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Aartsma-Rus, A.

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The Future of Exon Skipping for Duchenne Muscular Dystrophy

Annemieke Aartsma-Rus*

Department of Human Genetics, Leiden University Medical Center, Leiden, the Netherlands.

Antisense oligonucleotide (ASO)-mediated exon skipping can restore the open reading frame of dystrophin transcripts for Duchenne muscular dystrophy (DMD) patients. This allows production of internally deleted dystrophin proteins as found in the later onset, less severely progressive Becker muscular dystrophy. At present, ASOs that induce exon skipping and dystrophin restoration are approved for the treatment of DMD by the regulatory agencies of the United States and Japan. However, approval was based on restoration of very small amounts of dystrophin and the approved ASOs apply to only a subset of patients. This expert perspective evaluates ways to improve ASO efficiency that are currently in or close to clinical trials, as well as ways to improve applicability of this mutation-specific approach.

Keywords: oligonucleotides, splice modulation, therapy, dystrophin

EXON SKIPPING FOR DUCHENNE MUSCULAR DYSTROPHY

PATHOGENIC VARIANTS IN the dystrophin encoding *DMD* gene underlie both Duchenne and Becker muscular dystrophy (DMD and BMD, respectively). Dystrophin provides stability to muscle fibers during contraction by connecting the cytoskeleton within the fiber, to the extracellular matrix surrounding the fibers, via its N- and C-terminal domains, respectively.[1]

In DMD, variants cause a premature truncation of protein translation, resulting in nonfunctional dystrophins and an early-onset, rapidly progressive muscle wasting disease[2,3] (Fig. 1). Patients present with symptoms in early childhood, become wheelchair dependent by the age of ~10 years, need assisted ventilation around the age of 20 years, and die in the second to fourth decade of life owing to respiratory or cardiac failure. By contrast, variants that maintain the reading frame allow production of an internally deleted, but partially functional dystrophin[3] (Fig. 1). These variants are associated with BMD. The severity of BMD varies from patients with first symptoms in childhood to those with first symptoms in midlife. However, the onset is always later and progression slower than for DMD.[2]

The exon skipping approach is based on the facts that in-frame variants allow the production of partially functional dystrophins and that most DMD patients have the genetic capacity to produce a BMD-like dystrophin (Fig. 1).[4] Exon skipping utilizes antisense oligonucleotides (ASOs), small pieces of chemically modified RNA that hybridize to specific exons in the pre-mRNA. Upon binding, they hide the target exon from the splicing machinery, causing it to be skipped. This will enlarge the deletion, but restore the reading frame, thus allowing patients to produce a BMD-like dystrophin instead of a nonfunctional dystrophin.

Proof-of-concept that ASOs can induce targeted exon skipping and dystrophin restoration was given over 20 years ago in patient-derived muscle cell cultures and also *in vivo* in the *mdx* mouse model.[5–7] Furthermore, clinical trials in DMD patients have confirmed that ASO treatment restores dystrophin in patients, although at very

*Correspondence: Dr. Annemieke Aartsma-Rus, Department of Human Genetics, Leiden University Medical Center, Albinusdreef 2, Leiden 2333 ZA, the Netherlands. E-mail: a.m.rus@lumc.nl

