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AON-Mediated Exon Skipping for Duchenne Muscular Dystrophy

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1. Introduction

Duchenne muscular dystrophy (DMD) is a genetic, X-chromosome recessive, severe and progressive muscle wasting disorder, affecting around 1 in 3500 newborn boys. The onset of the disease is in early childhood and, nowadays, most children are diagnosed before the age of 5. The first signs of muscular weakness become apparent around the age of 2 or 3 years. In most patients the age at which the child starts to walk is delayed (retarded motor development). The children have less endurance and difficulties with running and climbing stairs (Moser, 1984). Gower’s sign is a reflection of the weakness of the muscles of the lower extremities (knee and hip extensors): the child helps himself to get upright from sitting position by using his upper extremities: first by rising to stand on his arms and knees, and then “walking” his hands up his legs to stand upright (Gowers, 1895). Muscle wasting is often symmetrical, however not all muscles are affected to the same extent. A prominent feature of the disease is enlargement of the calf muscle, caused by replacement of muscle fibres by connective and adipose tissue. Furthermore, the pelvic girdle, trunk and abdomen are severely affected and to a lesser extent the shoulder girdle and proximal muscles of the upper extremities. Progressive weakness and contractures of the leg muscles lead to wheelchair-dependency around the age of 10. Thereafter the muscle contractions increase rapidly leading to spinal deformities and scoliosis, often with an asymmetric distribution pattern. Involvement of the intercostal muscles and distortion of the thorax lead to respiratory failure and patients often require assisted ventilation in the mid to late teens. Thereafter dilated cardiomyopathy becomes apparent and most patients die before the age of 30. Another common feature is mental retardation (IQ less than 70) in around 20-30% of the patients (Emery, 2002).

Becker Muscular Dystrophy (BMD) is a related, but much milder, form of muscular weakness, affecting around 1 in 20 000 men. The phenotype varies between individual patients, from very mild to moderately severe, but the course of the disease is more benign compared to DMD. On average, the age of onset is around 12 years; however some patients remain asymptomatic until much higher ages. The age of wheelchair-dependency also shows more variability, but in general is in their second or third decade of life. The most severely affected patients die between 40 and 50 years of age, whereas patients with a mild
phenotype have (nearly) normal life expectancies. Around 50% of patients also develop cardiomyopathy (Emery, 1993).

The majority of female carriers shows no signs of disease. Only in 5 to 10% some degree of skeletal muscular weakness and enlarged calves are reported, but this is generally very mild and often does not affect daily activities. A small part of these carriers develops cardiomyopathy later in life; however most of the women with cardiac abnormalities on echocardiogram or ECG (left ventricular dilatation and decreased shortening fraction), are asymptomatic. There is no relation between the presence of skeletal muscle weakness and the development of cardiomyopathy (Grain et al., 2001).

At present there is no cure for DMD. However, during the past decades pharmacological interventions and improved care (e.g. physiotherapy and assisted ventilation) have led to increased function and quality of life and prolonged life expectancy for currently diagnosed patients into their forties. The current standard of care also consists of corticosteroids (mainly prednisolone or deflazacort). These are anti-inflammatory/immunosuppressive drugs that have shown to improve muscle function, prolong ambulation for around 3 years and to have a positive effect on cardiac function (Bushby et al., 2010).

2. DMD gene and dystrophin protein

2.1 Genetic defect in DMD

DMD is caused by a genetic defect in the DMD gene. In approximately 33% of cases this is a de novo (new) mutation. The DMD gene is located on the short arm of the X-chromosome (at Xp21). It is the largest gene in the human genome consisting of 2 220 223 base pairs. The coding sequence spans around 0.5% (11 058 bases) of the gene, dispersed over 79 exons. Mutations in the gene causing a disruption of the open reading frame or introducing a premature stop codon lead to a complete absence of a functional dystrophin protein. Dystrophin consists of 3 685 amino acids and has a molecular weight of 427 kDa (Muntoni et al., 2003). The protein is located inside the muscle fibres and forms a bridge between the actin cytoskeleton and the extracellular matrix (ECM). Thereby it provides mechanical stability to the muscle fibres during each contraction. The protein consists of 4 domains: first an N-terminus, containing 2 actin-binding domains (ABD), both consisting of a CH1-and a CH2-domain, which are bound to contractile structures (F-actin) inside the muscle cells. This is followed by a central domain, so called central rod domain, consisting of 24 spectrin-like triple helical coiled repeat units, interrupted by 4 proline-rich hinge regions. A third actin-binding domain is present between repeat 11 and 17 (Amann et al., 1998), while repeat 16-17 contain a binding site for neuronal nitric oxide synthase (nNOS) (Lai et al., 2009). Subsequently the protein contains a cysteine-rich part and finally a C-terminal domain. The cysteine-rich domain binds to β-dystroglycan, which is part of a membrane bound dystrophin-associated glycoprotein complex (DGC) (fig. 1). B-dystroglycan is a transmembrane protein that is bound to the extracellular α-dystroglycan, which in turn is bound to laminin-2, a part of the extracellular matrix (ECM). The central rod domain can absorb mechanical force. Hereby the protein transmits energy produced by the actin-myosin contraction machinery via the cell membranes to the connective tissue and tendons surrounding the muscles, to maintain the energy-balance and prevent overstressing of the muscle fibres (Ehmsen et al., 2002).
The dystrophin-associated glycoprotein complex (DGC) is composed of α- and β-dystroglycan, a sarcoglycan-sarcospan complex and the dystrophin containing cytoplasmic complex. Dystrophin (purple) forms the link between the actin cytoskeleton with its N-terminal domain and extracellular matrix component laminin-2 (lilac) via α- and β-dystroglycan (dark blue) with its C-terminal domain. B-dystroglycan is also bound to the sarcoglycan-sarcospan complex (light blue/black) and to caveolin-3 (orange), a scaffolding protein of skeletal muscle caveolae. Furthermore, the C-terminal domain of dystrophin is connected to α-dystrobrevin (green) and syntrophin (salmon pink), which recruits nNOS (yellow), a vasodilator, to the membrane. A-dystrobrevin, in turn, is linked to syncoilin (brown), forming a bridge between the DGC and the desmin intermediate filament protein network (brown).

