# General introduction

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and

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Chapter 1

## 1.1 Duchenne and Becker Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is a genetic, X-chromosome recessive, severe and progressive muscle wasting disorder, affecting around one in 5 000 new-born boys.[1,2] The onset of the disease is in early childhood and, nowadays, most children are diagnosed before the age of five. The first signs of muscular weakness become apparent around the age of two or three 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.[3] 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.[4] Muscle wasting is often symmetrical, however not all muscles are affected to the same extent. A prominent feature of the early stage of the disease is enlargement of the calve muscles (pseudohypertrophy), which are eventually replaced 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 ten. 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. Next dilated cardiomyopathy becomes apparent and most patients die before the age of thirty, most often due to respiratory or cardiac failure. Another common feature is mental retardation (IQ less than 70) in around 20-30% of the patients.[5]

At present there is no cure for DMD. However, during the past decades pharmacological interventions and improved care have led to increased function and quality of life and a prolonged life expectancy. Whereas for patients born in the 1960s the mean age of death was around 15, this has increased till 25 for those born between 1980 and 1990.[6] Pharmacological treatment involves mainly corticosteroids (predniso(lo)ne or deflazacort). It is not exactly known in which way steroids act. They are anti-inflammatory/immunosuppressive drugs, which may reduce muscle necrosis and inflammation. Furthermore, they might reduce muscle degeneration by enhancing proliferation of myogenic precursor stem cells and increase muscle regeneration due to their anabolic effects.[7,8] They have shown to improve muscle function, to prolong ambulation for about three years, to reduce the need for scoliosis surgery and to have a positive effect on cardiac function. However, they also induce considerable side effects, like weight gain, a decline in growth rate and delayed puberty.[9-13] Treatment with angiotensin-converting enzyme (ACE)-inhibitors, *e.g.* captopril and enalapril, has a positive effect on preventing/delaying of cardiomyopathy.[8,14,15] Importantly, the introduction of assisted ventilation has resulted in a major improvement in the survival during the last decades. The average age of death for non-ventilated patients is around 19 years, whereas for ventilated patients it increases till 25-30 years. Whilst till the 1980s respiratory failure was the main cause of death, this has now shifted towards cardiac failure.[6,16-19]

Becker muscular dystrophy (BMD) is a related, but much milder, form of muscular weakness, affecting around one 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, although some patients remain ambulant for much longer. The most severely affected patients die between 40 and 50 years of age, whereas patients with a mild phenotype have (nearly) normal life expectancies. The majority of patients eventually develops subclinical cardiomyopathy, which becomes symptomatic in over 70% of patients.[5,20,21]

Since DMD is an X-linked, recessive disease, females can be carriers. The majority of female carriers shows no signs of disease. The proportion of carriers displaying symptoms varies among different surveys between 2.5 to 22%.[22-24] These symptoms mainly comprise skeletal muscle weakness and enlarged calves, but this is generally very mild and often does not affect daily activities. A small part of these carriers (around 10%) develops symptomatic cardiomyopathy later in life; however cardiac abnormalities on echocardiogram or electrocardiogram (left ventricular dilatation and decreased shortening fraction) can be found in around 40% of the women. There is no relation between the presence of skeletal muscle weakness and the development of cardiomyopathy.[22,25] Cognitive impairment is also observed in some symptomatic carriers and is associated with mutations in the distal part of the gene, affecting the Dp140 and/or Dp71 isoforms involved in the development of cognitive function (see paragraph 1.2.2).[26]

Several mechanisms may be responsible for the symptoms in these carriers. First, and most reported, skewed (non-random) X-chromosome inactivation (XCI) can lead to higher expression of the mutated allele. The degree of skewed XCI might also correlate with the severity and age of onset of the symptoms, *i.e.* females with manifestations at muscle level show a higher degree of skewed XCI than manifesting carriers at cardiac level.[26,27] Secondly, the *DMD* gene can be disrupted by X-autosomal translocations. Due to non-random inactivation of the intact gene, which is commonly observed with X-autosomal translocations, this can lead to clinical symptoms.[28] Furthermore, rarely it is caused by co-occurrence with other genetic abnormalities, like X-chromosome monosomy, uniparental disomy and male pseudohermaphroditism.[22,29]

## 1.2 *DMD* gene and dystrophin protein

### *1.2.1 Genetic defects in DMD and BMD*

The X-linked, recessive inheritance pattern of DMD indicated that the gene responsible for the disease was located on the X-chromosome. Case reports of X/autosomal translocations in rare female DMD patients and genetic linkage studies narrowed the location of the gene to Xp21, which resulted in cloning of this region in the 1980s.[30-35] Unexpectedly, a linkage study in BMD patients revealed that the gene responsible for BMD was located in the same region.[36] Shortly thereafter the 14 kb cDNA corresponding to the complete transcript was cloned.[37] Deletions in the *DMD* gene were found in around 50% of DMD patients.[38] Furthermore the protein product of approximately 400 kDa was identified.[39] This protein could not or only in very low levels (<3%) be detected in DMD patients. In BMD patients on the other hands, dystrophin of reduced length and/or abundance was observed.[40,41] These observations led to the postulation of the reading frame hypothesis.[42,43] In DMD, mutations in the gene causing a disruption of the open reading frame or introducing a premature stop codon lead to a com-

plete absence of dystrophin. In BMD, the open reading frame stays intact (*i.e.* the size of the deletion in base pairs is divisible by three) thereby translation can continue and a shorter, but partly functional, dystrophin protein is present (fig. 1.1). This reading frame rule holds for over 90% of cases.[42,44] 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; see paragraph 1.2.2) 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, since if only mutations that are confirmed at RNA level are taken into account, 99.5% fits within the reading frame theory.[44]

