'Developments in reading frame restoring therapy developments for Duchenne Muscular Dystrophy'

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REVIEW

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Developments in reading frame restoring therapy approaches for Duchenne muscular dystrophy

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ABSTRACT

Introduction: Exon skipping compounds restoring the dystrophin transcript reading frame have received regulatory approval for Duchenne muscular dystrophy (DMD). Recently, focus shifted to developing compounds to skip additional exons, improving delivery to skeletal muscle, and to genome editing, to restore the reading frame on DNA level.

Areas covered: We outline developments for reading frame restoring approaches, challenges of mutation specificity, and optimizing delivery. Also, we highlight ongoing efforts to better detect exon skipping therapeutic effects in clinical trials. Searches on relevant terms were performed, focusing on recent publications (<3 years).

Expert opinion: Currently, 3 AONS are approved. Whether dystrophin levels are sufficient to slowdown disease progression needs to be confirmed. Enhancing AON uptake by muscles is currently under investigation. Gene editing is an alternative, but one that involves practical and ethical concerns. Given the field's momentum, we believe the efficiency of frame-restoring approaches will improve.

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Duchenne muscular dystrophy; exon skipping; antisense oligonucleotide; genome editing; therapy; clinical trials; personalized medicine

1. Introduction

Duchenne muscular dystrophy (DMD) is one of the most common inherited muscle wasting disorders [1]. Symptoms become apparent in early childhood and encompass delayed development of motor skills and muscle weakness. One of the most prominent early features is proximal muscle weakness, illustrated by the classic Gowers sign, where patients need the use of their arms to support their weight and legs while rising from the floor. Throughout the course of the disease, muscle fibers are gradually replaced by fibrotic and adipose tissue and patients lose motor functions, resulting in loss of ambulation in the early teens, the gradual loss of arm function during the teenage years and the need for assisted ventilation around the age of 20 [2–4]. Most patients die in the 2nd-4th decade due to respiratory or heart failure [2–4]. Currently, corticosteroids are often used as symptomatic treatment to slowdown disease progression though this comes at the costs of side effect [2–4].

The disease is caused by lack of dystrophin, a protein that provides stability to skeletal muscle fibers by linking the F-actin cytoskeleton to the extracellular matrix [5,6]. This is caused by mutations in the X-chromosomal *DMD* gene that disrupt the reading frame or cause premature stop codons. Consequently, dystrophin protein translation is prematurely truncated, and the linker function is lost. By contrast, mutations that maintain the open reading frame, generally allow the production of internally deleted dystrophin, that can still fulfill their linker function [7]. These partially functional dystrophins are found in Becker muscular dystrophy (BMD), a muscle-wasting disease that is milder compared to DMD, with a later onset of symptoms and a slower disease progression [1].

The exon skipping therapy is based on the difference between DMD and BMD mutations, where the aim is to restore the reading frame for DMD patients to allow them to make a BMD-like protein [8]. The exon skip therapy achieves this by 'hiding' a specific exon from the splicing machinery using antisense oligonucleotides (AONs). This induces a slightly larger deletion but does lead to restoration of the reading frame and the production of a partially functional dystrophin rather than a nonfunctional one (Figure 1).

AONs are RNA-homologues that bind to their target in Watson-Crick like manner. Chemical modifications are required to give the AONs druglike properties, e.g. increasing nuclease resistance, increasing bioavailability and improving the affinity to the target RNA transcripts. Many different chemical modifications exist that can be used for exon skipping purposes [9]. For DMD exon skipping 2'-O-methyl phosphorothioate (2OMePS) AONs and phosphorodiamidate morpholino oligomers (PMOs) have been the chemistries of choice for clinical development.

The exon skipping approach has been in development in cell and animal models and patients for over 20 years and the first exon skipping drug (eteplirsen) was approved in 2016 [10]. In a previous review paper we outlined the applicability and considerations of the exon skipping approach for different mutation types and provided an in-depth description of preclinical research [8]. In this current review, we focus on recent

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Article highlights

- Currently, 3 exon skipping compounds are approved by regulators for treatment of Duchenne muscular dystrophy and clinical trials are ongoing for additional compounds.
- Reading frame restoration is a mutation-specific approach.
- Measuring functional effects of dystrophin restoration in Duchenne patients is challenging.
- Efforts to improve exon skipping are ongoing preclinically and in clinical trials.
- Genome editing would offer a more permanent therapeutic effect and is studied preclinically.

This box summarizes the key points contained in the article.

developments in clinical testing and application of exon skipping therapies, as well as providing a comprehensive overview of preclinical work aimed at improving the delivery of the AONs into the skeletal muscle and heart and genome editing reading frame approaches to achieve a more permanent reading frame restoration effect.

2. Exon skipping compounds in clinical trials and approved

2.1. Mutation specificity

Exon skipping is a mutation-specific approach. Based on the location and extent of the mutations, different exons have to be skipped to restore the reading frame [11,12]. Since about two-thirds of patients carry a deletion involving one or more exons, the 'applicability' per exon is largely determined by the location of these deletions [12,13]. The majority of these deletions cluster in a hotspot region between exon 43 and 55, so it follows that most of the out-of-frame exons in this region feature in the top 10 most applicable exons (Table 1), with exon 51 applying to 14% of all mutations, exon 53 applying to 10% of all mutations and exon 45 applying to 9% of all mutations [11].

Notably, some very common deletions, such as a single exon deletion of exon 45 (4% of all mutations) and a single exon deletion of exon 52 (2% of all mutations) [12], can be reframed through the skipping of either the upstream or downstream exon (exon 44 and exon 46, and exon 51 and 53, respectively). Therefore, the total applicability of skipping additional exons is not simply the sum of the applicability of individual exons, as this would count some mutations twice. Interestingly, when one adjusts for this, by only including the additional mutations that would be applicable on top of the previously ranked exons (see footnate a in Table 1), the top 3 and the top 10 change significantly [12]. For instance, the top 3 becomes exon 51 (14%), exon 45 (an additional 9.1%) and exon 53 (an additional 8%). Furthermore, exon 46 is not included in the top 10 anymore, since the applicability is mostly caused by exon 45 deletions, which can be reframed by skipping of exon 44.

2.2. Exon 51 skipping

2.2.1. Eteplirsen

Because exon 51 skipping applies to the largest group of patients, initial clinical trials have focused on exon 51 AONs (Table 2). Eteplirsen, a PMO identified by an academic consortium [14] and developed by Sarepta Therapeutics, was the first DMD exon skipping compound to receive accelerated approval in 2016 from the USA Food and Drug Administration (FDA) [10,15]. Eteplirsen was first tested by intramuscular injection in the extensor digitorum brevis muscle in the foot, where local injection of 0.9 mg eteplirsen resulted in localized dystrophin restoration ([16], NCT00159250). Then, a dose-finding study was performed where patients were treated for 12 weeks with weekly intravenous infusions of 0.5, 1, 2, 4, 10, and 20 mg/kg ([17], NCT00844597). Increased expression of dystrophin was detected in 7 of the 21 included patients. In the next study 12 patients were treated with weekly infusions of 30 or 50 mg/kg for 48 weeks or 24 weeks (receiving placebo the first 24 weeks) ([18], NCT01396239), followed by open-label treatment (NCT01540409). Dystrophin positive fibers were detected in biopsies analyzed after 24 and 48 weeks of treatment. Quantification via western blotting analysis on biopsies obtained after 48 and 188 weeks of treatment revealed 0.4% and 0.9% increase, respectively, compared to biopsies from untreated patients [19]. Since the treatment was primarily done in an open-label setting, functional analysis was done using historic controls, which suggested a slower disease

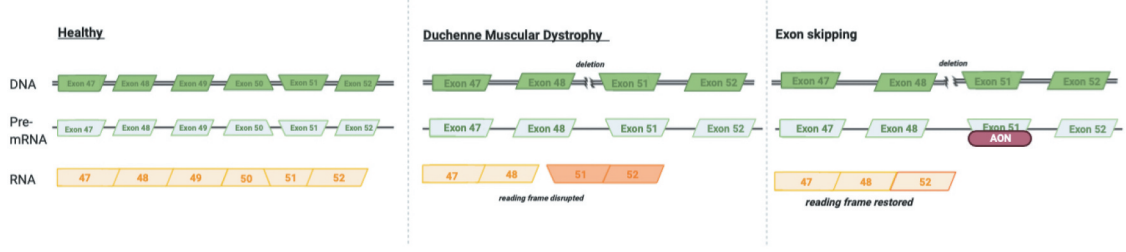

Figure 1. Schematic explanation of exon skipping therapy for DMD.