Fig. 1. The dystrophin-associated glycoprotein complex

In addition to its mechanical linker function, dystrophin is involved in the organisation of the DGC as well as many other proteins, the maintenance of the calcium homeostasis and control of the growth of the muscle cells (Hoffman et al., 1987). In the DGC, β-dystroglycan is connected to a complex of α-, β-, γ- and δ-sarcoglycans and sarcospan. This complex functions in maintaining membrane stability (Miller et al., 2007). B-dystroglycan is also bound to caveolin-3, a structural protein of skeletal muscle caveolae, small invaginations of the plasma membrane playing a role in, among others, signal transduction. Caveolins act as scaffolding proteins to compartmentalise and functionally regulate signalling molecules (Hezel et al., 2010). Furthermore, the C-terminal domain of dystrophin is connected to α-dystrobrevin and syntrophin. nNOS is recruited to the membrane by binding to dystrophin and syntrophin. In contracting muscles, nNOS produces NO to induce vasodilatation in order to increase the local blood flow necessary for the increased mechanical load. The absence of nNOS in DMD causes abnormal vasoconstriction and ischemic stress, which contributes to the muscle degeneration (Brenman et al., 1995). Syntrophin is also connected to sodium channels, which are involved in regulating the Na+ distribution. In DMD, defects in cardiac conduction systems are thought to be caused by disturbances in Na+ distribution (Gee et al., 1998). A-dystrobrevin is linked to syncoilin too, thereby forming a bridge between the DGC and the desmin intermediate filament protein network at the neuromuscular junction (Newey et al., 2001).
Furthermore, in addition to the most common form of the dystrophin protein found in muscles, additional full-length and shorter isoforms of dystrophin exist. This is due to the presence of at least 7 different promoters and alternative splicing events. Three full-length variants exist (including the muscle isoform), which only differ in their first exon. In addition to the muscle promoter expressed in skeletal muscle and cardiomyocytes, a brain promoter drives expression in the cortical neurons and hippocampus of the brain and a Purkinje promoter in the cerebellar Purkinje cells. Four internal promoters lead to the production of shorter dystrophin proteins, lacking the actin-binding domains, expressed in specific tissues. In addition, alternative splicing facilitates the expression of many more dystrophins with a tissue-specific function (Muntoni et al., 2003).

A. Normal situation

In the normal situation pre-mRNA is spliced to produce mRNA, which in turn is translated into the dystrophin protein. This fully functional protein forms a bridge between the actin cytoskeleton and the extracellular matrix.

B. Duchenne muscular dystrophy

In DMD mutations lead to a disruption of the open reading frame and translation into protein stops prematurely. A truncated, non-functional dystrophin protein (which is degraded) is formed and the bridge function is lost.

C. Becker muscular dystrophy

In BMD mutations do not disrupt the open reading frame and translation into a shorter, but largely functional protein can occur. The bridge function is maintained.

Fig. 2. The reading frame rule
2.2 Genetic defect in BMD
In contrast to DMD, suffering from a complete absence dystrophin, in BMD a shorter, but partly functional, dystrophin protein is present. This discrepancy can be explained by the type of mutation that affects the DMD gene. In DMD mutations cause a disruption of the open reading frame or a premature stop codon, whereby the transcription of the gene stops prematurely and no functional protein is formed. In BMD the open reading frame stays intact (i.e. the size of the deletion in base pairs is divisible by 3), thereby translation can continue and a shorter protein is formed (fig. 2). This reading frame rule holds for over 90% of the cases (Aartsma-Rus et al., 2006b; Koenig et al., 1989). Only in-frame deletions that are very large (>36 exons) or deleting essential parts of the protein (the complete actin-binding domain or part of the cysteine-rich domain) lead to DMD. Furthermore, a small number of mutations that do disrupt the reading frame, lead to BMD instead of DMD (2%). This is probably due to correction of the reading frame at RNA level (Aartsma-Rus et al., 2006b).

2.3 Animal models for DMD
2.3.1 Mouse models for DMD
The most widely used model for DMD is the mdx mouse model (C57Bl/10ScSn-DMD<sup>mdx</sup>/J). These mice have a single base substitution within exon 23, leading to a premature stop codon, so a truncated, non-functional dystrophin protein is formed (Sicinski et al., 1989). Despite the absence of dystrophin, the phenotype of the mdx mice is relatively mild compared to human DMD patients. However, compared to wild-type mice, their muscles are clearly dystrophic and functionally impaired (Chamberlain et al., 2007). Nevertheless, their life span is only slightly reduced and the muscular weakness is mild. This is probably due to compensatory mechanisms, like the upregulation of utrophin, a dystrophin homologue, which can partly take over its function. Mice that lack both dystrophin and utrophin (mdx/utrn<sup>-/-</sup>, double knock-out mice) show a very severe, progressive muscular dystrophy. Their muscles display several signs of damage and are rapidly replaced by fibrotic and adipose tissue. Furthermore, these mice are functionally impaired, have an arched spine and a life span of 20 weeks at maximum (Deconinck et al., 1997). Due to the very severe phenotype and short life span, mdx/utrn<sup>-/-</sup> mice are not practical as experimental model. An intermediate model is the mdx mouse with haploinsufficiency for utrophin (mdx/utrn<sup>+/+</sup>). Inflammation and fibrosis in both skeletal muscle and diaphragm are more severe than in the mdx mouse, but less than in the mdx/utrn<sup>-/-</sup> mouse. Their life span is significantly longer than that of mdx/utrn<sup>-/-</sup> mice (Zhou et al., 2008).

Next to the naturally occurring mutation in the mdx mouse, several DMD mutations have been induced in mice. For example, treatment of mice with the chemical N-ethyl-nitrosourea (ENU), a powerful mutagen in mice, resulted in several new mdx-like mouse models (B6Ros.Cg-Dmd<sup>mdx-Cv</sup>/J). Mdx<sup>2Cv</sup> has a mutation in a splice site in exon 43 (causing alternative splicing, resulting in out-of-frame transcripts), Mdx<sup>3Cv</sup> a mutation in intron 65 (inducing a new splice site, resulting in out-of-frame transcripts), Mdx<sup>4Cv</sup> a mutation in exon 53 (premature stop codon) and Mdx<sup>5Cv</sup> a mutation in exon 10 (frame-shift by introduction of a new splice site). All these mice have a phenotype comparable to the mdx mouse (Chapman et al., 1989). In addition, several mouse models have been generated that only affect 1 or a few of the different dystrophin isoforms.
2.3.2 Canine models for DMD

The Golden retriever muscular dystrophy (GRMD) dog is a spontaneously occurring canine model for Duchenne muscular dystrophy. These dogs have a single base substitution in the 3’ consensus splice site of intron 6, resulting in skipping of exon 7, thereby introducing a premature stop codon in exon 8. The course of the disease is more comparable to human patients than that of the mdx mouse. The dogs display rapid and fatal muscular dystrophy, characterised by muscle atrophy, myofibre degeneration, replacement by fibrotic and adipose tissue and cardiomyopathy (Sharp et al., 1992). Most affected animals die within a few years, mainly due to degeneration of the cardiac muscle (Howell et al., 1997). Although phenotypically the GRMD dog seems a better model for DMD, it shows a lot of interindividual variation in the severity of the pathology. Some animals die within days after birth, whereas others appear almost normal and live for years (Ambrosio et al., 2008). This makes the dogs less suitable for experimental use, due to standardisation problems.