The *DMD* gene is the largest gene in the human genome consisting of around 2.4 million base pairs, which is approximately 0.1% of the total human genome.[45,46] The coding

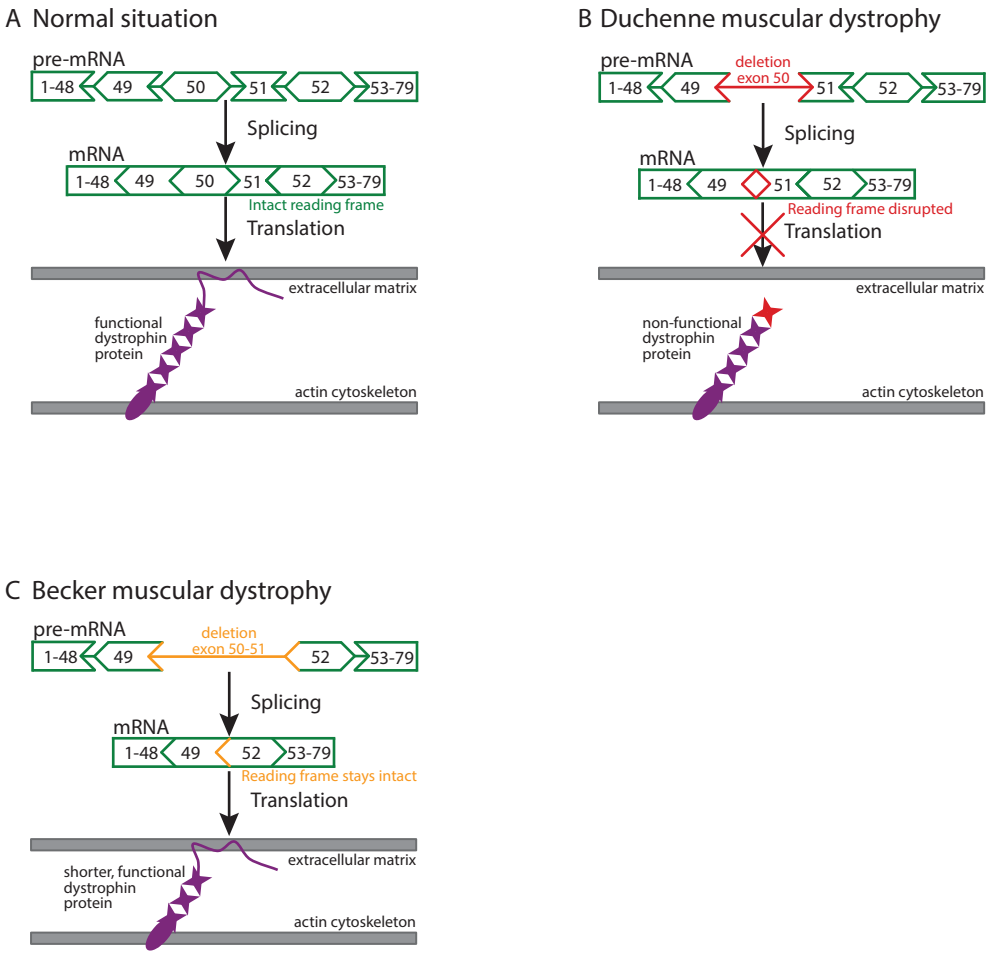


**Fig. 1.1:** The reading frame rule

a) 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) 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) 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.

sequence spans around 0.5% (14 kb) of the gene, dispersed over 79 exons.[45] The gene has a relatively high spontaneous mutation rate, as around one third of the mutations found in DMD and BMD are *de novo*.[47]

Two hotspot regions exist: a major spanning exon 45-53 and a minor spanning exon 2-20.[48] Intragenic deletions make up the largest part (65-70%) of all mutations. Most of them are found in the major hotspot. Duplications of one or multiple exons account for 7% of all mutations and are mainly found in the minor hotspot. Point mutations and small deletions or insertions account for around 20% of the mutations.[44,49]

The clinical phenotype is partly related to the amount of dystrophin detected in muscle biopsies. No or very low levels (≤3% of normal levels) are found in DMD patients, leading to a quite uniform severe phenotype. The severity of symptoms in BMD is highly variable. Studies about the relation between dystrophin quantity and disease severity show mixed results. Whereas some found higher levels in mildly affected patients compared to severely affected patients having <10% of dystrophin, others found less or no correlation.[40,41,50-53] Furthermore, the severity of the BMD phenotype is partly correlated to the location of the in-frame mutation. Mild BMD is often associated with deletions involving the central rod domain (see paragraph 1.2.2). A moderate (typical) phenotype is generally found in patients carrying deletions in the major hotspot (exon 45-53) and most severe phenotypes are often associated with deletions affecting the actin-binding domain (exon 2-8) of the protein.[54]