DOI: 10.1089/hum.2023.026

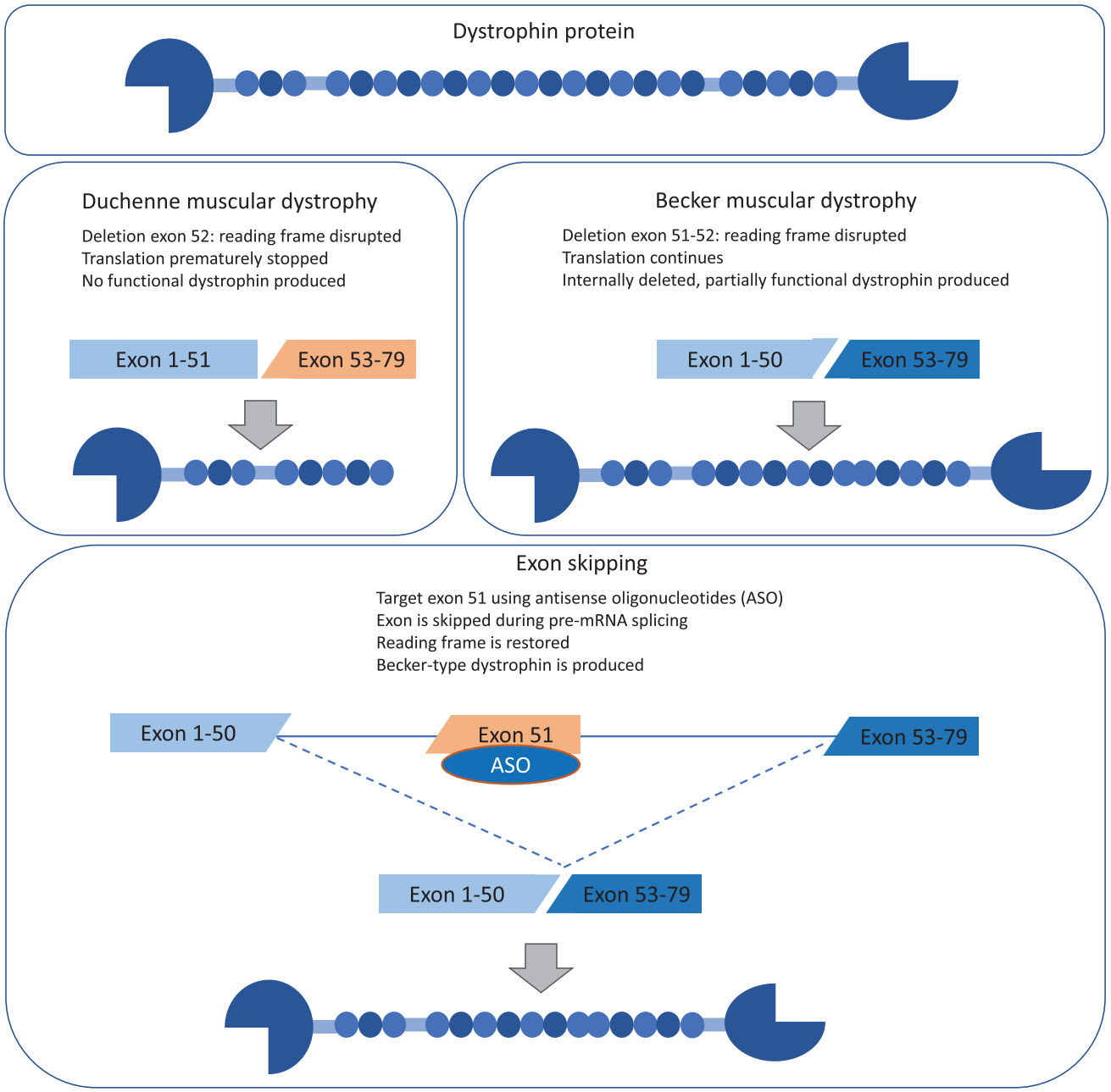

Figure 1. Schematic depiction of dystrophin in unaffected (*top panel*), Duchenne (*middle left panel*), and Becker (*middle panel right*) muscular dystrophy. In Duchenne, mutations (exon 52 deletion in this example) disrupt the open reading frame, resulting in premature truncation of protein translation and nonfunctional dystrophin protein. In Becker, mutations (exons 51–52 deletion in this example) maintain the open reading frame, resulting in internally deleted but partially functional dystrophin proteins. Exon skipping (*lower panel*) uses ASOs that target a specific exon in the pre-mRNA (exon 51 in this example for a deletion of exon 52). Upon this binding the exon is hidden from the splicing machinery, causing the reading frame to be restored and allowing the production of a Becker-type dystrophin. ASOs, antisense oligonucleotides.

low levels (0.4% to ~5%). This has resulted in marketing authorization by the Food and Drug Administration (FDA) and the Japanese Ministry of Health, Welfare and Labour (MHWL) of four ASOs: eteplirsen (targeting exon 51; FDA), casimersen (targeting exon 45; FDA), golodirsen (targeting exon 53; FDA), and viltolarsen (targeting exon 53; FDA and MHWL).[4,8] The labels of these ASOs specify that approval was based only on dystrophin restoration and it has yet to be confirmed whether treatment results in a slower disease progression.