Because of the large size of the golden retriever, the GRMD dog has been bred with a much smaller beagle to generate the canine X-linked muscular dystrophy (CXMD) model. These dogs have a milder phenotype compared to GRMD dogs and therefore have a longer life span (Shimatsu et al., 2003).

In addition to the above mentioned large phenotypical variation, experiments with dogs are very costly. Dogs have a long breeding time and the availability is low (a heterozygous breeding program is needed, due to the severity of the phenotype). Furthermore, for therapeutic studies the size of the dogs requires large amounts of compound.

3. Antisense oligonucleotide-mediated exon skipping

3.1 Introduction antisense oligonucleotides

Antisense oligonucleotides (AONs) are small synthetic pieces of DNA or RNA (15-30 bp), which are complementary to their target mRNA. Initially, DNA oligos were used for the specific knockdown of gene expression. These DNA oligos bind to the RNA to form DNA-RNA hybrids which activate RNase H. This enzyme cleaves the double-stranded mRNA, thereby preventing the translation into protein, thus decreasing protein expression. DNA oligos are fast degraded by endonucleases, therefore oligos with a phosphorothioate instead of a phosphodiester backbone (PS DNA oligos) were developed, which are more endonuclease-resistant. These led to very efficient expression knockdown of for example genes (UL36 or IL2) involved in CMV retinitis (85-95%) (Baker & Monia, 1999). In addition to activation of RNase H, AONs can also down regulate gene expression by inducing translational arrest through steric hindrance of ribosomal activity, interference with mRNA maturation by inhibiting splicing or destabilisation of pre-mRNA in the nucleus (Chan et al., 2006). Later, 2'O modified RNA oligos were developed, which have a higher affinity for mRNA and turned out not to induce RNase H-dependent cleavage (Sproat et al., 1989). The activation of RNase H is useful when down regulation of gene expression is required, but not when AONs are used for modulation of pre-mRNA splicing.

3.1.1 Antisense-mediated exon skipping for DMD

AON-mediated exon skipping for DMD is based on the reading frame rule (fig. 2), which underlies the phenotypic differences between DMD and BMD. Furthermore, in some DMD
patients rare, dystrophin-positive (so-called “revertant” fibres) were found, which are the result of spontaneous exon skipping or secondary mutations restoring the reading frame in these fibres and allowing dystrophin production. Therefore it was hypothesised that using AONs to induces skipping of specific exons could lead to the restoration of the reading frame and thereby production of slightly shorter dystrophin proteins, as found in BMD and revertant fibres (fig. 3) (van Ommen et al., 2008). This approach is mutation-specific and a large variety in mutations exists among DMD patients. Fortunately, 2 “hotspots” (a major around exon 43 to 53 and a minor spanning exons 2 to 20) exist, comprising a large proportion of the mutations (Aartsma-Rus et al., 2006b). In this Chapter we will describe the development of this therapeutic approach. We are aware that many excellent papers about exon skipping for DMD exist. Due to space constraints it was not feasible to cover them all. For a recent overview see Aartsma-Rus, RNA Biology 2010 (Aartsma-Rus, 2010).

In DMD mutations in the DMD gene lead to a disruption of the open reading frame (in this example a deletion of exon 50), thereby preventing production of a functional dystrophin protein. Binding of an exon-specific AON (in this example against exon 51) hides the exon from the splicing machinery. The exon will be ‘skipped’ and not incorporated in the mRNA. Thereby the reading frame is restored and translation of a shorter, but still largely functional dystrophin protein can occur, which is similar to the proteins found in BMD.

**Fig. 3. Antisense oligonucleotide-mediated exon skipping**

### 3.2 Backbone chemistries

To prevent activation of RNase H the 2’-O position of the ribose was modified (2’-O-methyl (2OME) or 2’-O-methoxyethyl (2OMOE)). Furthermore, various chemical modifications (fig. 4) have been developed, which differ in sugar and backbone chemistry and have different
biophysical, biochemical and biological properties. For a more detailed review see Chan et al., *Clin.Exp.Pharmacol.Physiol* 2006 (Chan et al., 2006). The 2OMePS chemistry has an increased affinity for RNA and nuclear uptake. Disadvantages are that the phosphorothioate backbone is toxic to some extent and some sequences elicit an immune response. This is partly counteracted by the 2OMe modification.

Peptide nucleic acids (PNA) contain a flexible, uncharged, achiral N-(2-aminoethyl)glycine backbone to which nucleobases are attached via methylenecarbonyl linkages in stead of the phosphodiester backbone of DNA oligos. PNAs have a high affinity for RNA, are not toxic even at high concentrations, are peptidase-and nuclease-resistant and have a high sequence-specificity. A disadvantage is the insolvency of PNAs, due to their hydrophobic nature, which makes transfection difficult. This can be solved by the attachment of carrier groups, which easily bind to the peptide backbone, or addition of cationic lysine residues to the C-terminus. Another disadvantage is the rapid clearance of PNAs *in vivo*. Their mechanism of action is mainly by steric hindrance (Larsen et al., 1999).

Locked nucleic acid (LNA) DNA oligos contain a 2'-O, 4'-C-methylene bridge in the β-D-ribofuranosyl configuration. They have a high hybridisation affinity towards target mRNA or DNA, thereby forming stable duplexes. This is an advantage, but also a disadvantage, since LNAs longer than 15 base pairs show self-annealing and are not very sequence-specific, which increases the chance of unwanted side effects (Aartsma-Rus et al., 2004b). However, currently mainly LNA/2'-O-methyl oligonucleotide mixmers are used, which show much more sequence-specificity (Fabani & Gait, 2008). LNAs have a good nuclear uptake and are nuclease-resistant.