Left panel: Unaffected individual. Middle panel DMD patient. A deletion of exon 49 and 50 in the *DMD* gene causes a frameshift leading to disruption of the reading frame. Protein translation is prematurely truncated. Right panel: Exon skipping in DMD. The exon adjacent to the deletion is hidden from the splicing machinery by an antisense oligonucleotide (AON) in the pre-mRNA. This exon is then skipped during splicing, which leads to restoration of the reading frame allowing the production of a partially functional dystrophin.

Table 1. Exon skipping applicability (adapted from [12]).

Position (Adjusted)[1]	Exon	Applicability	Adjusted[a] applicability
1 (1)	51	14%	14%
2 (3)	53	10%	8.1%
3 (2)	45	9.0%	9.0%
4 (4)	44	7.6%	7.6%
5 (6)	43	7.5%	3.1%
6 (outside top 10)	46	4.6%	<0.5%
7 (5)	50	3.8%	3.8%
8 (9)	52	3.6%	0.9%
9 (8)	55	2.7%	1.7%
10 (7)	8	2.0%	2.0%

[a]The adjusted applicability indicates how many extra mutations can benefit from the skipping of this exon on top of those ranked above it.

progression for eteplirsen-treated patients [20–23]. In September 2016 FDA granted eteplirsen accelerated marketing authorization based on increases in dystrophin expression, stating on the label that functional effects have not yet been confirmed. FDA mandated that Sarepta needs to present this evidence by 2021 [10].

Several clinical trials with eteplirsen are currently ongoing or have been completed since the accelerated approval. The first is a 96 week open-label trial, where patients with eligible mutations were treated with weekly intravenous infusions of eteplirsen, while the control group consisted of patients with mutations that could not be reframed by exon 51 skipping, but showing a similar pattern of disease progression and receiving the same level of multidisciplinary care (NCT02255552). These patients did not receive treatment and also did not have to undergo a muscle biopsy at baseline and after 96 weeks, but they were followed up with the same functional analyses in a clinical trial setting (control group). This study is now complete and results have been deposited to clinicaltrials.gov (https://clinicaltrials.gov/ct2/show/results/NCT02255552?term = eteplirsen&cond = Duchenne±Muscular ±Dystrophy&draw = 2&rank = 10). A total of 109 patients were included in the trial, 79 in the eteplirsen treatment group, and 30 in the control group. At baseline, the groups were well matched for age (9.1 vs 8.8 years old, respectively). At 96 weeks, one patient from the eteplirsen group had withdrawn from the study, while 15 patients from the control study had withdrawn, 11 to participate in interventional studies. At 96 weeks, data on functional analyses were available for 9 patients for the control group, and for 61–67 of the eteplirsen-treated patients. For the primary endpoint (6-min walk distance), the eteplirsen-treated group on average walked 117.91 meter less than at baseline, while the control group on average walked 133.56 m less. The difference (15.65) is less than the clinically meaningful cutoff of 30 m [24,25].

Several secondary endpoints were included as well. Dystrophin quantification was performed in muscle biopsies obtained from 16 eteplirsen-treated patients at baseline and after 96 weeks of treatment, and revealed an average increase of 0.516%. The total score for the North Star Ambulatory Assessment (NSAA) decreased 7.23 and 8.44 in the eteplirsen and control group, respectively, while 33/61 (54%) assessed patients in the eteplirsen-treated group were able to independently rise from the floor as assessed by the NSAA compared to 3/9 (33%) of the control group. Forced vital capacity decreased by 3.413% and 2.461% in the eteplirsen and control groups, respectively. Finally, 12/67 (18%) eteplirsen-treated patients lost ambulation during the 96 week treatment period, compared to 1/12 (5%) in the control group.

With regard to safety, there were no deaths during the trial. Adverse events and severe adverse events were reported more frequently in the eteplirsen group than the control group (13.9% vs 6.7% for severe adverse events and 98.7% vs 80% for adverse events). Each of the severe adverse events occurred in only 1 or 2 of the 79 treated patients. Adverse events occurring in >20% patients in the eteplirsen group but not in the 30 controls include vomiting (49%), nausea (22%), procedural pain (32%) and skin abrasions (27%).

While it is difficult to draw strong conclusions due to the very small number of patients in the control group, it seems that treatment was well tolerated. There appears to be no strong treatment effect of eteplirsen, as there was clear functional decline in the eteplirsen-treated group over the course of 96 weeks measured with several functional outcome measures.

There can be several reasons for this. Possibly, the dystrophin levels induced by treatment (<1%) are too low to significantly slowdown disease progression in these patients. Notably, the patients in this trial were on average 9 years old, meaning the disease had already advanced to a moderate extent. It is likely that restoring dystrophin in younger patients with a less advanced disease stage will have more of an impact. To study this, clinical trials in young (4–6 year old, NCT02420379) and very young (6–48 months old, NCT0318995) patients were initiated. Furthermore, it is possible that the dose used is too low and that higher doses will lead to higher levels of dystrophin restoration and more therapeutic effects. To this end a clinical trial with higher doses has been initiated (NCT03992430). Finally, it is possible that there are effects on arm function, which is something that can also be measured in patients with a more advanced disease. An open-label clinical trial to evaluate safety in with more advanced disease (aged 7–21) is conducted as well (NCT02286947). Results have been submitted to clinicaltrials.

Table 2. Overview of clinical trials with AONs for DMD exon skipping.

Exon	Compound	Chemical modification	Status	Clinical trials	References
51	Drisapersen	2′-*O*-methyl phosphorothioate	Development terminated	NCT01153932 (Placebo-controlled phase 2 trial using subcutaneous injection of 6 mg/kg drisapersen per week or saline for 48 comparing two different regimen, completed) NCT01462292 (Placebo-controlled phase 2 trial comparing weekly treatment with 3 or 6 mg/kg drisapersen or saline with weekly subcutaneous injections for 24 weeks, completed) NCT01254019 (Placebo-controlled trial phase 3 trial, comparing weekly subcutaneous treatment of 6 mg/kg drisapersen or placebo for 48 weeks, completed) NCT01128855 (Open label single dose escalation safety study testing subcutaneous injection of 3, 6, 9 and 12 mg/kg drisapersen, completed) NCT01910649 (Open label extension study for patients involved in a previous dose finding study for drisapersen, using weekly subcutaneous or intravenous doses of 6 mg/kg drisapersen, terminated) NCT02636686 (Open label extension study using 6 mg/kg drisapersen subcutaneously per week for patients previously involved in other drisapersen trials – alternative regimens: 3 or 6 mg/kg intravenous dosing or 8 weekly subcutaneous doses of 6 mg/kg followed by a 4 week treatment break, terminated) NCT01803412 (Open label extension study for North American patients previously involved in drisapersen trials, using weekly dosing of 6 mg/kg drisapersen subcutaneously, or 3 mg/kg or 6 mg/kg intravenously, terminated) NCT01803412 (Open label extension study for patients previously involved in drisapersen studies, receiving 6 mg/kg drisapersen subcutaneously using weekly or intermittent dosing, terminated)	[26–30,32]
	Eteplirsen	Phosphorodiamidate morpholino oligomer	FDA approved	NCT00159250 (intramuscular injection; completed) NCT00844597 (dose finding study (0.5, 1, 2, 4, 10 and 20 mg/kg weekly IV for 12 weeks, completed) NCT01396239 (30 and 50 mg/kg weekly IV for 24 or 48 weeks, completed) NCT01540409 (open label extension study for NCT01396239 study, completed) NCT02255552 (96 week open label, untreated control with non-eligible mutations; completed) NCT02420379 (open label study 96 weeks with weekly 30 mg/kg eteplirsen IV in 4–6 year old patients, completed) NCT03218995 (in patient dose titration (2, 4, 10, 20 and 30 mg/kg eteplirsen) with weekly IV injections for 96 weeks, in 6–48 months old patients, ongoing, recruitment complete) NCT03985878 (open label extension study for patients previously treated with eteplirsen in trial NTC03218995) NCT03992430 (double-blind study assessing weekly IV injections of 30 mg/kg and two higher doses of eteplirsen for 144 weeks, ongoing) NCT02286947 (96 week open label study in 7–21 year old patients, receiving weekly injections of 30 mg/kg eteplirsen, completed) NCT04179409 (by invitation, for single exon 45, 51 and 53 duplications; patients receive weekly intravenous injections with the relevant compound for 1 year, ongoing)	[10,15–22]
	Suvodirsen	Stereopure 2′-O-methyl phosphorothioate based	Development terminated	NCT03508947 (Dose escalation trial comparing different doses (0.5, 1, 2, 5, 7 and 10 mg/kg) of weekly intravenous suvodirsen injections for 12 weeks, completed) NCT03907072 (Placebo controlled trial comparing 3.5 and 5 mg/kg suvodirsen using weekly intravenous injections or placebo for 48 weeks, terminated)	
	SRP50-51	Peptide conjugated eteplirsen	Dose finding clinical trials ongoing	NCT03375255 (Open label single treatment dose finding study evaluating intravenous injection of one of 5 different doses of SRP-505, completed) NCT03675126 (Open label extension study for NCT03375255 using a selected intravenous dose at an as yet unspecified dosing frequency, ongoing) NCT04004065 (Two stage study, involving dose finding study using monthly intravenous injections of different doses of SRP-5051 for 12 weeks, followed by a second stage where stage 1 patients and additional patients will be treated with monthly intravenous injections of the selected dose for 24 weeks, ongoing)	