So in summary, over two decades after *in vitro* proof-of-concept, there is proof-of-concept also in humans that exon skipping restores production of dystrophin in DMD patients. Although it is not yet clear whether these low levels of dystrophin are sufficient to slow down disease progression, it is clear is that there is room for improvement.

This perspective article is directed at those involved in exon skipping therapy development for DMD and outlines the most important challenges for improving the efficiency and applicability for exon skipping for DMD and how the field is currently working on overcoming them. For more background content on the disease and the exon skipping approach we refer the reader to previous reviews.[2,4]

Limitations of currently approved ASOs

The main reason for the low efficiency of currently approved ASOs is their limited uptake by the target tissue: skeletal muscle and heart. The approved ASOs are all of the phosphorodiamidate morpholino oligomer (PMO) chemistry, which is uncharged and does not bind to serum proteins. As such, the bioavailability of these ASOs is low and after intravenous dosing, they are rapidly cleared by the kidney.[9] Only very limited amounts are taken up by the dystrophic skeletal muscle, resulting in low levels of exon skipping and dystrophin restoration, even with high intravenous doses (weekly doses of 30 mg/kg for eteplirsen, casimersen, golodirsen and weekly doses of 80 mg/kg for viltolarsen). The dystrophin levels in heart have not been measured in patients, but based on animal model studies, it can be assumed they are negligible at the doses currently used.[9–12]

OVERCOMING THE CHALLENGE OF POOR DELIVERY TO SKELETAL MUSCLES

Delivery to skeletal muscle is particularly challenging, because it is a highly abundant tissue (30–40% of the bodyweight) and it is dispersed over hundreds of larger and smaller individual muscles. This makes it more challenging to safely achieve a specific drug concentration in muscles, as this requires a higher systemic dose than for less abundance tissues. Furthermore, both skeletal muscle and heart have dense endothelium, although the endothelium is more permeable in dystrophic skeletal muscle, owing to chronic inflammation and ongoing regeneration.[13,14]

Conjugates for improved delivery

To improve delivery to skeletal muscle and heart, conjugates are used. First, short arginine-rich peptides linked to PMOs (pPMO) have been shown to improve delivery of the PMO to all tissues. Studies in *mdx* mice have revealed extremely efficient exon skipping and dystrophin restoration in skeletal muscle and heart for a variety of arginine-rich peptides.[11,15,16] However, there is a threshold effect in dose–response studies, where below a certain dose (usually $\sim$20–40 mg/kg), little dystrophin restoration is seen, whereas above this dose, extremely efficient dystrophin restoration is seen with most fibers being positive and total dystrophin levels being up to 80% in skeletal muscle and $\sim$40% in heart.[11,17]

At present, three different pPMOs are in, or close to, clinical trials: vesleteplirsen, containing a linear peptide conjugated to a PMO targeting exon 51, developed by Sarepta; PGN-EDO51, containing a linear peptide conjugated to a PMO targeting exon 51, developed by PepGen and ENTR-601-044, containing a cyclic peptide conjugated to a PMO targeting exon 44 and being developed by Entrada Therapeutics. Vesleteplirsen has been tested in DMD patients in monthly doses up to 30 mg/kg and PGN-EDO51 in healthy volunteers in a single dose up to 20 mg/kg. In DMD patients, dystrophin levels of up to 6.5% have been reported in a press release after 3 monthly doses of 30 mg/kg vesleteplirsen.[18]

However, in both patients and healthy volunteers[19] hypomagnesemia was reported, which may be an early marker of kidney toxicity and dysfunction.[20] The pPMOs were well tolerated in mice, but hypomagnesemia had also been reported in the nonhuman primate studies for PGN-EDO51. The development of ENTR-601-044 has been placed on clinical hold by the FDA.[21]