Ethylene bridged nucleic acids (ENA) contain an ethylene bridge between the 2'-O and the 4'-O-C of the ribose. They have similar properties to LNAs, but have a higher affinity to RNA, are very stable and more nuclease-resistant (Morita et al., 2002; Yagi et al., 2004).

Phosphoroamidate morpholino oligomers (PMO) have a six-membered morpholino ring instead of the ribose sugar and the phosphodiester bond is replaced by a phosphoroamidate linkage. They do not activate RNase H, are very resistant to nucleases and are non-toxic. Furthermore, they are uncharged, which prevents undesired binding to proteins. However, this also results in limited nuclear uptake, where pre-mRNA splicing takes place. Their neutral charge also makes them hard to transfect in cell cultures, but *in vivo* PMOs can be taken up by tissues after local injection. This is probably due to the fact that the neutral nature does not form interactions with other cellular components. In general, PMOs are often a bit longer than 2OMePS AONs (25 nucleotides or more compared to around 20 nucleotides for 2OMePSs). They primarily act by steric prevention of ribosomal assembly (Aartsma-Rus et al., 2004b; Chan et al., 2006; Heemskerk et al., 2009b). PMOs have been linked to arginine-rich cell-penetrating peptides (pPMOs) to increase uptake and efficiency. These conjugates indeed have higher efficacy, but there are toxicity concerns and the peptide might evoke an immune response (Moulton & Moulton, 2010), though this has not yet been observed. Conjugation of PMOs with a dendrimeric octaguanidine polymer (vivo-morpholino) improves the delivery of the compound *in vivo*. Since this polymer is not a peptide, the risk of an immune response is small and has not been observed so far (Wu et al., 2009), though the polymer is toxic at higher concentrations as well.
3.3 AON design and targets

Target sites for exon skipping AONs are splice sites (SS), exonic splicing enhancer (ESE) sites or exon inclusion sequences (EIS). Splice sites are required for the correct identification of exons by the spliceosome, a catalytic complex that coordinates the splicing process and consists of 5 small nuclear ribonucleoproteins (snRNP) and hundreds of other splicing factors. The 5’ (donor) splice site (beginning of an intron), the branch point (just upstream of the acceptor splice site) and the 3’ (acceptor) splice site (end of an intron) contain consensus sequences that are bound by snRNPs and splicing factors to bring about the removal of introns and ligation of exons. Blockage of splice sites or the branch point prevents incorporation of the exon in the mRNA. Exon recognition is further facilitated by ESE sites, which are exonic sequence motives to which certain splicing factors (Ser-Arg-rich (SR) proteins) can bind. AONs targeting ESEs have been proposed to sterically hinder the binding of SR proteins (Aartsma-Rus et al., 2005; Aartsma-Rus et al., 2009b; Kole et al., 2004; Tanaka et al., 1994).
4. Antisense-mediated exon skipping in vitro

4.1 Single exon skipping

First proofs-of-principle for the feasibility of restoring the reading frame by exon skipping have been shown in vitro in cultured primary human myoblasts, derived from DMD patients and in mdx-cell cultures.

In the early nineties, a DMD patient (named “DMD Kobe”) was identified carrying a deletion of 52 base pairs within exon 19, which led to the skipping of the whole exon. The authors hypothesised that this sequence might be important for splicing. An AON targeting part of this exon induced exon skipping in human control lymphoblastoid cells (Pramono et al., 1996; Takeshima et al., 1995). In cells derived from a patient with a deletion of exon 20, PS AONs (able to activate RNase H) against the aforementioned sequence, resulted in exon 19 skipping and the restoration of dystrophin in ~20% of treated cells (Takeshima et al., 2001). By that time, exon skipping with 2OMePS AONs (unable to activate RNase H), had also been explored. In 2 patients with an out-of-frame exon 45 deletion treatment with AONs resulted in exon 46 skipping, which should restore the open reading frame. Exon skipping levels were ~15%, which restored the synthesis of functional dystrophin in more than 75% of the cells (van Deutekom et al., 2001). Subsequently, the skipping of different exons has been reported for patient-derived cells with other deletions, point mutations and duplications. For an overview see Aartsma-Rus, RNA Biology 2010 (Aartsma-Rus, 2010).

Restoration of dystrophin synthesis was detectable at the membrane and the (at least partial) functionality of these BMD-like proteins was suggested by the reformation of the dystrophin-glycoprotein complex, shown by increased membrane expression of DGC-associated proteins. Another interesting finding were the higher exon skipping levels observed in these patient cell lines, than previously seen in control cell lines. This can be explained by nonsense-mediated decay (NMD) of the original out-of-frame transcripts, which are less stable than the newly formed in-frame transcripts (Aartsma-Rus et al., 2003).

4.2 Double and multiple exon skipping

In theory, skipping of a single exon would be beneficial for approximately 64% of the known mutations in DMD patients. However, there still is a large population which requires the skipping of 2 or more exons for reading frame restoration (Aartsma-Rus et al., 2009a). The theoretic applicability of exon skipping could be extended to 79% by double exon skipping and around 90% of patients by multiple exon skipping. Feasibility of double exon skipping was first shown in 2 different patient cell lines. One patient had a nonsense mutation in exon 43, for which dystrophin synthesis could be restored by skipping of exon 43 and 44. The second, carrying an exon 46-50 deletion, was successfully treated with a combination of AONs against exon 45 and 51. Dystrophin synthesis was found in 70% of the myotubes, which is almost as high as after single exon skipping (75-80%) (Aartsma-Rus et al., 2004a). Subsequently, successful double exon skipping has been proved by other groups (reviewed in Aartsma-Rus, RNA Biology 2010 (Aartsma-Rus, 2010)). The dog model for DMD needs double skipping to bypass the mutation and cells derived from these dogs have been used to show double exon skipping in vitro (see below) (McClorey et al., 2006).