### *1.2.2 Dystrophin protein*

The dystrophin protein consists of 3 685 amino acids and has a molecular weight of 427 kDa.[49] 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 four domains: first an N-terminus, containing two actin-binding domains (ABDs), 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, the so-called central rod domain, consisting of 24 spectrin-like triple helical coiled repeat units, interrupted by four proline-rich hinge regions. A third ABD is present in repeat 11 to 17, while repeats 16-17 contain a binding site for neuronal nitric oxide synthase (nNOS).[55,56] Subsequently the protein contains a cysteine-rich part and finally a C-terminal domain. The cysteine-rich domain binds to $\beta$-dystroglycan, which is part of a membrane bound dystrophin-associated glycoprotein complex (DGC) (fig. 1.2). B-dystroglycan is a transmembrane protein that is bound to the extracellular $\alpha$-dystroglycan, which in turn is bound to laminin-2, a part of the 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.[57]

In addition to its mechanical linker function, dystrophin is involved in the organisation of the DGC as well as many other proteins and the control of the growth of the muscle cells.[39,58] The DGC is important in the maintenance of calcium homeostasis, both by maintaining membrane integrity, thereby preventing ‘leakiness’, and by proper localization of receptors (*e.g.* ryanodine receptors (RyRs)) involved in the functioning of $Ca^{2+}$-channels.[59] In the DGC, $\beta$-dystroglycan is connected to a complex of $\alpha$-, $\beta$-, $\gamma$- and $\delta$-sarcoglycans and sarcospan. This complex functions in maintaining membrane stability.[60] B-dystroglycan is also

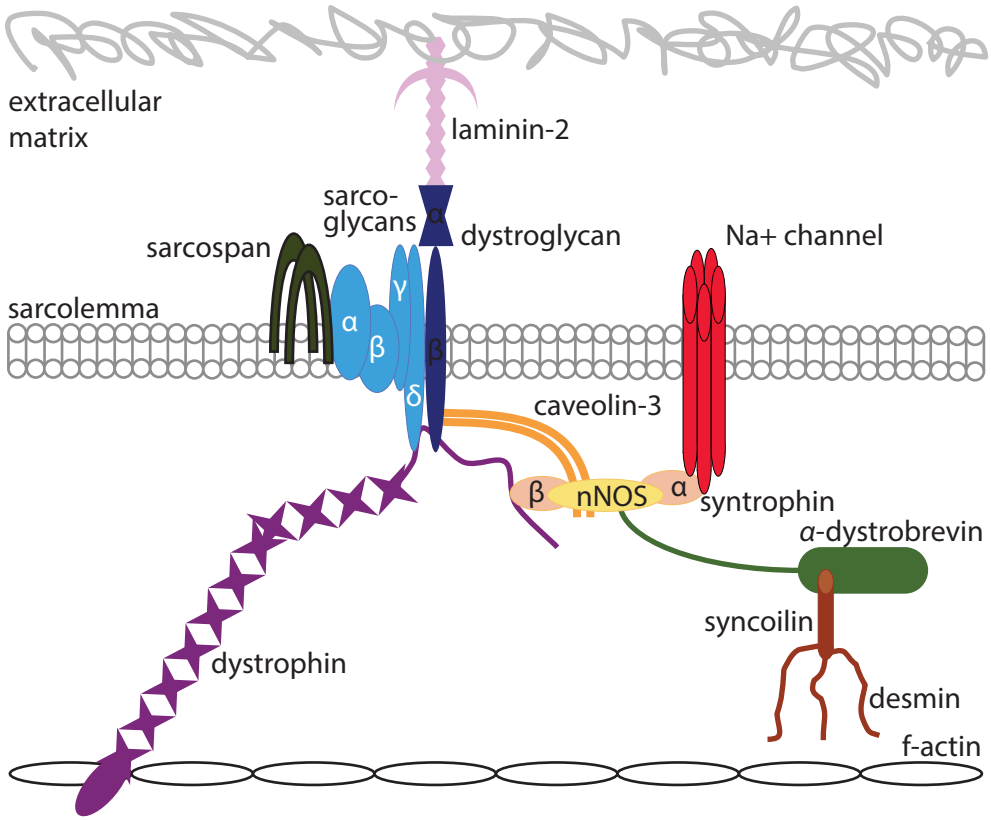


**Fig. 1.2:** The dystrophin-associated glycoprotein complex
The dystrophin-associated glycoprotein complex (DGC) is composed of *α*- and *β*-dystroglycan, a sarcoglycan-sarcospan complex and the dystrophin containing cytoplasmic complex. Dystrophin (purple) forms the link between the actin cytoskeleton 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).