The currently outstanding question is, whether the pPMOs will be sufficiently tolerable at a dose where they increase dystrophin protein compared with the unconjugated PMOs. An open-label clinical trial treatment with monthly doses of 30 mg/kg vesleteplirsen is evaluated for up to 2 years is currently ongoing.[22]

Improving muscle homing

To improve muscle-specific uptake, several companies are exploring the conjugation of antibodies targeting transferrin 1 receptors, which are highly expressed on skeletal muscle. Dyne Therapeutics is evaluating an exon 51 skipping PMO conjugated to their proprietary ''FORCE'' system in clinical trials. This system includes an antibody fragment conjugated to an ASO and improved exon skipping and dystrophin restoration significantly compared with unconjugated PMOs targeting exon 23 in the *mdx* mouse model.[10] Meanwhile, Avidity is preparing for a clinical trial with AOC 1044, where a full transferrin 1 antibody is conjugated to PMOs targeting exon 44.[23]

Improving delivery by chemical modifications

In addition to conjugation, companies are also exploring additional chemical modifications to obtain ASOs with higher exon skipping efficiencies. BioMarin is preparing for a clinical trial with an exon 51 ASO BMN351 that is negatively charged and contains phosphorothioate modifications.[24] Wave Life Sciences is developing WVE-N531, a stereopure negatively charged ASOs. Their first stereopure ASO targeting exon 51, suvodirsen, did not result in increased expression of dystrophin in a clinical trial in Duchenne patients.[25] Wave recently initiated a dose finding study to assess the efficiency and safety of a new stereopure exon 53 ASO, that contains a further optimized backbone.[26,27]

After three biweekly doses of 10 mg/kg, exon 53 skipping was confirmed in muscle biopsies from DMD

patients.[28] No dystrophin was detected. However, it is known that the dynamics of dystrophin restoration differ from exon skipping, where exon skipping precedes protein production, so it is possible that dystrophin restoration will be observed in biopsies taken at a later timepoint.[29]

Considerations for improved delivery

It is clear there is need for improved ASO delivery and efficacy and reduced treatment frequency. However, this should be achieved in a safe manner. The challenge is that the new chemical modifications and conjugates as outlined in the previous sections have not been tested yet in humans. This means pharmacokinetic and pharmacodynamic studies in animal models and patients have to be more extensive as there is no prior art on which to base dose selection. Furthermore, there is a risk that the novel modifications and conjugates are not tolerated in humans as underlined by the finding of hypomagnesemia seen for the peptide-conjugated PMOs, or not effective, as underlined by the suvidirsen development.

Reducing treatment frequency by delivery an antisense gene

The dosing regimen for currently approved exon skipping ASOs for DMD is weekly intravenous infusion. In an effort to have a more permanent treatment effect, developers are exploring delivering an antisense gene. Here antisense sequences targeting a specific exon are introduced in a modified U7 small nuclear ribonucleoprotein (snRNP), which is then delivered with adeno-associated viral vectors (AAV).[30] A clinical trial evaluating a U7 snRNA targeting exon 2 is ongoing at the Nationwide Children's Hospital (Columbus, OH) in DMD patients with a duplication of exon 2,[31] after preclinical optimization in an exon 2 duplication mouse model.[32]

So far, three DMD patients with an exon 2 duplication have been treated. Results have been presented at scientific conferences, showing dystrophin levels of 7% and 1%, 12 months after treatment of 8- and 11-year-old DMD patients, respectively.[33] The third patient was dosed at 7 months and had 88% dystrophin 12 months after treatment. It is possible that the youngest patient responded better as his muscle quality was better at the time of treatment than for the older two patients. However, as numbers are very small as yet, it is premature to draw strong conclusions about this.

As this is a gene therapy approach, long-lasting effects are anticipated. However, AAV transgenes are cytoplasmic and will disappear with muscle turnover. Furthermore, the muscle fiber size of the patient dosed at 7-month-old patient will increase several fold when he grows and this will likely result in a dilution of transgenes and therefore of dystrophin levels. The question is how long this will take, years or decades. Given that patients are immunized against AAV when receiving gene therapy, repeating AAV treatment is likely not an option. However, if a single treatment would suffice to stabilize or slow down disease progression for a number of years, that would have a major impact on the disease course.