A surprising finding in control myotubes was that combinational treatment with 45AON and 51AON caused the skipping of the entire stretch of exons from 45 through 51. This
Exon skipping is in theory useful for the majority of patients. Exceptions are mutations that involve regions in the gene that are essential for the function of the dystrophin protein: all actin-binding N-terminal parts, the cysteine-rich C-terminal part (binding to the DGC-complex), the promoter region or the first exon. Furthermore it is not applicable to translocations. Fortunately these kind of mutations make up only a small part (~8%) of all known mutations (Aartsma-Rus et al., 2009a). The largest part of mutations is made up by deletions and small mutations. A minor part consists of exon duplications (double or multiple). In the case of single duplications, skipping of one of these exons would in theory generate wild-type dystrophin transcripts. However, this turned out to be challenging. In cells with an exon 45 duplication this was indeed possible, but in other cases the skipping was so efficient that both exons were skipped, leading to an out-of-frame transcript (Aartsma-Rus et al., 2007). Skipping of an additional exon could restore the reading frame again. For example for an exon 18 duplication, successful skipping of exon 17 and both exon 18s resulted in restoration of the reading frame (Forrest et al., 2010). Successful skipping of multiple exon duplications has not yet been achieved (Aartsma-Rus et al., 2007). In total 6% of patients could benefit from single or multiple skipping of exon duplications.

5. Antisense-mediated exon skipping in vivo in animal models

5.1 AONs in mouse models for DMD

5.1.1 AONs in the mdx mouse model

After the promising in vitro results, AONs have been tested in vivo in animal models. As mentioned before, the mdx mouse is most widely used. The target site for exon 23 was first optimised in mdx myotube cell cultures. This resulted in a 5′ splice site targeting AON with a 2′-O-methoxyethyl phosphorothioate (2OMePS) backbone, which was tested locally in the mdx mouse. A single intramuscular injection of this compound in the tibialis anterior of both young (2 or 4 weeks old) and aged (6 months old) mice resulted in marked dystrophin expression 2 weeks after injection, which persisted up to 3 months after injection. The functionality of the dystrophin protein was suggested by the re-expression of dystroglycans, sarcoglycans and nNOS at the membrane. It also resulted in partial restoration of physiological function, maximum isometric titanic force, of the treated muscles. Importantly no auto-immune response against the newly formed dystrophin protein was observed (Lu et al., 2003). Of course, since DMD affects body-wide musculature, including heart and diaphragm, injection of every muscle separately is not feasible and systemic treatment is required. Three intravenous injections at weekly intervals resulted in dystrophin expression, highest in gastrocnemius, intercostal muscles and the diaphragm, without signs of toxicity or damage to other organs. However dystrophin could not be detected in the cardiac muscle (Lu et al., 2005). To optimise delivery and efficiency, different administration routes have
been compared. Intravenous injection resulted rapidly in high plasma levels, which were quickly cleared. Peak plasma levels were twofold lower after subcutaneous and intraperitoneal injection, but clearance was much slower. Furthermore, intravenous injection resulted in very high AON levels in the kidney and liver, which might induce toxicity after long term treatment. Skipping levels were highest after intravenous injection and slightly lower for both subcutaneous and intraperitoneal injection. Dystrophin expression followed a similar pattern. Importantly, all 3 routes resulted in exon skipping and dystrophin expression in the heart, albeit at low levels. Due to the better pharmacokinetic profile of subcutaneous versus intravenous injection and slightly higher exon skipping compared to intraperitoneal administration, subcutaneous injection seemed to be the delivery method of choice. After subcutaneous treatment also a decrease in serum creatine kinase (CK) levels was observed. Creatine kinase is an enzyme that leaks out of the muscles into the bloodstream when muscles are damaged, so a decrease indicates an improvement of muscle integrity (Heemskerk et al., 2010).

Morpholino (PMO) AONs have been shown to be effective in vivo as well. Intramuscular injection in the tibialis anterior elicited a dose-dependent increase in dystrophin expression in the majority of muscle fibres and dystrophin protein levels up to 60% of levels found in healthy muscle. Efficiency was comparable in both young (3 weeks old) and aged (6 months old) mdx mice. Repeated systemic (intravenous) injections induced exon skipping and expression of dystrophin protein body-wide, albeit with large variations between individual muscles. Highest levels were found in the quadriceps, abdominal and intercostal muscles. Lower levels were found in the tibialis anterior and diaphragm. CK levels were decreased and muscle function was improved as well. As with 2OMePS AONs, targeting of the cardiac muscle appeared difficult, since exon skipping and dystrophin expression were undetectable (Alter et al., 2006). Wu et al. showed that dystrophin restoration could be achieved (up to 30% of healthy levels) by systemic PMO treatment, although extremely high doses (up to 3 g/kg bodyweight) were required (Wu et al., 2010). Furthermore, a dosing regime of multiple low doses seems to be preferable above a few high doses to reduce the risk of toxicity and increase the efficiency, since both AONs and dystrophin protein show an accumulation over time (Malerba et al., 2009).

In the mdx mouse model PMOs appeared more effective and at lower doses compared to 2OMePS AONs. A direct comparison revealed that this was indeed the case for mouse exon 23 in the mdx mouse. Intramuscular injection of both AONs in the gastrocnemius, resulted in much higher skipping levels for PMOs than for 2OMePS AONs at the same molar amount. Systemic (intravenous) comparison in the mdx mouse showed, as had been noticed before, that most of the 2OMePS AONs are taken up by the liver and kidney. However the PMOs were almost exclusively taken up by the kidney. A possible explanation is that 2OMePS AONs bind to serum proteins, which prevents renal clearance (Geary et al., 2001), whereas PMOs do not, which explains their high renal clearance (Oberbauer et al., 1995). 2OMePS AON uptake was higher for all skeletal muscles, diaphragm and heart. In contrast to the biodistribution, exon skipping efficiency was much higher for the PMO AONs in skeletal muscle and diaphragm (approximately 40% versus 10%). Skipping levels in the heart were much lower and almost comparable between both compounds (2.5% for the PMOs versus 1.5% for the 2OMePS AONs). Protein levels followed the same pattern (Heemskerk et al., 2009b).
A PMO conjugated to a cell-penetrating peptide (pPMO) showed to be more effective than the naked PMO AON. Systemic (intravenous) treatment of mdx mice was very potent in both skeletal muscle, diaphragm and, importantly, heart. pPMOs lead to a decrease in CK levels (Jarawirirayapaisarn et al., 2008). Another study confirmed that the long term systemic treatment with pPMOs was effective in restoring dystrophin expression in skeletal muscle, improving muscle function and preventing heart failure (Wu et al., 2008). These pPMOs were also able to rescue the severe mdx/utrn-/- mouse model by systemic (intraperitoneal) treatment. Considerable improvement of muscle function was observed, combined with dystrophin expression in almost all muscles, except for the heart (Goyenvalle et al., 2010). Peptides might elicit an immune response, but no signs of such a response or toxicity were found in the mouse models so far. Unfortunately, when a pPMO compound was tested in primates, there were toxicity concerns. In cynomolgus monkeys pPMO doses equivalent to the ones used in mice, were not toxic, but also had little exon skipping effect. Higher doses were effective, but also caused tubular degeneration in the kidneys, a sign of renal toxicity (Moulton & Moulton, 2010). Yin et al. generated a chimeric fusion peptide consisting of a muscle-targeting heptapeptide (MSP) fused to an arginine-rich cell-penetrating peptide (B-peptide), which they conjugated to a PMO oligomer (B-MSP-PMO). These B-MSP-PMOs were already efficient at very low doses in restoring high levels of dystrophin expression body-wide (Yin et al., 2009). Novel cell-penetrating peptides have been discovered by inducing modifications to a Drosophila melanogaster-derived R6-Penetratin peptide. These peptides are called PNA or PMO internalisation peptides (Pips). A conjugate of Pip2b and a PNA AON (Pip2b-PNA) resulted in approximately threefold higher dystrophin-positive fibres compared to the naked AON after local injection in the tibialis anterior of mdx mice (Ivanova et al., 2008). More and improved Pips have been developed. Pip5e fused with a PMO (Pip5e-PMO) showed high exon skipping efficiency after a single intravenous injection in the mdx mouse. Most importantly it also efficiently targeted the heart, leading to dystrophin levels of more than 50% of wild-type levels (Yin et al., 2011).