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 compartmentalize and functionally regulate signalling molecules.[61] 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. Especially the spectrin-like repeat 16 and 17 of dystrophin turned out to be important for targeting nNOS to the sarcolemma.[56] 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.[62] 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.[63] 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.[64]

Next, in addition to the most common form of the dystrophin protein (called Dp427m) found in skeletal muscles, cardiomyocytes and in low amounts in glial cells, additional full-length and shorter isoforms of dystrophin exist. This is due to the presence of at least seven different promoters and alternative splicing events.[49,65] Three full-length variants exist (including the muscle isoform Dp427m), which only differ in their first exon. Dp427c (sometimes referred to as Dp427b) is expressed in the brain (in the cortical neurons and hippocampus) and the retina. Dp427p is expressed in cerebellar Purkinje cells and to a small extent in skeletal muscle.[66,67] Four internal promoters lead to the production of shorter dystrophin proteins, expressed in specific tissues, which are named after their molecular weight. These isoforms lack the actin-binding terminus but have retained the cysteine-rich part, associated with the DGC, and the C-terminal domain. Dp260, transcription starting in intron 29, is expressed in the retina.[68] In the brain, retina and kidney Dp140 is found, which has its promoter in intron 44 and ATG start site in exon 51. The promoter of Dp116 is located in intron 55, producing an isoform expressed in Schwann cells in adult peripheral nerves.[69] Finally, the two shortest isoforms are starting from a unique first exon in intron 62, which is spliced to exon 63. Dp71 is ubiquitously expressed in non-muscle tissues (most notably brain, retina, kidney, liver and lung) and in cardiomyocytes, but not in skeletal muscle.[70] Different Dp71 isoforms exist, due to the exclusion of exon 78 in some isoforms, resulting in a frame-shift, thereby inducing a unique, hydrophilic C-terminus in contrast to the hydrophobic C-terminus of the other dystrophin isoforms. Dp71 also interacts with the DGC in numerous cell types and plays a role in several cellular processes and during embryonic development. Its absence is associated with cognitive impairment and retinal dysfunction; two commonly observed non-muscular DMD phenotypes.[71] However this cannot account for all patients with cognitive dysfunction, since most mutations do not affect the Dp71 isoform or Dp140 isoform, which is also expressed in the brain. This is probably due to more general changes in the central nervous system (CNS), since full-length dystrophin and the DGC play a role in the development and functioning of the CNS.[72,73] Nevertheless, mutations affecting the distal part of the gene are more often associated with mental impairment, suggesting an additive negative effect when all dystrophin isoforms are deleted.[72,74] Dp40 lacks the normal C-terminal end due to an alternative polyadenylation site in intron 70. It is widely expressed in non-muscle tissues, most notably the brain.[75,76] In addition, alternative splicing facilitates the expression of many more dystrophin protein isoforms of which has been hypothesized to have a tissue-specific function. All these different isoforms might partly explain the variation in symptoms and involvement of specific tissues among DMD and BMD patients, depending on the specific location of the mutation. For example, mutations in the distal part of the gene, *e.g.* distal to exon 63, are associated with (severe) mental retardation and often also affect the transcription of the Dp71 isoform,[49,77] whereas retinal involvement is associated with mutations that affect Dp260.[78]

### *1.2.3 Animal models for DMD*

The most widely used model for DMD is the *mdx* mouse model (C57Bl/10ScSn-DMD$^{mdx}$/J). These mice have a single base mutation within exon 23 (a C to T substitution at position 3185), leading to a premature stop codon, so a truncated, non-functional dystrophin protein is formed.[79] 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, *mdx* muscles are clearly dystrophic and functionally impaired. Furthermore *mdx* mice show an impaired ventricular heart function, most prominently in the right ventricle, compared to wild type mice

as assessed by magnetic resonance imaging (MRI).[80] Nevertheless, their life span is only slightly reduced and the muscular weakness is mild. This is probably due to an improved regeneration, upregulation of muscle transcriptional factors (*e.g.* MyoD) and compensatory mechanisms, like the upregulation of utrophin, a dystrophin homologue, which can partly take over its function.[81] The gene encoding for utrophin (*UTRN*) is smaller than the *DMD* gene (1 Mb versus 2.4 Mb), contains 72 exons and is widely expressed. Like dystrophin, utrophin forms a link between the cytoskeleton and the ECM. Structurally utrophin is very similar to dystrophin: the N-terminal, cysteine-rich and C-terminal domains show ~80% similarity and the spectrin-like repeat domain ~35%. Utrophin is ubiquitously present in muscle in early foetal stages, but levels decrease during development and in adult muscle it is solely found at neuromuscular junctions, to retain their structural integrity. In developing and regenerating fibres it its present along the entire sarcolemma, where it is later replaced by dystrophin. In the absence of dystrophin, utrophin can be found along the entire muscle fibre membrane where it recruits most of the proteins normally associated with dystrophin.[82-84] In both DMD patients and *mdx* mice, utrophin is upregulated, albeit to a larger extent in *mdx* mice.[85] Mice that lack both dystrophin and utrophin (*mdx*/*Utrn*$^{-/-}$; double knockout mice) show a very severe, progressive muscular dystrophy. Their muscles display severe signs of damage and are rapidly replaced by fibrotic and adipose tissue. Ventricular dysfunction is already prominent at two month of age. Furthermore, these mice are functionally impaired, have an arched spine (kyphosis) and a life span of 20 weeks at maximum.[80,86] Due to the very severe phenotype and short life span, *mdx*/*Utrn*$^{-/-}$ mice are not practical as an experimental model. An intermediate model is the *mdx* mouse with haploinsufficiency for utrophin (*mdx*/*Utrn*$^{+/-}$). Their life span is significantly longer than that of *mdx*/*Utrn*$^{-/-}$ mice. However, inflammation and fibrosis in both skeletal muscle and diaphragm are more severe than in the *mdx* mouse.[87] The functional performance of *mdx*/*Utrn*$^{+/-}$ mice is also significantly worse compared to *mdx* mice with two *Utrn* copies.[88]