(*Continued*)

Table 2. (Continued).

Exon	Compound	Chemical modification	Status	Clinical trials	References
53	Golodirsen	Phosphorodiamidate morpholino oligomer	FDA approved	NCT02310906 (two-stage clinical trial, stage 1: placebo-controlled in patient dose escalation study (4, 10, 20 and 30 mg/kg intravenous) for 12 weeks, stage 2: all patients from stage 1 receive weekly 30/mg/kg golodirsen for up to 168 weeks, control arm is an untreated group not benefiting from exon 53 skipping but evaluated with the same functional outcome measures; completed) NCT02500381(placebo controlled trial comparing weekly intravenous treatment with 30 mg/kg golodirsen or 30 mg/kg casimersen or placebo for 96 weeks in eligible patients; all patients receive golodirsen/casimersen from week 97–144 in the open label part of this study, ongoing) NCT03532542 (open label extension study for patients previously treated with golodirsen or casimersen, involving weekly intravenous dosing of 30 mg/kg for up to 144 weeks, ongoing) NCT04179409 (by invitation, for single exon 45, 51 and 53 duplications; patients receive weekly intravenous injections with the relevant compound for 1 year, ongoing)	[35,36,38]
	Viltolarsen	Phosphorodiamidate morpholino oligomer	Approved by Japanese Ministry for Health, Labor and Welfare	NCT02081625 (open label dose finding study testing 1.25, 5 and 20 mg/kg weekly intravenous dosing of viltolarsen for 12 weeks, completed) NCT02740972(placebo-controlled dose comparison study testing weekly dosing of 40 mg/kg or 80 mg/kg viltolarsen for 24 weeks, the placebo group receives saline in week 1–4, followed by 20 weeks of weekly treatment of viltolarsen, completed) NCT04060199 (placebo-controlled trial testing weekly 80 mg/kg viltolarsen infusions or placebo for 48 weeks, ongoing) NCT04337112 (open label study for patients previously treated with viltolarsen, weekly doses at 80 mg/kg intravenous, ongoing)	[37,39]
	PRO-053	2′-*O*-methyl phosphorothioate	Development terminated	NCT01957059 (Dose finding study with weekly subcutaneous or intravenous dosing of PRO-053, terminated)	
45	Casimersen	Phosphorodiamidate morpholino oligomer	New drug application submitted to FDA	NCT02530905 (dose escalation study using unspecified doses of casimersen or placebo, followed by an open label phase in all patients, completed) NCT02500381(placebo controlled trial comparing weekly intravenous treatment with 30 mg/kg golodirsen or 30 mg/kg casimersen or placebo for 96 weeks in eligible patients; all patients receive golodirsen/casimersen from week 97–144 in the open label part of this study, ongoing) NCT03532542 (open label extension study for patients previously treated with golodirsen or casimersen, involving weekly intravenous dosing of 30 mg/kg for up to 144 weeks, ongoing) NCT04179409 (by invitation, for single exon 45, 51 and 53 duplications; patients receive weekly intravenous injections with the relevant compound for 1 year, ongoing)	
	Renadirsen (DS-5141b)	2′-O-methyl/ethylene bridged-nucleic acid chimera with a phosphorothioate backbone	In trials	NCT02667483 (dose escalation study testing 0.1, 0.5, 2 and 6 mg/kg weekly subcutaneous injections for 2 weeks, followed by 12 weekly injections of 2 selected doses, followed by an open label phase where patients receive weekly subcutaneous doses of renadirsen for up to 48 weeks, ongoing) NCT04433234 (open label extension study for patients previously treated with renadirsen, patients will be treated with weekly subcutaneous injections of 2 or 6 mg/kg renadirsen, not yet recruiting)	
	PRO-045	2′-*O*-methyl phosphorothioate	Development terminated	NCT01826474 (dose escalation study comparing 0.15, 1, 4, 6 and 9 mg/kg weekly subcutaneous injections of PRO-045, terminated)	
44	PRO-044	2′-*O*-methyl phosphorothioate	Development terminated	NCT01037309 (dose escalation study comparing 5 weekly doses of 0.5, 1.5, 5, 8 10 and 12 mg/kg PRO-044 subcutaneous or 1.5, 5 or 8 mg/kg intravenous, completed) NCT02958202 (open label extension trial for patients previously treated with PRO-044, using weekly subcutaneous treatment of 6 mg/kg or intravenous treatment of 6 or 9 mg/kg, terminated)	
2	AT702	Modified U7 snRNP delivered using adeno associated viral vectors	In trials	NCT0420314 (open label trial where patients will be treated with a single intravenous injection of 2.10^{13} viral genomes/kg, ongoing)	

gov, showing a similar safety profile compared to that observed in younger patients.

In an effort to improve delivery, Sarepta is performing dose escalation safety trials with SRP-5051 (NCT03375255, NCT03675126, NCT04004065), which consists of eteplirsen conjugated to an arginine rich peptide (see section 3 for more details on conjugates).

2.2.2. Drisapersen and suvodirsen

Drisapersen and suvodirsen have been evaluated in clinical trials as well, but clinical development of these compounds was stopped. Drisapersen was a 2OMePS AON developed by Prosensa Therapeutics, GlaxoSmithKline and Biomarin and tested in over 300 DMD patients, in various placebo-controlled trials (see Table 2). While treatment seemed to slowdown disease progression in early-stage patients, no benefit was observed in a phase 3 clinical trial (NCT01254019) involving patients in the early and late ambulant stages [10,26–31]. Furthermore, injection side reactions and proteinuria were observed in almost all patients treated for longer durations, while thrombocytopenia was observed in a subset [26,28,29,32,33]. Regulatory approval was sought but not given by FDA and the application to EMA was withdrawn and the clinical development terminated [10].

Suvodirsen is a stereopure 2OMePS AON containing also undisclosed nucleotide modifications, that was developed by Wave Life Sciences. The phosphorothioate modification gives each nucleotide chirality. When this is not controlled, the backbone can be in the D- or S-orientation at each phosphorothioate linkage in an AONs [34]. Thus, a 20mer AON actually is a mix of 2^{19} different isomers. For suvodirsen, the chirality of each phosphorothioate bond was specified, which was proposed to result in a more effective AON, as suggested by preclinical studies in cell models and mice. However, dystrophin analysis in biopsies from patients treated for 12 weeks with weekly intravenous injections revealed no increase in dystrophin levels (NCT03508947). Following this result, the clinical development of suvodirsen was terminated.