Another modification of the PMO is conjugation to a dendrimeric octaguanidine polymer (vivo-morpholino). This modification also significantly improved the delivery and dystrophin production in mdx mice after intravenous injection. Repeated treatment resulted in dystrophin expression in almost 100% of the skeletal muscle fibres and levels of protein up to 50% of wild-type levels. Importantly, levels of ~10% of those found in healthy hearts were found in the cardiac muscle. In these mice no signs of an immune response or toxicity were observed (Wu et al., 2009).

5.1.2 AONs in the other mouse models

Both 2OMePS and PMO AONs have also been tested in the mdx4Cv mouse. These mice require skipping of both exon 52 and 53 to remove the mutation and maintain the reading frame. Immortalised myoblast cell cultures from these mice were used to design the most effective AONs against exon 52 and 53, which were then tested in vivo in the mdx4Cv mice. 2OMePS AONs induced exon skipping in these cell cultures, but no dystrophin protein was observed. Intramuscular injection of the cocktail of AONs in the tibialis anterior resulted in sporadic exon skipping in this muscle, but no detection of dystrophin protein. A combination of PMO AONs against both exons resulted both in vitro and in vivo (after injection in the tibialis anterior) in exon skipping and restoration of dystrophin expression (Mitrpant et al., 2009).
AONs are sequence-, and therefore species-, specific. So, to be able to test human-specific AONs, a mouse containing the full-length human DMD gene was generated \( (hDMD) \). These mice have a fully functional \( hDMD \) transgene integrated on mouse chromosome 5. The functionality of the transgene was proven by rescuing the severe dystrophic phenotype of the \( mdx/utrn/-/\) mouse after crossing of both models \( ('t \ Hoen \ et \ al., \ 2008) \). Intramuscular injection (gastrocnemius) of 2OMePS AONs against exon 44, 46 or 49, induced specific skipping of the targeted human exons. It also highlighted the sequence-specificity of the AONs, since in the corresponding mouse sequences, with only 2 or 3 mismatches, no detectable skipping was observed \( (Bremmer-Bout \ et \ al., \ 2004) \). As described before, PMOs were more efficient in the \( mdx \) mouse than 2OMePS AONs. However in the \( hDMD \) mouse, AONs targeting human exon 44, 45, 46 or 51 were comparably effective or only marginally different between both chemistries. This indicated that the differences between PMO and 2OMePS AONs are probably more due to sequence differences than to chemistry differences. Furthermore, it also suggested important differences in sequence-specificity. 2OMePS AONs with 2 mismatches had a greatly reduced efficiency, whereas PMO AONs remained equally effective. This can increase the risk of off-target side effects \( (Heemskerk \ et \ al., \ 2009b) \).

Studies in these \( hDMD \) mice revealed that the uptake of AON by the healthy \( hDMD \) muscle fibres is much lower than by dystrophic \( mdx \) fibres. This can probably be explained by the dystrophic nature of the \( mdx \) fibres: the lack of dystrophin results in damage to the muscle fibres, leading to leakage of the muscle enzyme creatine kinase into the bloodstream. It has been proposed that the AONs migrate into the muscle fibres through these same holes \( (Hoffman, \ 2007) \). In this way the disease is facilitating delivery of the potential therapeutic compound. Indeed AON uptake and skipping in the \( hDMD \) mouse is more difficult. The exon skipping levels observed after intramuscular injection with either 2OMePS or PMO AONs were lower than previously observed in the \( mdx \) mouse and in cell cultures \( (Heemskerk \ et \ al., \ 2009a) \). A pilot experiment with systemic (intravenous) injection of 2OMePS AONs targeting exon 51 in the \( hDMD \) mouse resulted in very low or undetectable exon skipping in the muscles \( (Heemskerk \ et \ al., \ 2010) \). Recently, vivo-morpholinos against exon 50 were shown to be able to achieve high levels of exon skipping after systemic (intravenous) injection in the healthy skeletal muscles of the \( hDMD \) mouse and even low levels in the cardiac muscle. There were no large signs of toxicity or adverse effects, only a small increase in serum CK levels, which could reflect a bit of membrane integrity disturbance \( (Wu \ et \ al., \ 2011) \). The influence of the nature of the muscle fibres on AON delivery efficiency might also explain why targeting of the heart is so difficult. The heart muscle is structurally and pathologically different from skeletal muscle, since it is made up of individual cardiomyocytes, which do not become ‘leaky’.