In addition, many more double mutants have been created to study the role of other factors involved in muscle functioning and during development of dystrophic pathology.[89,90] Several double knockouts have been generated lacking components of or proteins associated with the DGC. MyoD is a myogenic regulatory factor that plays an important role during muscle differentiation and regeneration. *Mdx*/*MyoD*$^{-/-}$ mice exhibit marked muscle dystrophy and develop severe cardiomyopathy during ageing.[91] Additional knockout of myocyte nuclear factor (MNF), a transcription factor selectively expressed in satellite cells, (*mdx*/*Mnf*$^{-/-}$) causes a severe phenotype and death within a few weeks as a result of satellite cell dysfunction.[92] In the absence of the DGC component $\alpha$-dystrobrevin, the DGC stays largely intact, but DGC-dependent signalling is impaired, resulting in a moderately more severe pathology compared to *mdx* mice in mice also lacking the DGC-component $\alpha$-dystrobrevin (*mdx*/*Adbn*$^{-/-}$) mice, *i.e.* life expectancy of eight to ten months and increased fibrosis/necrosis. Triple mutants lacking utrophin and $\alpha$-dystrobrevin (*mdx*/*Utrn*$^{-/-}$/*Adbn*$^{-/-}$) display a phenotype comparable to *mdx*/*Utrn*$^{-/-}$ mice.[93] In addition to the DGC, $\alpha_7\beta_1$-integrin connects the ECM to the cytoskeleton and *mdx*/*Itga7*$^{-/-}$ animals are born normally, but die within one month.[94] Calcium-buffer parvalbumin (PV) is present in high concentrations in skeletal muscles of rodents compared to humans. *Mdx*/*Pv*$^{-/-}$ mice were generated to test whether this explains the relatively mild phenotype of *mdx* mice. However, these mice were only slightly more affected than *mdx* mice, indicating this factor might contribute to the mild pathology but is certainly not the only cause.[95] The additional loss of the vasodilator NO producing nNOS in *mdx* mice (*mdx*/*nNOS*$^{-/-}$) does not alter the *mdx* phenotype, indicating that relocalization of

nNOS to the cytosol does not contribute significantly to *mdx* pathogenesis.[96]
To show the effect of genetic background on the severity of the dystrophic phenotype the DBA/2-*mdx* mouse has been generated by crossing *mdx* mice (C57Bl/10 background) with mice with a DBA/2 background. DBA/2 mice exhibited a lower self-renewal efficiency of satellite cells than C57Bl/6 mice. Skeletal muscles of DBA/2-*mdx* mice exhibited lower muscle weight, fewer myofibres, and increased fat and fibrosis. This results in a more severe phenotype, showing severe muscular weakness.[97]

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-ethylnitrosourea, a powerful mutagen, resulted in several new *mdx*-like mouse models on a C57Bl/6 background (B6Ros.Cg-Dmd$^{mdx\text{-}cv}$/J).[98] In *mdx* mice only the full-length Dp427 dystrophin isoform is affected, while in some of these *mdx*$^{cv}$ mice one or more additional isoforms are also affected. *Mdx*$^{2cv}$ mice have an A to T single base substitution in a splice acceptor sequence in intron 42, causing disrupted splicing, resulting in out-of-frame transcripts, which also affects the Dp260 isoform.[99] *Mdx*$^{3cv}$ mice have a mutation (T>A) in intron 65, which induces a new splice site, resulting in a frame-shift. In these mice all isoforms, including the brain Dp71 isoform are affected.[100] *Mdx*$^{4cv}$ mice have a C to T mutation at position 7 916 in exon 53, introducing a premature stop codon and affecting the Dp260 and Dp140 isoform. *Mdx*$^{5cv}$ mice have a 53 base pair deletion in exon ten due to an A tot T substitution at position 1 324, which introduces a cryptic splice donor site causing a frame-shifting deletion.[99] All these mice have a phenotype roughly comparable to the *mdx* mouse, although some minor differences exist. Compared to *mdx* mice, *mdx*$^{3cv}$ mice display a relatively high number of revertant fibres, whereas very low levels are present in *mdx*$^{4cv}$ and *mdx*$^{5cv}$ mice.[101] Probably due to the low level of near full-length dystrophin expression (~5%), the *mdx*$^{3cv}$ mice show higher forelimb grip strength than *mdx*$^{4cv}$ mice, although muscle pathology is comparable.[102] Additional knockout of utrophin in *mdx*$^{3cv}$ mice (*mdx*$^{3cv}$/*Utrn*$^{-/-}$), rendering them deficient in all dystrophin isoforms and utrophin, are even much milder affected compared to *mdx*/*Utrn*$^{-/-}$ mice, reflected by increased survival and stronger specific muscle force. Furthermore the *mdx*$^{5cv}$ model exhibits a slightly more severe phenotype, reflected by variances in gene expression, and histological differences and worse functional performance.[103]