2.3. Exon 44, 45 and 53 skipping

Exon 45 and exon 53 skipping trials have been initiated as well (see Table 2). Two exon 53 skipping PMOs, golodirsen (Sarepta Therapeutics) [35,36] and viltolarsen (Nippon Shinyaku) [37], are currently approved by the FDA and the Japanese Ministry for Health, Labor and Welfare, respectively. Also, here approval was based on increases in dystrophin expression in biopsies obtained after treatment. Weekly intravenous treatment with 30 mg/kg golodirsen for 48 weeks resulted in an average increase of 0.9% of dystrophin as quantified by Western blotting [38]. For viltolarsen, weekly treatment with 40 mg/kg or 80 mg/kg for 24 weeks resulted in 5.3–5.4% dystrophin [39]. Notably, the two companies do not use identical Western blotting protocols, or normal control references (dystrophin levels can vary up to sixfold between healthy individuals [40]). As such, it is difficult to directly compare the two percentages. Furthermore, since viltolarsen is 4 nucleotides shorter than golodirsen, the 80 mg/kg viltolarsen dose is more than threefold higher than the 30 mg/kg golodirsen dose at a per molecule basis. Placebo-controlled trials to assess functional effects of golodirsen and viltolarsen treatment are ongoing (NCT02500381 and NCT04060199).

For exon 45 skipping, casimersen, a PMO from Sarepta Therapeutics is developed furthest. In patients treated with weekly intravenous doses of 30 mg/kg casimersen dystrophin levels increased by 0.8% (https://investorrelations.sarepta.com/news-releases/news-release-details/sarepta-therapeutics-announces-positive-expression-results). Treatment was generally well tolerated. Transient iron deficiency and flushing were observed in some patients. Sarepta has filed for a new drug application with the FDA based on this finding. FDA has accepted this application and will provide regulatory action on casimersen in February 2021. In parallel placebo-controlled trials to evaluate functional effects of casimersen treatment are ongoing (NCT02500381).

Renadirsen, a 2′-O-methyl, ethylene bridged nucleic acid chimera with a phosphorothioate backbone targeting exon 45 is in development by Daiichi Sankyo [41]. In a first trial using 12 weekly subcutaneous infusions of 2 or 6 mg/kg in 7 patients, renadirsen was well tolerated and induced exon 45 skipping in all patients but failed to induce dystrophin expression (https://www.daiichisankyo.com/media_investors/media_relations/press_releases/detail/006840.html). Currently an open-label study where patients are treated for 48 weeks is ongoing (NCT02667483).

PRO-053, PRO-045, and PRO-044, 2OMePS AONs targeting exon 53, exon 45 and exon 44 were being evaluated in a dose escalation trials (Table 2). However, when the development of drisapersen was terminated, BioMarin terminated the development of the other compounds as well.

2.4. Clinical development for small mutations and duplications

So far, the focus has been on patients carrying the most common mutation type for DMD, a deletion of one or more exons. Exon skipping can also restore the reading frame for other mutation types (as outlined in detail in [8]). For small mutations that disrupt the reading frame or substitutions that introduce stop codons, skipping the mutated exon can bypass the mutation, while maintaining the reading frame if the mutated exon is in-frame. However, the small mutations are dispersed over the exons and therefore skipping individual in-frame exons applies to very small groups of patients and is currently not in clinical development. For small mutations in out-of-frame exons, generally a combination of two or three exons needs to be skipped. Thus far, no clinical trials are conducted using a combination of AONs.

For duplications, the situation is more complicated, since the AONs will target both the original and the duplicated exon. There are rare cases where the duplication can be reframed skipping an unduplicated exon [42]. However, generally, the target exon will be present twice, where skipping the one of the duplicated exons will restore the reading frame,

while skipping the other exon will disrupt it. The exception is a single exon duplication. Here skipping either one of the exons will restore production of normal dystrophin mRNA. However, when skipping is too efficient and both exons are skipped, the reading frame will be disrupted. In cell cultures, generally both exons are skipped [43,44], although for some exons using lower AON doses can increase the amount of single exon skipping [44,45]. It is anticipated that in vivo the efficiency of AON delivery is much more limited and that it is very unlikely that two AONs target the same dystrophin transcript in one nucleus [8]. As such, single exon duplications make good targets for exon skipping. Sarepta is currently conducting a clinical trial where patients with a single exon duplication of exon 45, 51, or 53 are treated with casimersen, eteplirsen, and golodirsen, respectively (NCT04179409).

The most common duplication mutation is a duplication of exon 2 [12,46]. Here, the suitability of exon skipping may be even better, since skipping both exons 2 still allows dystrophin production, since in a dystrophin mRNA lacking exon 2, an alternative translation initiation site in exon 5 is activated [47]. Deletion of one of the exons even results in the production of a normal dystrophin transcript in cells where the exon 2 duplication was present [45] Audentes and Astellas are jointly conducting a trial for an exon 2 skipping strategy (NCT04240314). Here, an adeno-associated viral (AAV) vector will be used to deliver a *U7 snRNP* gene, where the regular antisense sequence hybridizing to histone RNA, is replaced by a sequence targeting an exon of choice [48], in this case exon 2. This approach precludes repeated dosing, since the 'antisense gene' will be expressed as long as the transgene is present in the skeletal muscle cells. Audentes is also performing preclinical optimization for exon 51 and exon 53 skipping constructs using the same platform (https://www.audentestx.com/duchenne/).

3. Improving delivery of exon skipping compounds

While 3 AONs have now been approved, it is clear that delivery is suboptimal, as evidenced by the low levels of dystrophin restoration in skeletal muscle biopsies. Notably, preclinical studies show that most likely the effects in cardiac muscle is even lower since AON uptake by heart is very poor [49–55]. To achieve improved uptake of AONs into skeletal and heart muscle several approaches have been tested over the years. The most important ones are using different oligonucleotide chemistries, conjugating peptides, or ligands to the AONs and using nanoparticles.

3.1. Chemical modifications and formulations

As mentioned, different chemical modifications can be used to induce exon skipping, each having their own properties. One modification that has shown promising pre-clinical results is the tricyclo-DNA (tcDNA). tcDNA nucleotides have an extremely high affinity for target RNA, allowing the use of very short AONs, of 13–15 nucleotides. Systemic treatment of *mdx* mice with 200 mg/kg of a 15-mer tcDNA with a phosphorothioate backbone for 12 weeks resulted in high exon skipping and dystrophin levels 20–40% in skeletal muscle, but importantly also diaphragm and heart [56]. Treatment also resulted in exon skipping and dystrophin levels in brain. While these levels were very low (<5%), this did improve the behavior phenotype of the *mdx* mice, e.g. reducing the fear response. Treatment of the severely affected dystrophin-utrophin double knockout mouse rescued the phenotype [56]. While treatment with the exon 23 tcDNA was well tolerated by the *mdx* mouse model [57,58], a tcDNA specific for human exon 51 resulted in acute toxicity after intravenous injection, characterized by complement activation, platelet activation, and increased coagulation times. These toxic effects appear to be the result of the sequence-specific formation of homo-dimers. By generating targeted mismatches in the tcDNA, the formation of homo-dimers could be avoided. Due to the very high affinity of tcDNA to the target, these mismatches do not prevent efficient exon skipping [59]. Currently, preclinical and toxicity studies are performed to prepare for clinical trials.

It has also been shown feasible to increase muscle delivery of AONs by formulating them in hexose solutions [60,61] or glycine solutions [62]. This increase was observed for both PMOs and 2OMePS AONs for hexose and, the effect was shown to be related to the metabolic state of the *mdx* muscle, which due to active and continuous muscle regeneration has a very high demand for nutrients. The increased uptake was not observed in wild type mice [61]. While DMD patients show some hypertrophy at the earliest stages of their disease, the regenerative capacity of human muscle is unfortunately much less than that of the *mdx* mouse model. As such, most likely the uptake benefit will be much less in humans than mice. For the glycine formulation, only PMOs were used. Here uptake is facilitated by the facts that glycine increases regeneration in *mdx* mice [62], and that PMOs rely on active regeneration for uptake [63].