### 5.2 AONs in the canine models

First AON experiments with the canine model have been performed \textit{in vitro} in myoblast cell cultures of the GRMD dog. The nature of the mutation requires the skipping of 2 exons (exon 6 and exon 8) to restore the reading frame, thereby making it more challenging. \textit{In vitro}, 2OMePS AONs induced higher exon skipping levels than the PMOs, but only for a short term and without induction of detectable dystrophin protein. PMOs could restore a low level of dystrophin production, but only at very high concentrations. pPMOs could
induce slightly higher exon skipping levels and restored dystrophin expression (McClorey et al., 2006). Further testing of these AON cocktails in vivo by intramuscular injections, revealed that the AONs targeting exon 8 were effective, but the AONs targeting exon 6, which showed effectiveness in vitro, were not (Partridge, 2010). Another small experiment (in a 6 months old and a 5 years old dog) with cocktails of 2OMePS AONs or PMOs, resulted in high skipping levels of the desired exons and restoration of dystrophin protein to near normal levels after a single injection in the tibialis anterior with the highest test dose. The structure of the dystrophin-positive cells was reported to be improved. Furthermore, both backbone chemistries showed comparable results and results were better in the younger dog than in the older dog (Scheuerbrandt, 2009).

Systemic (intravenous) treatment of CXMD dogs with a cocktail of 3 PMO AONs targeting exon 6 (2 PMOs) and exon 8 (1 PMO), generated body-wide production of functional dystrophin. In the heart there was only modest production of dystrophin, as observed in mice. Furthermore, an interindividual variation between dogs and intra-individual variation between different muscles of the same dog was seen. Functional improvement could be shown too and no signs of toxicity were observed (Yokota et al., 2009).

6. Clinical trials with antisense-mediated exon skipping

6.1 Local treatment with AONs

After the promising preclinical results in vitro and in vivo, the first clinical trials were initiated. These trials used local (intramuscular) injections to obtain proof-of-principle in humans and examine possible adverse effects. Normally, the first human trials are done in healthy volunteers (phase I). However, this is not possible in this case, since exon skipping in healthy persons would result in disruption of the reading frame. Therefore this phase was skipped and AONs were tested immediately in DMD patients (phase I). These first trials focused on skipping of exon 51 for both 2OMePS (in 2006) and PMO AONs (in 2008), since this would be applicable to the relatively largest group of known mutations (13%) (Aartsma-Rus et al., 2009a).

A single injection in the tibialis anterior with 0.8 mg of a 2OMePS AON (called PRO051) in 4 patients resulted in specific exon 51 skipping without adverse effects. It restored dystrophin expression at the sarcolemma in 64-97% of the myofibres and restored protein levels till 17-35% of control levels. However, it also clearly indicated the importance of muscle quality since the target of AONs, the dystrophin transcript, is only expressed in muscle fibres and not in adipose and fibrotic tissue, which replaces the muscle tissue when the disease progresses. The patient with the lowest dystrophin levels had the most advanced disease state and relatively little muscle tissue left (van Deutekom et al., 2007).

For PMO AONs a placebo-controlled, single-blinded study was performed. Seven patients received an injection with a PMO AON (called AVI-4658) into their extensor digitorum brevis (EDB) and saline into the contralateral muscle. In 2 patients receiving the lowest dose (0.09 mg) this resulted in low levels of exon 51 skipping, but no observed increase in dystrophin expression. However, a clear dystrophin restoration was observed in the higher dose (0.9 mg) group. As for the PRO051 study no adverse events, like an inflammatory response, were observed. Immunofluorescent staining for dystrophin indicated 11-21% higher intensity levels in the AON-treated muscle compared to the contralateral saline-
treated muscle, and levels of 22-32% of control dystrophin levels (Kinali et al., 2009). Since both studies studied different muscles and used different techniques for quantifying immunocytochemistry the results are not directly comparable (Aartsma-Rus & van Ommen, 2009). However both studies showed unequivocal effectiveness of the used compound in the absence of side effects.

6.2 Systemic treatment with AONs

The next step towards clinical application of exon skipping are systemic clinical trials. The first pilot experiment has been conducted in Japan. Takeshima et al. treated 1 DMD patient intravenously with a weekly dose of 0.5 mg/kg bodyweight of a PS AON against exon 19 for 4 weeks. Only very low levels of exon skipping and dystrophin protein were observed in a muscle biopsy (Takeshima et al., 2006). This is not surprising, as the dose used was very low and the PS backbone chemistry is not ideal for exon skipping purposes (see above). Furthermore, this was only 1 single patient, so no real, reliable conclusions can be drawn from this experiment.

More extensive, open-label, dose-escalation, phase I/IIa studies have recently been completed for both 2OMePS and PMO AONs. The first was a study with a abdominal subcutaneous injections of PRO051 (2OMePS AON, now called GSK2402968)) in 12 patients testing 5 weekly doses (0.5, 2, 4 and 6 mg/kg bodyweight) in groups of 3 patients. Doses of 2 mg/kg bodyweight or higher resulted in specific exon 51 skipping. In 10 out of 12 patients dystrophin expression in a tibialis anterior biopsy could be observed in 60-100% of the muscle fibres at levels up to 15.6% of healthy levels in a dose-dependent manner. After analysis of this first phase (6 to 15 months later), all patients entered an open-label extension study in which they received weekly injections of the highest dose. After 12 weeks, this resulted in functional improvement as measured by the 6-minute walk test. Since a placebo group is lacking, interpretation of this improvement must be done with caution. Nevertheless, the overall results are encouraging and only mild adverse events, like irritation at the injection side and mild proteinuria, were observed (Goemans et al., 2011).