Immunodeficient *mdx* or *mdx*-like transgenic mice have been developed to allow testing of transplantation of healthy donor cells (see paragraph 1.4.4). *Mdx nu/nu* mice are *mdx* mice bred onto a nude background (*Foxn1*$^{-/-}$), lack functional T-cells and display other skin defects. Their pathology is largely comparable to *mdx* mice, but the collagen content and fibrogenesis is affected by the nude mutation in skeletal limb muscle.[104,105] *Scid* mice are deficient in both T and B lymphocytes, due to a mutation in the *Scid* gene.[106] They were crossed with *mdx* mice (*Scid*/*mdx*), combining the *Scid* mutation with the *Dmd* mutation.[107] A disadvantage of this model is that the mice still have functional NK cells, limiting their use. Therefore recently the *NSG-mdx*$^{4cv}$ model has been generated, whose disease severity is comparable to *mdx* mice. This model contains both the *Scid* mutation and an *IL2Rg* mutation, which depletes NK cells and suppresses thymic lymphomas at the same time, in an *mdx* background.[108]

Additionally, several mouse models or cell lines have been generated often affecting only one or a few of the different dystrophin isoforms. *mdx52* mice have been generated by deleting exon 52 of the murine *Dmd* gene using a homologous recombination technique. In these mice the Dp140 and the retinal Dp260 isoform are absent, resulting in retinal abnormalities. Furthermore the muscle pathology is comparable to *mdx* mice, showing muscle hypertrophy and severe degeneration in the diaphragm.[109] In *Dp71*$^{-/-}$ mice the expression of the Dp71 iso-

form is specifically inactivated by deleting its first unique exon and replacing it by a reporter gene, which revealed differential activity of the Dp71 promoter during various stages of development and cell differentiation and between cell types.[110]

Transgenic *mdx* mouse models expressing dystrophin transgenes have been generated to reveal which parts of dystrophin are essential for functionality. In *mdx* mice in which full-length dystrophin was overexpressed under a muscle-specific promoter, pathology was completely prevented,[111] whereas *mdx* mice carrying a transgene with the non-muscle Dp71 isoform expressed in muscle, only showed restoration of the DGC, but no amelioration of muscle pathology. This indicates that the N-terminal actin-binding and/or central spectrin-like repeat domains are required for the functionality of the dystrophin protein.[112,113] Furthermore expression of a minigene missing exons 71 to 74, mimicking a dystrophin isoform normally only present in brain and not in muscle, in transgenic *mdx* animals resulted in normal muscle morphology and physiology, suggesting these exons are not crucial for dystrophin function.[114] Myofibres from *mdx* mice expressing a microdystrophin transgene (ΔR4-R23) showed less damage after lengthening contractions than *mdx* and even wild type muscles. This is probably due to molecular and cellular adaptations in these mice. Compared with wild type myofibres an increase in $\alpha_7$-integrin and utrophin expression was seen due to chronic myotendinous strain (*e.g.* partial tears) caused by over-stretching of muscle to which they are more susceptible.[115]

Next to the *mdx* mice models, expressing (almost) no dystrophin and transgenic *mdx* mice expressing normal or higher levels of (truncated) dystrophins, several models exist expressing different levels of dystrophin. Transgenic mice expressing low levels of full-length dystrophin or minidystrophins of murine or human origin have been generated.[116] First of all, these mice are useful to determine which part(s) of the protein are crucial for its function. Mice expressing around 50% of the aforementioned truncated dystrophin lacking exons 71-74 in skeletal muscle and 10-20% in diaphragm displayed a markedly milder phenotype compared to *mdx* mice.[114] In another transgenic *mdx* model, expression of minidystrophins by a muscle-specific promoter resulted in around 50% of dystrophin in fast-twitch skeletal muscle, but almost no expression in slow-twitch muscle, heart and diaphragm, which ameliorated the dystrophy in muscles in which it was expressed.[117] Secondly, they provide an indication which quantity of dystrophin protein restoration is required to (partly) prevent pathology. Injection of wild type embryonic stem cells (ESCs) into *mdx* blastocysts generated chimeric mice expressing more variable levels, which resulted in a dose-dependent amelioration of the muscle pathology and function; *i.e.* no improvement is seen in mice with >5% dystrophin, whereas chimeric mice with 10-30% appeared almost indistinguishable from wild type.[118] A more sophisticated model expressing various levels of dystrophin is the *mdx-Xist*$^{\Delta hs}$ mouse, generated in our own group, where dystrophin levels range from 3-47%. These mice have been generated by crossing non-dystrophic female mice with skewed X-inactivation, due to mutations in the *Xist* promoter resulting in preferential inactivation of the mutated *Xist* allele, with *mdx* males. This leads to female offspring in which the X-chromosome containing the wild type *Dmd* gene is preferentially inactivated. In these mice the level of dystrophin needed to improve the phenotype is dose-dependent, but varies between different aspects of the disease phenotype. Low levels (3-14%) are already sufficient to ameliorate some dystrophic symptoms, including cardiomyopathy, but levels >30% are needed for full recovery, especially when mice are subjected to forced exercise.[119] When these mice were crossed with mice also lacking utrophin (*mdx*/*Utrn*$^{-/-}$/*Xist*$^{\Delta hs}$) low dystrophin levels (<4%) greatly improved survival and motor function, which is severely comprised in *mdx*/*Utrn*$^{-/-}$ mice, and higher levels

improved histopathology too.[120]