3.2. Conjugates

Cell-penetrating peptides (CCPs) are peptides able to penetrate cell membranes without the need of receptor binding. This can be achieved either by endocytosis or in an energy-independent manner [64]. When conjugated to an AON, CCPs could therefore help deliver the AON more efficiently. Positively charged, arginine-rich, short CPPs were found to significantly improve the uptake of PMO AONs in skeletal muscle and heart, leading to vastly improved exon skipping and dystrophin levels in DMD mouse models [49,52,55,65–68]. An overview of conjugates reported to be promising can be found in Table 3. Notably, it is only possible to conjugate positively charged peptides to charge neutral AONs, as conjugating them to negatively charged AON is obviously challenging.

One of the earlier studies demonstrating that an arginine-rich peptide conjugated to a PMO restored dystrophin levels in both skeletal muscles and heart after a single intravenous injection in *mdx* mice involved the peptide (RXRRBR)2XB, (R = arginine, X = 6-aminohexanoic acid, and B = β alanine)

Table 3. Overview of peptides tested for AON conjugation to increase uptake in target tissues.

Peptide (alias)	CCP or homing peptide	Comments	Reference
RxR4	Arginine rich CCP	First CCP to be conjugated to a PMO targeting exon 23 in *mdx* mice. Increased exon skipping in the heart and restored dystrophin levels were observed.	[68]
(RXRRBR)2XB (PMOE23)	Arginine rich CCP	Conjugation to PMO resulted in high levels of dystrophin expression in skeletal muscle and heart in *mdx* mice.	[66]
ASSLNIA	Muscle and heart homing peptide	One of the first peptides found with high affinity to skeletal and cardiac muscle. This homing peptide conjugated to a PMO could not increase AON uptake or exon skipping in the *mdx* mouse.	[67,79]
Pip2 (a and b) a: ((R-Ahx-R) 3IdKILFQNdRRMKWHKBC) b: (R-Ahx-R) 3IHILFQNdRRMKWHKBC	Pip series CCP	Pip2a and pip2b conjugates showed increased number of dystrophin positive fibers compared to unconjugated control and RxR4 conjugates and pip1 conjugates in the *mdx* mouse.	[72]
Pip5 (e) RXRRBRRXR-ILFQY-RXRBRXRB	Pip series CCP	Pip5e-PMO resulted in restored dystrophin levels in skeletal and cardiac muscle in the *mdx* mouse.	[55]
Pip6 (a) RXRRBRRXR-YQFLI-RXRBRXRB	Pip series CCP	Pip6-a-PMO could significantly increase dystrophin levels in heart compared to Pip5e-PMO in *mdx* treated mice.	[65,69]
SKTFNTHPQSTP (T9)	Muscle and heart homing peptide	Limited internalization in muscle cells was found.	[82]
LGAQSNF (P4)	Muscle and heart homing peptide	Conjugation of this peptide to a 2OMePs AON improved uptake in muscle and heart and significantly increased exon skipping in heart in the *mdx* mouse.	[80]
PGAQSNF (P5)	Heart and muscle homing peptide	This peptide was not taken up by myoblasts efficiently and not further tested in vivo	[80]
RRQPPRSISSHP (M12)	Muscle Homing peptide	A PMO-M12 conjugate restored dystrophin levels in skeletal muscles of the *mdx* mouse.	[83]
CQLFPLFRC (CyPep10)	Heart Homing peptide	Conjugated to an a 2OMePs AON increased the uptake of the AON in *mdx* mice.	[81]

[66]. Immunohistochemical analysis two weeks after a single dose of 30 mg/kg conjugated-PMO showed homogenous dystrophin expression in all skeletal muscle fibers analyzed, whereas only 5% or less fibers showed dystrophin expression when unconjugated PMO was used. Western blot analysis revealed that levels of dystrophin in muscles were up to 91–100% after treatment of PPMOE23. For the cardiac muscle high levels of dystrophin expressing fibers were also found (94%), but expression was not in all areas; myofibers near the ventricles and papillary muscles lacked high dystrophin expression. Western blot analysis revealed 58% normal dystrophin levels in heart. This same study also showed that increased dystrophin levels resulted in restored functionality of skeletal muscles and heart function. In a more severely affected mouse model for DMD, the dystrophin-utrophin double knockout mouse, these peptide conjugated-PMOs were also studied. Intraperitoneal injections of a exon 23 targeting PPMO at 25 mg/kg/week for 6 weeks induced dystrophin levels up to ~80% in muscle tissues but not heart. In severely affected *mdx* utrophin knockout mice, treatment increased survival of the treated mice by over a year [70]. This showed the clear potential of CCPs in a more severely affected model. Initial positive results using arginine-rich CCPs initiated research into additional peptides for improving AON delivery. A vast array of CCPs has been identified over the years of which Guidotti and colleagues give a structured overview of the most characterized peptides [71].

A family of CCPs developed later, are the PMO internalization peptides (Pips), which are peptides with a hydrophobic core flanked by arginine-rich domains containing amino hexanoic (X) and β-alanine (B) spacers [72,73]. Several series of Pips have been synthesized to test which sequence could enhance AON uptake best. Pip5 and Pip6 have shown most promising results, demonstrating high levels of exon 23 skipping in the *mdx* mouse. Single-dose administration of 25 mg/kg of a pip5 conjugated to a PMO (Pip5e-PMO) resulted in restored dystrophin levels in skeletal and importantly also cardiac muscle [55]. In the heart, more than 90% of the fibers were dystrophin positive and >80% dystrophin positive fibers were detected in skeletal muscle groups analyzed. Western blot analysis confirmed dystrophin expression restoration, showing >50% of normal levels of dystrophin protein in the heart. Subsequently, Pip6 peptides were developed by implementation of different modifications of the Pip5 series with the aim to be more efficient at lower doses. These studies revealed that the core sequence length is more important for the activity of the Pip as opposed to core sequence itself [65]. Since AONs generally reach cardiac muscle less easily than skeletal muscle, the field has tried to find a conjugate that improved uptake mainly in heart. It was reported a single dose of 12.5 mg/kg of Pip6-a conjugated to PMO increased

dystrophin expression significantly more in heart than the best performing Pip5-PMO [65]. These positive results using arginine-rich peptide conjugates in mice suggested that this would be a promising approach to improve AON delivery in DMD patients. However, most of the positively charged CCPs induce toxicity in higher animals, i.e., monkeys at doses needed for a therapeutic effect [74]. A delicate balance needs to be found between the electrostatics of the peptide conjugates, the ability to deliver the AON and the safety aspects. The Pip7, 8 and 9 series contain less arginine residues, hoping to reduce toxicity, while maintaining good delivery. In vivo studies are still being carried out testing these newest generations of Pips [75]. The aforementioned SRP50-51 (Table 2) was developed by Sarepta Therapeutics with the same aim. The critical question is whether these CPPs will achieve exon skipping at good levels at a dose that does not result in toxicity in humans.

3.3. Muscle and heart homing peptides

While CPPs improve delivery in general, they do not prevent that most AONs are taken up by liver and kidney. Conjugating a ligand to the AONs that directs them to skeletal muscle and heart is an obvious way to increase delivery. However, the challenge lies in finding these peptides. One way to achieve this is using phage display libraries and biopanning experiments. Here a library of phages, each displaying a unique peptide on their surface is incubated with a target molecule, cell cultures, or injected into an animal model. Generally, libraries are enriched with multiple biopanning rounds, after which the library is sequenced and overrepresented peptides are selected as molecule or tissue-specific peptides. Using this approach, a number of muscle and heart homing peptides have been identified (Table 3).