Genome editing to reduce treatment frequency

When using ASOs, owing to turnover of the ASOs, the skipped transcripts and the dystrophin proteins, repeated treatment is required, with, as mentioned, a current regimen of weekly intravenous infusions. The hope is that improved ASOs allow less frequent dosing. However, when the reading frame would be restored on DNA level with genome editing approaches, the effect would be expected to persist much longer. A full expose on how different genome editing tools can be utilized to allow dystrophin restoration is beyond the scope of this perspective. For more detail, we refer the reader to a review by Chey et al.[34]

Although the potential of the genome editing approach is large, there are many hurdles that are yet to be overcome, including but not limited to safety and specificity of long-term expression of nucleases, risk of off-target edits and preexisting immunity to the viral vectors used to deliver genome editing components and the nucleases that are often from bacterial origin. Furthermore, although the perception may be that genome editing would allow permanent expression of dystrophin in edited muscle fibers, this is not necessarily true. At present, nuclei in muscle fibers are edited, whereas satellite cells are not, or very inefficiently, reached (<1%).[35,36] As the dystrophin that is expressed will be partially functional, it will not fully prevent muscle turnover, and as such with time the edited nuclei will be lost to be replaced by expanded satellite cells that are not edited.

OVERCOMING THE CHALLENGE OF MUTATION SPECIFICITY

Applicability

Exon skipping for DMD is mutation specific: for different mutations, different exons have to be skipped to restore the reading frame.[37] This means that skipping a specific exon only applies to a subset of patients. Owing to the clustering of large deletions between exon 45 and 55, skipping certain exons applies to relatively large groups of patients (Table 1).[38] The approved ASOs jointly apply to ~31% of patients. Developers are currently performing confirmatory clinical trials for the approved ASOs, as well as improved versions of exon 51 and exon 53 skipping ASOs, arguing that larger groups of patients have to be available to confirm efficacy in clinical trials.

However, currently there are five trials planned or ongoing for the 14% of patients benefitting from exon 51 skipping,[39] and three trials ongoing for the 8% of patients

benefitting from exon 53 skipping. For exon 44 skipping there is an ongoing trial of Nippon Shinyaku for NS-089, a PMO targeting exon 44,[40] and two upcoming trials for ENTR-601-044 and AOC 1044 for a subset of <7% of patients. This means that each trial will have <3% of patients who fit the inclusion criteria, which is less than the applicability for exon 50 (almost 4%) and exon 43 (3%). For exon 50, Nippon Shinyaku is preparing clinical trials, whereas for exon 43 skipping no clinical trials are currently planned.

Humanized mouse models

A challenge for ASO development in general is that generally human-specific ASOs cannot be tested in animal models. The *mdx* mouse model requires skipping of mouse dystrophin exon 23. However, these ASOs will not induce exon 23 skipping in DMD patients owing to sequence differences between mouse and man. My institute has generated a mouse model containing copies of the full-length human *DMD* gene integrated in the mouse genome, crossed into the *mdx* background.[41] At present, a mouse model carrying a deletion of exon 52 has been produced (hDMDdel52/*mdx*), which allows evaluation of human-specific ASOs targeting exon 51 or exon 53 *in vivo*.[42,43] Although this models facilitates testing of human-specific ASOs in a dystrophic setting, dystrophin restoration and functional effects can only be assessed after exon 51 or exon 51 skipping. Additional models are needed to evaluate ASOs targeting other exons (exons 44, 45, 50, and 52) and are currently being generated (Hohenstein and Aartsma-Rus, manuscript in preparation).

Multiexon skipping

As a method to increase applicability, the skipping of stretches of exons has been proposed, mostly the multi-exon skipping of exon 45 to exon 55 as this would apply to >40% of patients and the deletion of exon 45–55 is associated with relatively mild BMD.[44,45] However, skipping multiple exons is challenging, as it requires a cocktail of ASOs, which makes preclinical toxicity studies exponentially more complex due to having to test safety of individual ASOs and possible mixtures. Furthermore, using a mixture means that for most patients, not all the ASOs in the cocktail will have a target, as part of the target exons are deleted. Under current regulations, it is not allowed to treat an individual with a medicinal product known to not be effective. Developers are trying to lessen these challenges by reducing the number of ASOs in their cocktail.[46] However, then the question is whether they will be equally applicable to all mutations as depending on the deletion breakpoints the dynamics of splicing may have changed.