Finally, a mouse line carrying an intact and functional copy of the complete human dystrophin gene (*hDMD*) integrated on mouse chromosome five has been made. These mice have been generated by fusion of yeast spheroplasts containing an artificial chromosome carrying this *hDMD* gene with murine ESCs and injecting these into mouse blastocysts. Dystrophin expression of the different isoforms in a tissue-specific manner is maintained in these *hDMD* mice. Crossing of these mice with *mdx* or *mdx*/*Utrn*$^{-/-}$ mice prevented muscular dystrophy completely, indicating the functionality of the transgene.[121] These mice are very useful for testing of therapies specific for the human *DMD* gene. However these mice carry an intact version of the human *DMD* gene; therefore studies on the effect of therapies on muscular dystrophy are not possible. Attempts to generate dystrophic mouse models carrying *hDMD* genes with specific mutations have not been successful so far [Veltrop *et al.*, personal communication].

Several spontaneously occurring canine X-chromosome linked muscular dystrophy with dystrophin deficiency (CXMD) models similar to DMD in humans have also been described. The golden retriever muscular dystrophy (GRMD) dog is most well described. These dogs have a single base substitution in the 3' consensus splice site of exon seven, resulting in skipping of exon seven, thereby introducing a premature stop codon in exon eight. 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.[122] Most affected animals die within a few years, mainly due to degeneration of the cardiac muscle.[123] 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.[124,125] This makes the dogs less suitable for experimental use, due to standardisation problems. Furthermore, 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. 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 ($CXMD_J$) model. The phenotype of this dog is less severe compared to GRMD; cardiac involvement is milder and survival rate increased.[126-128]

Next to the GRMD and $CXMD_J$ dystrophin deficiency has been described in several other dog strains, carrying various mutations in the *DMD* gene. For some of these the exact mutation has been characterized. Rottweilers carrying a substitution in exon 58 creating a premature stop codon and German short-haired pointers with a large deletion encompassing the entire *DMD* gene have been described.[129] Cavalier King Charles Spaniels with dystrophin-deficient muscular dystrophy have a point mutation in the 5' donor splice site of exon 50 that results in deletion of exon 50 on RNA level, thereby causing a frame-shift.[130] Furthermore the absence of dystrophin is reported in several other strains, such as Belgian Shepherd Brittany Spaniel, Irish Terrier, Labrador Retriever, Miniature Schnauzer and Rat Terrier.[131-136] In Japanese Spitz dogs full-length dystrophin is absent, but a 70-80 kDa protein is present on immunoblotting with antibodies against the C-terminal domain of dystrophin. These dogs display clinical signs comparable to other CXMD models, indicating that the protein of this size is not large enough to protect against dystrophy.[137]

Another natural animal model occurs in cats: hypertrophic feline muscular dystrophy (HFMD). This is caused by a deletion of around 200 kb in the *DMD* gene, involving the muscle (Dp427m) and Purkinje (Dp427p) promoter. Dp427c is present in low levels in the muscles.[138,139] Unlike humans, canine and murine models, these cats exhibit little fibrosis, but display extensive muscle hypertrophy and $Ca^{2+}$-deposits in the muscle fibres, becoming apparent from an age of around 10-14 weeks. Eventually these animals die of compression of the oesophagus by hypertrophic diaphragm muscles or renal failure, due to impaired water uptake caused by hypertrophy of the tongue.[140] Although hypertrophy is also present in the heart, these cats only occasionally display heart failure.[141]

Since the dystrophin protein is highly conserved during evolution, homologues are also found in invertebrates, like the worm *Caenorhabditis elegans*, zebra fish *Danio rerio* and fruit fly *Drosophila melanogaster*.[142] In *C. elegans* null mutations of the dystrophin-like gene *dys-1* cause only subtle changes like hyperactive locomotion, but muscle histology appears normal and shows no signs of degeneration, probably due to the short life-span of these worms. However if *dys-1* mutated worms are crossed into an *hlh-1* background, carrying a weak mutation in the *hlh-1* gene (the *C. elegans* homologue of *MyoD*; also called *CeMyoD*) extensive muscle degeneration is observed.[143,144] Several dystrophin null mutations, called *Sapje* or *Sapje*-like, have also been identified in *D. rerio*. These zebra fish display progressive muscle degeneration due to failure of somatic muscle attachment, causing 'tearing' of muscle.[145,146]

The most recently developed animal model is the DMD pig, carrying a deletion of exon 52.[147] The rationale behind choosing a pig is that pigs closely resemble human size, anatomy and physiology.[148] These pigs display a severe, progressive muscular dystrophy, impaired mobility and death within three months. Three months-old pigs show similarities with DMD patients in gene expression profiles reflecting inflammation, de- and regeneration and fibrosis. However some pigs die shortly after birth and their profiles are more like those seen after acute muscle injury. At the moment male DMD pigs are generated by nuclear transfer of male pigs cells with a deletion of exon 52, but the generation of female $DMD^{+/\Delta exon52}$ cells will be used to produce heterozygous females for future breeding.[147]