However, several precautions have to be taken when performing biopanning experiments, to avoid identifying false positives. Databases like PepBank [76] and SAROTUP [77] can be consulted to find peptides that are presently known be non-target specific binders, e.g. those with a preference to plastics that are used throughout the biopanning experiments. Finally, the sequencing depth can be increased using NGS, allowing for a lower amount of biopanning rounds, and the unselected (naïve) library can be compared to experimental libraries after biopanning, thus reducing advantage biases due to overrepresentations of phages in the naïve library [78]. Moving forward it is important to keep record of the peptides that have been found and tested, and their results so that future studies testing conjugates can select as effectively as possible for new tests.

A study in 1999 identified a candidate muscle-homing peptide with the amino acid sequence ASSLNIA, using a biopanning experiment in mice [79]. Unfortunately, it was later found that conjugating this peptide to a PMO did not increase delivery of PMOs or exon skipping levels in *mdx* mice [67]. Aiming to find specific heart homing peptides for conjugation to negatively charged 2OMePS AONs our own lab identified the peptide LGAQSNF using biopanning. Conjugating this peptide to a 2OMePS AON significantly increased exon skipping levels in heart and diaphragm in the *mdx* mouse, albeit at modest amounts for ~20% [80]. Levels of this AON-conjugate were higher in all analyzed tissues, but the relative uptake in skeletal muscle and heart, compared to liver and kidney was improved.

In another effort to find muscle homing peptides, the focus of our group was shifted to cyclic peptides, which were thought to have higher binding affinity due to their conformational restrictions [81]. One cyclic peptide (CQLFPLFRC) was identified as a candidate for muscle homing. Conjugation of this homing peptide increased the uptake of a 2OMePS AON by a twofold in several skeletal muscles and near threefold for heart and diaphragm. Exon skipping levels were also increased by a twofold compared to the unconjugated AON. Dystrophin levels were too low to be reliably quantified for control and conjugated AONs. Interestingly, a similar increase in uptake was seen for another peptide (CLNSLFGSC) conjugated to the 2OMePS. However, here no increases in exon skipping were detected, suggesting that the AON was not delivered to the functional compartment of the cell (nucleus), but likely stuck in the interstitium of the heart and skeletal muscle.

Another muscle and heart homing peptide that was identified but unfortunately resulted in limited internalization in muscle cells is SKTFNTHPQSTP [82]. A muscle homing peptide (RRQPPRSISSHP) conjugated to a PMO AON has also been tested [83]. The peptide-PMO conjugate was found to preferentially bind to skeletal muscles in *mdx* mice after systemic administration. In skeletal muscles dystrophin levels were 3 to 10-fold higher compared to a control PMO after *mdx* mice were treated for 3 weeks with a weekly 25 mg/kg injection. Grip strength was enhanced for the peptide-PMO treated mice. Unfortunately, no increase was observed present for cardiac muscle, limiting the clinical applicability of this homing peptide.

3.4. Nanoparticles

The use of nanoparticles to act as vehicle for delivery has also been under investigation for enhanced AON uptake. T1 nanospheres consist of a core of polymethylmethacrylate (PMMA) and shell with cationic groups. These characteristics make T1 ideal for binding to AONs, eliminating the need for encapsulation. Encapsulation systems rely on degradation of the matrix for compound release, which can be slow and can reduce drug activity because of degradation inside the delivery system [84]. Using T1 nanoparticles to deliver a 2OMePs AON to the *mdx* mouse model resulted in restored dystrophin expression in skeletal muscles and to some extent in heart after weekly intraperitoneal (IP) injections, which was not found when only the AON (i.e. naked AON) was administered using the same dose [85]. Researchers assume that the ability to use low doses and still induce effect, was partly because of the slow release that T1 particles are responsible for. However, the T1 nanoparticles also biodegrade slowly, which could potentially lead to accumulation and therefore

possible adverse effects. Furthermore, T1 nanoparticles formed aggregates, which make them unsuitable for intravenous administration.

ZM2 nanoparticles also consist of a core of PMMA and have shell containing random copolymers derived from N-isopropylacrylamide (NIPAM) and reactive methacrylate-bearing cationic groups. Intraperitoneal delivery of ZM2 encapsulated AONs also resulted in higher exon skipping and dystrophin restoration levels in skeletal muscle and heart in *mdx* mice than after delivery of naked AONs did not [86]. The percentages of exon skipping were roughly doubled compared to T1-AON treatment [85]. Additionally, it was later found that after a 7 week course of weekly intraperitoneal administration of ZM2-AON in *mdx* mice, dystrophin restoration was still detectable 90 days later in skeletal muscles [87]. However, despite these early promising results, no further reports have appeared for ZM2 nanoparticle mediated delivery of AONs for DMD.

4. Genome editing

The challenge of AON-mediated exon skipping is that the effect is transient. With turnover of the AONs, targeted transcripts and restored dystrophin protein, repeated AON treatment is required. Currently, patients receive weekly intravenous infusions. Targeting the DNA would generate a permanent effect, since then each transcript generated from the targeted gene would be in-frame.

Several methods to edit the *DMD* gene have been studied (Table 4). These methods all make use of the fact that generating double-stranded DNA breaks trigger the DNA repair system. Since skeletal muscle is post-mitotic, only the non-homologous end joining (NEJM) pathway will be utilized. NEJM does not allow an error-free repair, but can be used to reframe the reading frame, by, e.g., corrupting a splice site or deleting an exon. Homology-directed repair (HDR) in combination with a donor clone has been studied, since this approach would be less error-prone and restore production of normal dystrophin. A recent study revealed that efficiency of gene repair using HDR in DMD models is very low [88]. Myoblasts from a golden retriever muscular dystrophy dog model were treated with genome editing compounds and a homology template, which resulted in minimal or no dystrophin production.

The first attempt at gene editing in the *DMD* gene used meganucleases. Proof of principle was shown by insertion of meganuclease target sites in the middle of a dog microdystrophin plasmid containing a frame-shift mutation that was transfected into 293 FT cells [91]. Overexpression of this meganuclease was able to induce small indels at these target sites, which restored the reading frame and microdystrophin. This same approach was applied later in rag/*mdx* mouse and human myoblasts where restoration of microdystrophin in the muscle fibers and myoblasts was observed [92]. Later a meganuclease was designed to cleave within intron 44 in patient-derived myoblasts, just upstream of the deletion hotspot. Repair matrixes carrying exon 45–52 were co-transduced into patient-derived myoblasts carrying a deletion of exon 45–52. This resulted in the production of full-length dystrophin mRNA [93]. However, an important drawback of meganucleases is that the recognition site has to be introduced in the desired gene. Whilst this does ensure low off-target nuclease activity, first having to genetically modify the target gene has its own risk of off-target effects and is very impractical in a clinical setting.

DNA binding domains that are amply present in the human genome are those targeted by zinc fingers. Zinc finger nucleases are thus a more straightforward approach to achieve targeted editing. For DMD, zinc finger nucleases have been used to delete exon 51 from the *DMD* gene in myoblasts of DMD patients [94]. However, some off-target effects were also found. To increase specificity, several zinc finger domains can be combined, but this does not eliminate non-specificity completely due to context dependency. Recognition and cleavage of the target sequence is not only determined by the matching zinc finger domain but also by neighboring sequences. Furthermore, zinc finger nucleases sometimes have a bias for recognition of a particular sequence, making it harder to design them for any given target. Moreover, efficiency of the zinc finger delivery has proven to be relatively low [95].

Table 4. Overview of different genome editing studies for DMD therapy.