AVI-4658 (PMO AON, also called eteplirsen) was tested by 12 weekly intravenous infusions of different doses (0.5, 1, 2, 4, 10 and 20 mg/kg bodyweight) in a total of 19 patients, without serious adverse events. In a biceps biopsy, exon 51 skipping and restoration of protein expression was observed starting at a dose of 2 mg/kg bodyweight, albeit variable between individual patients. The responding patients showed dystrophin levels of 8-16% of healthy controls by immunofluorescent staining. Notably, there were 3 patients who responded very well, with up to 55% of dystrophin-positive fibres by immunofluorescent staining and dystrophin levels up to 18% by western blot. In 4 other patients some improvement was observed. Furthermore, the functionality of the newly formed proteins was confirmed by the restoration of DGC-associated proteins at the sarcolemma. In addition, a reduction of inflammatory infiltrates was observed in the highest dose group, which probably indicates a reduction in necrosis and an increased resistance to mechanical load (Cirak et al., 2011). Not all patients responded equally well, which may be explained by the short serum half-life of PMOs. Since PMOs do not bind to plasma proteins (see above), they are rapidly filtered out by the kidney (accounting for 40-60% of total plasma clearance). Thus, the amount available for uptake by other tissues (e.g. muscles) is low. Therefore further optimisation (e.g. higher doses) is needed.
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The next steps are larger randomised, placebo-controlled studies and targeting of other exons. For GSK2402968 a phase III study was initiated in January 2011. 180 ambulant patients will receive 6 mg/kg bodyweight AON once weekly for 1 year or placebo (http://clinicaltrials.gov/ct2/show/NCT01254019?term=GSK2402968&rank=1). This study will tell us whether long-term treatment is safe and leads to functional improvement or slowing down of disease progression (compared to placebo-treated patients). In parallel, a study in non-ambulant patients with different AON doses, primarily to determine the pharmacokinetical profile in older patients, and a study in ambulant patients where different treatment regimes are compared, are conducted (http://clinicaltrials.gov/ct2/show/NCT01128855?term=GSK2402968&rank=3 and http://clinicaltrials.gov/ct2/show/NCT01153932?term=GSK2402968&rank=2). In addition a clinical trial for AVI-4658 (etepirsen) with higher doses (30 mg/kg and 50 mg/kg bodyweight) for 24 weeks has been initiated to assess its efficacy and safety (http://clinicaltrials.gov/ct2/show/NCT01396239?term=eteplirsen&rank=1). These trials focus on skipping of exon 51, applicable to the relative largest group of patients. Skipping of exon 44 would be useful for another large group of patients (6.2%) (Aartsma-Rus et al., 2006b). A phase I/IIa study with PRO044 (2OMePS AON against exon 44) with the same set-up as the phase I/IIa study for PRO051 is currently ongoing (http://clinicaltrials.gov/ct2/show/NCT01037309?term=PRO044&rank=1). Furthermore, preclinical studies with other 2OMePS AONs (against exon 45, 52, 53 and 55) are performed by Prosensa Therapeutics. In addition to this, preclinical tests with AVI-5038 (pPMO AON against exon 50) are ongoing, although toxicity issues with this pPMO have been reported (http://investorrelations.avibio.com/phoenix.zhtml?c=64231%20&p=irol-newsArticle&id=1406001&highlight=).

7. Improvement of AON delivery and efficiency

The efficacy of AONs depends partly on the amount of AON that reaches its target, i.e. the muscle fibre nuclei. Several strategies to improve muscle-specific uptake are under investigation, like muscle-homing peptides and cell-penetrating peptides (see above). Due to AON clearance and turnover, the effect of AONs is only temporarily, thus repeated, life-long, injections are required, should this approach prove to be efficacious. The first clinical trials showed that the average serum half-life was 29 days for 2OMePS AONs and around 1.5 hours for PMOs. A way to allow a more prolonged effect is the use of viral vectors stably expressing modified small nuclear ribonucleoprotein (snRNP) genes, in which the normal antisense sequence is replaced by an antisense sequence of choice. snRNPs are small protein-RNA hybrids that are amongst others involved in pre-mRNA splicing and histone processing. The U1 and U7 snRNPs have been most in splicing modulation experiments (Brun et al., 2003). Exon 51 targeting U1 snRNPs induced effective skipping of exon 51 and rescue of dystrophin synthesis in a patient-derived cell line (De Angelis et al., 2002). Adeno-associated viruses (AAVs) are very efficient at transferring genes into skeletal muscles. Injection of AAV vectors expressing U7 or U1 snRNPs targeting mouse exon 23 resulted in sustained production of functional dystrophin in the mdx mouse after intramuscular injection and body-wide dystrophin expression and reduced muscle wasting after systemic treatment (Denti et al., 2008; Goyenvalle et al., 2004). However serious problems with the use of AAV vectors are the possibility of an immune response against the viral capsid and the difficulty to produce them on a large scale under good manufacturing practice (GMP), necessary for implementation in
the clinic. Another problem is the translation from mice to larger animals or humans. In mice it is feasible to treat a whole muscle, but transfection of whole muscles body-wide is more challenging in larger animals and humans.

8. Conclusion

In summary, Duchenne muscular dystrophy is caused by genetic defects in the gene encoding the dystrophin protein. These mutations cause a premature stop codon or disrupt the reading frame, leading to a non-functional protein. In most cases this can be overcome by specific skipping of the mutated exon with AONs, to produce a slightly shorter, but largely functional dystrophin protein, as found in the related, but much milder Becker muscular dystrophy. Over the past years major steps have been made in development of this therapy. Proof-of-principle has first been shown in vitro in cultured muscle cell lines and in vivo in several animal models (e.g. mdx mice and GRMD dogs). Recently the first clinical trials with AONs of 2 different chemistries, targeting exon 51, applicable to the largest group of patients, have been completed with positive results. Larger trials are ongoing or planned for the near future. Although the results obtained in the past few years are very encouraging, precaution is needed and several problems still exist. First of all, this is not a cure, but a potential treatment that will hopefully lead to an improvement of the phenotype. Secondly, the approach is mutation-specific, i.e. requiring different AONs for different mutations. Luckily most mutations cluster in 2 hotspots (see above). However, development and application in the clinic of the therapy for rare mutations will be difficult, since at the moment each AON is considered as a new drug, therefore has to go through all (pre)clinical steps before it can be registered. For these rare mutations simply not enough patients are available for these studies. At the moment efforts to discuss this with the regulatory authorities are coordinated by the TREAT-NMD Network of Excellence. For example, it may be possible to reduce the toxicity trials for an AON with similar backbone chemistry, if 1 or 2 of this kind have been proven to be safe (Muntoni & Wood, 2011). Thirdly, the approach will not be useful for mutations affecting the essential parts (actin-or dystroglycan-binding domains) of the protein. Fortunately these make up only a small percentage of all known mutations. Furthermore, restoration of the reading frame is more challenging when double and especially multiple exon skipping is required. Finally, the preclinical studies and first clinical trials have shown that muscle quality is very important for the therapeutic success, since dystrophin transcripts are only produced in muscle cells and not in the fibrotic and adipose tissue that replaces the muscle cells when the disease progresses. Therefore early start of treatment will probably be required.

In conclusion, AONs are currently a promising therapeutic approach for DMD and major steps towards clinical implementation have been made over the past years, but further improvements are necessary for increasing therapeutic effectiveness and more research for broader clinical application of the technique.

9. References

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