Creating a multiexon deletion with genome editing is more straightforward, as it requires only 2 guide RNAs.[47] However, at present, efficiencies are low because of the spatial distance between exon 45 and exon 55 (>300 kb). As mentioned earlier, genome editing is in a preclinical stage and there are multiple additional challenges to be overcome. For this specific application there is the added hurdle that systems where two double-stranded breaks are induced, risks for unintended cuts will increase.

Table 1. *Overview of antisense oligonucleotides that are approved an in clinical development*

Target Exon	*Cumulative Applicability*	*Approved*	*Ongoing Trials*	*Planned Trials*
51	14%	Eteplirsen	Vesleteplirsen, eteplirsen high dose, PGN-EDO51, Dyne-051	BMN351
45	9.1%	Casimersen	Casimersen	
53	8.1%	Golodirsen, Viltolarsen	Golodirsen, Viltolarasen, Wave	
44	7.6%		NS-089/NCNP-002	ENTR-601-044, AOC 1044
50	3.8%			
43	3.1%			
—				
2[a]	<1%		AAV U7snRNP targeting exon 2	

[a]Exon 2 applicability is very low, but the exon is included because of the exon 2 skipping trials.

AAV, adeno-associated viral vectors.

FUTURE PERSPECTIVE

Two decades after exon skipping was shown to restore dystrophin in patient-derived cells, it is now clear that also in DMD patients, exon skipping can restore dystrophin. However, the levels are currently very low. Although these low levels may be able to slow down disease progression, the question is whether this difference can be detected robustly in clinical trials lasting 1, 2, or even 3 years. While DMD is a progressive disease, the loss of function is a process that takes years, so clinical trials to assess impact on disease milestones like loss of ambulation or the start of assisted ventilation, take too long. Instead, decline of muscle function of legs or arms is measured over time. However, there is individual variation between patients' trajectories,[48] making is challenging to pick up a small treatment effect.

The hope is that ASOs that are in development will result in higher dystrophin restoration levels, and therefore a more significant impact on disease progression. At present, it is not yet known which level of dystrophin restoration is required to slow down disease progression at a given disease stage.[49] Once this is known, this will also facilitate the development of ASOs for exons that apply to very small groups of patients, where placebo-controlled trials are not feasible.

ACKNOWLEDGMENTS

A.A.-R. is a member of the Netherlands Neuromuscular Center, the Duchenne Centrum Netherlands and the

European Reference Network for rare neuromuscular diseases EURO-NMD.

AUTHOR DISCLOSURE

A.A.-R. discloses being employed by LUMC, which has patents on exon skipping technology, some of which has been licensed to BioMarin and subsequently sublicensed to Sarepta. As co-inventor of some of these patents A.A.R. is entitled to a share of royalties. A.A.R. further discloses being *ad hoc* consultant for PTC Therapeutics, Sarepta Therapeutics, Regenxbio, Alpha Anomeric, Lilly, BioMarin Pharmaceuticals, Inc., Eisai, Entrada, Takeda, Splicesense, Galapagos, and Astra Zeneca. A.A.R. also reports being a member of the scientific advisory boards of Eisai, Hybridize therapeutics, Silence therapeutics and Sarepta therapeutics. Remuneration for these activities is paid to LUMC. LUMC also received speaker honoraria from PTC Therapeutics, Alnylam Netherlands, Pfizer, and BioMarin Pharmaceuticals and funding for contract research from Italfarmaco, Sapreme, Eisai, Galapagos, Synnaffix, and Alpha Anomeric. Project funding is received from Sarepta Therapeutics through an unrestricted grant.

FUNDING INFORMATION

No funding was received for this article.

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Received for publication February 21, 2023;
accepted after revision March 11, 2023.

Published online: March 14, 2023.