Approach	Study	Reference
Meganucleases	Proof of principle using of Meganucleases for DMD Knock in repair using meganuclease system	[91,93]
Zinc Fingers	Deletion exon 51 from the *DMD* gene in myoblasts of DMD patients.	[94]
TALENs	Targeting exon 51 in DMD patient derived fibroblasts or immortalized myoblasts cell lines which carried a deletion of exon 48–50. Targeting exon 45 used to correct the dystrophin gene of DMD patient induced pluripotent stem cells (iPSCs) that lacked exon 44.	[96,97]
CRISPR-Cas9	CRISPR-Cas9 mediated dystrophin restoration in patient-derived cell cultures Restoration of dystrophin function in hiPSC-Derived muscle cells. Local and systemic delivery of CRISPR-Cas9 components to target exon 23 in the adult *mdx* mouse. Resulting in successful (partial) recovery of dystrophin protein in muscle Successful gene editing in specifically skeletal and cardiac muscle in *mdx* mouse by utilizing CK8 regulatory cassette. Dystrophin protein expression restoration in cardiac muscle of *mdx* mice. Restored dystrophin expression after intramuscular injection and systemic administration in canine model for DMD. Creation of a DMD mouse model where exon 50 was removed which was then corrected by targeting exon 51. Postnatal treatment of mdx mice restores dystrophin production in heart and skeletal muscle and improves muscle function	[89,90,97–101,101–104]

Transcription activator-like effector nucleases (TALENs) offer an alternative. TALE domains consist of a repeat variable residue, which are tandem repeats of 34 amino residues. Each repeat variable residue can recognize only one nucleotide and TALEs suffer a lot less from context-dependency than zinc fingers. The TALEN system was utilized to target exon 51 in DMD patient-derived fibroblasts or immortalized myoblasts cell lines which carried a deletion of exon 48–50. Successful introduction of a small deletion in exon 51 could restore the reading frame and resulted in restored dystrophin expression in myoblasts [96]. TALEN was also used to correct the reading frame in DMD patient-derived induced pluripotent stem cells (iPSCs) that lacked exon 44 [97]. In this scenario, TALEN targeted exon 45 and successfully introduced a 1 bp deletion, restoring the dystrophin reading frame. However, this deletion was present for only 40 of the 229 iPSC clones analyzed, indicating that this process is not efficient.

With the development of the CRISPR/Cas9 system, it is now much more straightforward to delete desired exons on DNA level (Figure 2). The advantages of CRISPS/Cas9 compared to meganucleases and zinc finger nucleases are: easier design, because the target specificity is dependent on RNA–DNA interactions and not protein–DNA interaction. Furthermore, this system offers the possibility to modify several genomic sites at once, thus allowing the possibility to generate smaller and larger genomic deletions.

The first report of CRISPR/Cas9 mediated dystrophin restoration in patient-derived cell cultures was in 2015 [97]. Since then, many reports confirming this in cell and animal models have followed, including 3 back-to-back-to-back papers in Science in 2016 [98–100] (Table 4). In de *mdx* mouse model successful gene editing in specifically skeletal and cardiac muscle was reported when Bengtsson and colleagues used the muscle-specific CK8 regulatory cassette expressing guide-RNAs targeting either exon 52 or 53. Unique shorter transcript were identified though RT-PCR for both approaches and dystrophin protein levels were ~23% and ~8,5%, respectively, compared to wild type dystrophin levels [101]. Refeay and colleagues reported restoration of dystrophin in cardiac muscles of up to 40%, after they treated neonate *mdx/utrn*+/− mice with single systemic delivery of recombinant AAV to deliver CRISPR/Cas9 compounds targeting exon 23 [102].In a canine model for DMD, CRISPR/Cas9 targeted exon 51 and could restore dystrophin expression after intramuscular injection. Systemic delivery has yet only restored dystrophin levels in 1 dog when high doses of the AAV was used [103].

In 2016 a large deletion of 725 kb mimicking and exon 45–55 deletion was achieved in hiPSCs through using guide RNAs targeting intron 44 and 55, which restored dystrophin in both skeletal muscles and cardiomyocytes derived from the treated hiPSCs [104]. Notably, the majority of DMD mutations are located between exon 45 and 55 and therefore this approach would apply to a larger group of mutations, as was confirmed in patient-derived myoblast cultures with varying mutations [105].

CRISPR/Cas9 has also been applied to create a DMD mouse model where exon 50 was deleted. This model displayed severe muscle dysfunction, which researchers were able to correct by targeting genomic sequences adjacent to the splice acceptor of exon 51, which resulted in restoration of 90% of normal dystrophin expression in skeletal and cardiac muscles of the mouse [105]. Later, CRISPR/Cas9 was used to generate a mouse model lacking exon 44 [106]. Then, the CRISPR/cas9 approach was used to induce single-cut CRISPR gene editing to restore dystrophin protein expression in muscles and also heart. Four 4 weeks after treatment with a single dose of gene-editing components, dystrophin levels of up to 90% were measured. Notably, off-target effects were evaluated as well. A list of top 10 potential off-target sites was developed and off-target effects were measured by sequence analysis of these sites. No off-target effects were found at these sites.

For successful CRISPR/Cas9 mediated genome editing, the Cas9 protein and guide RNAs need to be delivered to the same cell. Since AAV have only a limited capacity, often delivery is accomplished with separate vectors. However, researchers have accomplished delivering both gRNA and Cas9 using an adenoviral vector [107].

While results are promising, several challenges for genome editing using the CRISPR-Cas9 system are still to overcome. First, the Cas9 often induces also off-target effects, where DNA is altered at undesired locations [108,109]. Furthermore, adenoviral vectors, which can accommodate both the Cas9 and guide

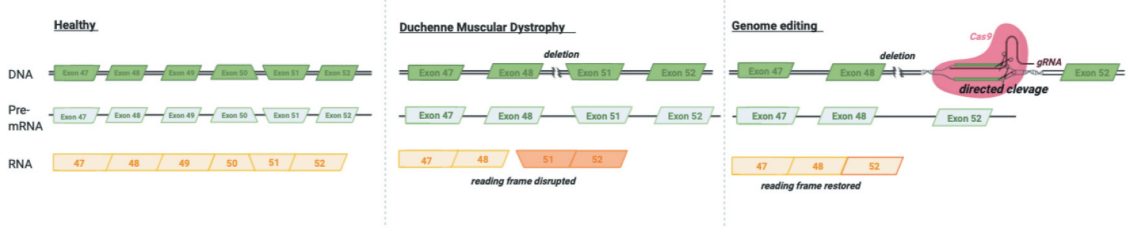

Figure 2. Genome editing for DMD.

Left panel: Unaffected individual. Middle Panel: DMD patient. A deletion of exon 49 and 50 in the *DMD* gene causes a frameshift leading. Protein translation is prematurely truncated. Right panel: Genome editing the *DMD* gene using the CRISPR/Cas9. DNA adjacent to the deletion site is cleaved out of the DNA by usage of a guide RNA (gDNA) and the Cas9 protein, therefore the reading frame is restored in each transcript produced, allowing the production of partially functional dystrophin.

RNAs, have reduced tropism for muscle cells and muscle progenitor cells and are therefore suboptimal for targeted delivery in mature skeletal and cardiac muscle cells [107]. Also, the promise of permanent change can be debated. Restoring the reading frame using gene editing would result in partially functional dystrophin protein production, which cannot completely impede muscle damage. Muscle damage will hopefully progress at slower rates but at some point, accumulated muscle damage becomes so high that genome-edited nuclei will be lost [110].

5. Expert opinion

Clear advances have been made since reading frame restoration was first proposed as a possible therapy for DMD, with three approved AONs and many clinical trials ongoing. However, there is definitely room and need for improvement as currently approved AONs restore only very low dystrophin levels. The question is whether these levels are sufficient to slowdown disease progression. Unfortunately, this is not a question with a straightforward answer [40]. Often estimates are made based on dystrophin levels seen in BMD patients, who have a slower disease progression even when they produce levels of ~10% dystrophin. The fact that some dystrophin is better than none, is further underlined by the fact that DMD patients who have a deletion flanking exon 44, which is spontaneously skipped at low levels, have a slower disease progression compared to other DMD patients, due to very low dystrophin levels [40]. However, these BMD and DMD patients express dystrophin since birth, while in DMD patients treated with AONs, dystrophin is restored only from the time of intervention. Reading frame restoration therapies rely on the expression of the *DMD* gene, which occurs in skeletal and heart muscle but not in fibrotic and adipose tissues. Thus, with time, the amount of target transcripts will reduce due to muscle wasting. It is therefore important to intervene as early as possible. Furthermore, muscle quality in patients will vary due to e.g. genetic variations that reduce inflammation or the tendency to form fibrosis [111]. Another factor that may influence the therapeutic effect is the type of dystrophin that is produced. Exon 51, for example, can generate a variation of internally deleted dystrophins, that likely will not all be equally functional [40].

