, F= 23F, C= untreated control, S= saline, Gastr= gastrocnemius, Qua= quadriceps, Tib.A= tibialis anterior, Tric= triceps, Dia= diaphragm, (*T-test for significant P<0.05). Bars represent means ± SD.
Notably, 23F AON treated mice had significantly lower increases in body weight over time compared to 23M AON and saline treated mice (fig. 3E). Additionally, we found a significantly higher spleen/bodyweight ratio for mice treated with 23F AON compared to the other groups at sacrifice (fig. 3F).

To assess whether the discrepancy between in vitro and in vivo results observed for mouse exon 23 AONs also occurred for other exons, we compared a 2FPS and 2OMePS AON targeting human exon 45 in vivo in the hDMD mouse model. This mouse model carries the human DMD gene integrated in the mouse genome, which compensates for lack of mouse dystrophin resulting in healthy muscle17. Since AON uptake in mdx mice is facilitated by the dystrophic phenotype we pretreated hDMD gastrocnemius and triceps muscles with IM cardiotoxin injections to induce muscle necrosis and enhance AON uptake18. Two days later treated muscles were injected with 2.9 nmol of the most potent 2OMePS AON targeting human exon 45 (45-2M) or isosequential 2FPS AON (45-2F) for 2 consecutive days into gastrocnemius and triceps muscles. One week after the last injection, RNA was isolated, and exon skip levels were determined by nested RT-PCR and visualized on an agarose gel. Results suggested enhanced exon skipping for 2FPS AON over the 2OMePS AON, which was most pronounced in the triceps muscle (fig. 4). These results demonstrate that 2FPS AONs are in fact capable of exon skipping in vivo after IM injections and the failure of the 2FPS AON to induce mouse dystrophin exon 23 skipping in vivo is not due to an inability of 2FPS AONs to be active in vivo per se.

4. Discussion

Exon skipping is a therapeutic approach using AONs to reframe dystrophin transcripts for DMD and is currently evaluated in clinical trials11. 2FPS AONs have shown unanticipated enhanced exon skipping in a model of SMA due to recruitment of ILF2/3 proteins to the 2F/pre-mRNA duplex13. For DMD, exon skipping is a desired feature, making 2FPS AONs potentially useful tools for reframing dystrophin transcripts. In this study, we demonstrate in vitro that 2FPS AONs have enhanced exon skipping in human and mouse myotube cultures over 2OMePS AON counterparts. The increased efficiency was most pronounced for human exon 45 AONs and least for mouse exon 23 AONs. A possible explanation in vitro is the difference in AON target sites and parameters such as AON sequence composition and secondary structure of the target region. For example, the AONs used here to target...
human exon 45 or 53, target intralexonic sites whereas the mouse AON targets a donor splice site. It is likely that the added bulkiness of recruited ILF2/3 by fluoro modified AONs is less effective when targeting exon intron boundaries where direct competition takes place with U1 snRNP binding sites than when targeting predicted ESE in intralexonic regions. This is consistent with our previous finding that exonic AONs appear to outperform splice site targeting AONs.

In vivo we demonstrated that 2FPS AONs were not capable of skipping exon 23 in mdx mice, in contrast to 2OMePS AONs. A possible explanation for the observed difference between 2OMePS and 2FPS AON in vivo is that uptake of 2FPS by skeletal muscles after systemic treatment in mdx mice yielded insufficient concentrations to allow exon skipping. However, this does not explain why no exon 23 skipping was detected after IM injections in mdx mice. Potentially lower in vivo stability of 2FPS AONs could lead to lower tissue levels, but the fact that human exon 45 skipping 2FPS AONs were effective in vitro after IM injections argues against this possibility. It should be noted however, that the exon 45 AON was tested in healthy muscle, while it is possible that in the mdx mouse the underlying pathology, such as the chronic inflammation and high muscle turnover caused increased 2FPS degradation.

Recently, Shen et al., showed in vitro that 2FPS modified AON interfered with splicing proteins and that 2FPS AON treatment of cultured cells resulted in a general disruption of normal splicing. We evaluated the accuracy of dystrophin splicing for exons 46 to 53 and the splicing of other genes (Beta-2-Microglobulin, Transforming Growth Factor beta 1, Alpha-1 type I collagen and Activin, chosen because of their involvement in immunogenicity, proliferation, differentiation etc.) in muscle RNA isolated from saline, 2OMePS and 2FPS treated mice, but did not observe any differences between the groups (data not shown). This suggests that the splicing disruptions as observed by Shen et al., might not underlie the lack of exon 23 skipping we observed in the mdx mouse. However only a deep analyses of the full dystrophin transcript can rule out the occurrence of splicing abnormalities completely.

Since the first encouraging 2F results were published more than 20 years ago, only short term in vivo experiments (1-3 weeks) have been reported for 2F-modified AONs. Furthermore, only a limited number of tissues was evaluated after intraperitoneal (IP) administration in Spinal Muscular Atrophy (SMA) or normal mice. To the best of our knowledge, no long term in vivo follow up studies have ever been reported for this chemistry.

With respect to safety, in our study of systemic AON treatment targeting mouse exon 23, no clear indications of toxicity were seen in markers for liver, kidney or muscle damage. However, mice treated with 2FPS AON had a significantly higher spleen/bodyweight ratio compared to 2OMePS AON and saline treated mice. This corroborates the finding of a dose-dependent increase in spleen weights for mice treated with 2F AONs with flanking and or alternating 2'-O-methoxyethyl nucleotides after three weeks treatment with 6.25-50 mg/kg twice a week. Lastly, we also noticed that the 2FPS treated mdx mice gained significantly less bodyweight than saline and 2OMePS treated mice. On average the 2FPS treated mice weighed 17% less at the end of our experiment. Taken together this suggests that mdx mice did not tolerate the treatment with fully modified 2FPS AONs targeting mouse exon 23 very well.

In summary our data shows that 2FPS modified AONs had an improved effect in vitro and were effective in vivo on DMD exon skipping when targeting human exons, but this effect was minimal or absent in vitro and in vivo targeting mouse DMD exon 23. The exact reason for a lack of exon skipping in vivo by 2FPS modified AON targeting mouse DMD exon 23 is still unclear. In addition, 2FPS AONs revealed possible safety issues for long term in vivo application, which needs to be further addressed when one wants to use such AONs in future studies. However, our results do not support a clinical application for 2FPS AON.

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Author disclosure statement

AAR reports being a coinventor in patents of the LUMC on exon skipping, licensed by LUMC to Prosensa Therapeutics, and being entitled to a share or royalties. AAR also declares being an ad hoc consultant for Global Guidepoint, GLC consulting, Deerfield Institute, PTC Therapeutics and BioMarin and being a member of the scientific advisory board of ProQR. Remuneration for these activities go to LUMC. PCdV and RV report being employed by Prosensa Therapeutics B.V./BioMarin the Netherlands.
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