Unfortunately, studies in mouse models are only of limited use when answering these questions. At face value, it may appear that there is a poor translatability between mice and humans, since treated *mdx* mice show functional improvements and the dystrophin-utrophin double knockout model shows increased survival after AON treatment. However, one has to bear in mind that in most of these mouse studies the exon skipping and dystrophin restoration levels are much higher than observed in humans. Indeed, when restoring lower dystrophin levels in *mdx* models, functional effects are more modest [112–114]. Another consideration is that most *mdx* models regenerate extremely well. As such the quality of the muscle will generally be better than in DMD patients. More severely affected models may offer an alternative. These include the d2/*mdx* model, and the larger rat, dog, and pig models [115–118]. However, these models come with their own challenges. The d2/*mdx* mouse and DMD rat models are relatively new developments and have not yet been fully characterized. This makes planning optimal proof-of-concept and pre-clinical studies challenging. The dog and pig models are expensive, precluding experiments in larger groups.

However, though there will be variation between patients, we believe that restoring even low amounts of dystrophin will slowdown the disease progression to some extent. The challenge lies in measuring this slower progression in a clinical trial setting [25]. The rate of functional decline as measured with a certain outcome measure is generally not linear, but is stable in some patients, while it cannot be measured in others, who have already lost that function. One thus has to identify the patients able to perform the functional tests for the duration of the clinical trial but who without intervention would show a measurable decline. Only then can a slower decline be picked up reliably. Two collaborative initiatives (the collaborative trajectory analysis project (cTAP) and the Duchenne muscular dystrophy regulatory science consortium (D-RSC)) are generating models to facilitate optimize patient selection for clinical trials [119–122].

Current phase 3 registration trials generally last 96 weeks, with weekly hospital visits, which is a burden on patients and families (Table 2). Efforts are made to find alternatives for the use of placebo groups, e.g. using historic controls. However, this poses many risks, since the standards of care are improving with time, and steroid regimens vary between different sites. Finding a perfect contemporary match for each patient, with similar baseline parameters for the primary endpoint, comparable care and similar steroid regimen is very challenging. Creative solutions have been used, such as the described clinical trial for eteplirsen, where patients not eligible for eteplirsen treatment from the same clinics were followed up with the same functional outcome measures and checked for the occurrence of 'adverse events.' However, the challenge here is that while these 'controls' are not eligible for eteplirsen, they are eligible for many of the other therapies currently tested for DMD. It is therefore in hindsight not unexpected, that the majority of the control group dropped from the trial prematurely. A shared placebo-group as is happening for the currently ongoing clinical trials for golodirsen and casimersen is probably a more workable solution. Furthermore, the models generated by cTAP and D-RSC will also facilitate optimal matching between contemporary natural history controls.

As described, multiple approaches are ongoing to increase the efficiency of AON delivery to muscle and heart, to increase dystrophin restoration levels. The challenge here is that these approaches have not yet been tested in human subjects and that many of them may not be safe in humans. It is clear that the therapeutic window for mice is much wider than for humans for e.g. the arginine-rich CPPs. Hopefully, it will be possible to identify a CPP that achieves therapeutic levels of exon skipping at a dose that is safe in humans. The fact that AON treatment does not lead to permanent effects also poses burdens on treated patients, their families and treating clinicians. Currently, weekly intravenous dosing is required, which

involves a weekly hospital visit for most families. Home dosing is being set up as well to reduce the travel burden but does not avoid the burden of a weekly infusion that can take up to a couple of hours. The hope is that with an effective CPP-PMO the dosing frequency can be reduced. However, as mentioned, it remains to be seen whether these compounds are safe.

Genome editing poses a more permanent effect, since each restored *DMD* gene will produce restored transcripts. However, since DMD affects skeletal muscle and heart, systemic treatment with CRISPR/Cas9 will be required. This involves very high doses of viral vectors due to the abundance of skeletal muscle [123]. It is known from ongoing clinical trials with AAV gene therapy in patients with muscle diseases, that this is not without risk, as side effects involve transient renal failure in treated DMD patients and unfortunately even three deaths in patients with myotubular myopathy due to immune responses triggered by treatment with high doses of viral particles (https://medcitynews.com/2020/08/astellas-audentes-therapeutics-discloses-third-patient-death-in-gene-therapy-trial/). Furthermore, only patients without a preexisting immune response to the AAV serotype used are eligible for treatment.

In addition to these practical concerns, genome editing involves ethical concerns, due to the risk of off-target effects not only in the target tissues, but also in other tissues reached by the viral vector used [109]. As long as it cannot be excluded that also reproductive organs are reached, ethical and moral concerns will likely preclude using this approach systemically. While the genome editing approach is seen by many DMD patients as a therapy that will be available in the near future, we believe this is not likely, as the scientific field first needs overcome both practical problems and ethical issues. Furthermore, the scientific field needs to provide a realistic picture of the potential benefit of the genome editing approach. For DMD, genome editing will not be a 'CRISPR cure' as some patients and parents believe and some websites proclaim (https://www.technologyreview.com/2018/08/24/2157/a-crispr-cure-for-duchenne-muscular-dystrophy-trial-in-dogs-exonics/). Like AON-mediated exon skipping, the therapeutic effect will depend on the time of intervention and the restored dystrophin will be only partially functional. Reframing a *DMD* gene in adipose or fibrotic tissue will not facilitate dystrophin production, as these tissues do not express dystrophin. Furthermore, functions lost will not return.

One thing that has been gaining more attention is the fact that processing of dystrophin transcripts is very challenging [124–126] and that mutated dystrophin transcripts are instable, especially for DMD, but also for in-frame mutations found in BMD patients [124–126]. Our group recently reported that this instability is not due to nonsense-mediated decay but due to chromatin changes at the DNA level [127–128]. Further research is needed to fully elucidate this process. In either case, this suggests that less transcripts will be available for exon skipping in DMD patients and that even when the reading frame is restored on DNA level, likely the dystrophin expression levels will be lower than for unmutated *DMD* genes.

As mentioned exon skipping is a mutation-specific approach, where a few AONs can benefit relatively large subgroups of patients, but where the majority of patients would require the skipping of exons that apply to less than 1% of all mutations [11]. Hopefully, development of AONs for additional exons will benefit from the lessons learnt from the AONs that are currently approved and in clinical trials [129]. However, when groups of patients are very small, it will be very challenging to show benefit in placebo-controlled settings. Here, a shared placebo group or the matching models in development by c-TAP are potential solutions.

It is clear that reading frame restoration still faces many challenges that need to be overcome to improve this therapy. However, we would like to end on a positive note. In our previous paper in 2017, there was one approved AON for DMD, now there are 3, underlining that the field is moving forward. Even though these therapies are not yet optimal, they achieve dystrophin restoration, showing that the mechanism of reading frame restoration works. Furthermore, over the past few years, we have obtained a lot of new insights in patient trajectories for different functional outcome measures, and therefore future clinical trials can be designed more optimally. Currently, there are 11 oligonucleotide drugs approved, 3 of which are for DMD. We are confident that more and better DMD AONs will follow.

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Declaration of interest

A Aartsma-Rus 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 Aartsma-Rus is entitled to a share of royalties. A Aartsma-Rus further discloses being ad hoc consultant for PTC Therapeutics, Sarepta Therapeutics, CRISPR Therapeutics, Summit PLC, Alpha Anomeric, BioMarin Pharmaceuticals Inc., Eisai, Astra Zeneca, Santhera, Audentes, Global Guidepoint and GLG consultancy, Grunenthal, Wave and BioClinica, having been a member of the Duchenne Network Steering Committee (BioMarin) and being a member of the scientific advisory boards of ProQR, hybridize therapeutics, silence therapeutics, Sarepta therapeutics and Philae Pharmaceuticals. Remuneration for these activities is paid to LUMC. LUMC also received speaker honoraria from PTC Therapeutics and BioMarin Pharmaceuticals and funding for contract research from Italpharmaco and Alpha Anomeric. Project funding is received from Sarepta Therapeutics. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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