## 1.3 Antisense oligonucleotide-mediated exon skipping

Antisense oligonucleotides (AONs) are typically small synthetic pieces of DNA or RNA (15-30 base pairs), which are complementary to their target mRNA. Initially, DNA AONs were used for the specific knockdown of gene expression. These DNA AONs bind to the (pre-)mRNA to form DNA-RNA hybrids, which are recognized by RNase H. This enzyme cleaves the RNA at the location of the bound AON, thereby preventing the translation into protein, thus decreasing protein expression. DNA AONs are degraded quickly by endonucleases. Therefore AONs with a phosphorothioate (PS) instead of a phosphodiester (PO) backbone were developed, which are more endonuclease-resistant. These led to very efficient expression knockdown (85-95%) of, for example, genes (UL36 or IL2) involved in cytomegalo virus (CMV) induced retinitis.[149] In addition to activation of RNase H, AONs can also downregulate 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.[150] 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.[151] The activation of RNase H is useful when downregulation of gene expression is required, but not when AONs are used for modulation of pre-mRNA splicing.

For DMD, AON-mediated exon skipping is based on the reading frame rule (fig. 1.1), which underlies the phenotypic differences between DMD and BMD. Furthermore, in some DMD patients rare, dystrophin-positive (so-called "revertant" fibres) are found, which are the result of spontaneous exon skipping or secondary mutations restoring the reading frame in these fibres and allowing dystrophin production. 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 (fig. 1.3), as found in BMD and revertant fibres.[152] This approach is mutation-specific and a large variety in mutations exists among DMD patients. Fortunately the aforementioned two hotspots comprise a large proportion of the mutations.[44]

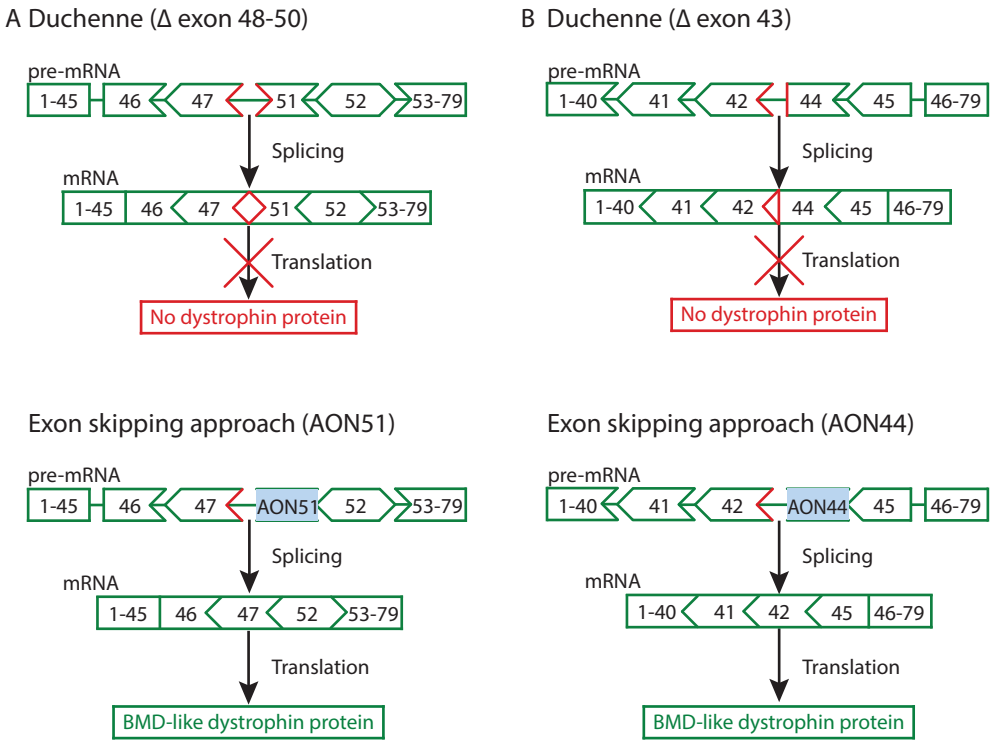


**Fig. 1.3:** The exon skipping approach
In Duchenne muscular dystrophy (DMD), mutations in the DMD gene cause a disruption of the open reading frame, causing translation into protein to stop prematurely and loss of functional dystrophin. The exon skipping approach aims to restore the open reading frame by using an antisense oligonucleotide (AON) to bind to the pre-mRNA, thereby hiding one (or multiple) exons for the splicing machinery, which causes the exon(s) to be 'skipped' and not being incorporated in the mRNA. This enables the translation into a shorter, but largely functional 'BMD-like' dystrophin protein. Since this approach is mutation-specific, different mutations require the skipping of different exons. a) Example of a deletion of exon 48–50, where the reading frame can be restored by the skipping of exon 51. b) Example of a deletion of exon 43, where the reading frame can be restored by the skipping of exon 44.

### *1.3.1 Backbone chemistries*

Knowing that RNA/RNA duplexes are more stable than DNA/RNA duplexes led to the use of RNA in AONs. As RNA is even less stable in biological systems than DNA, the 2'-OH position of the ribose was modified (2'-*O*-methyl (2OMe) or 2'-*O*-(2-methoxy)ethyl (2OMOE)). During the last decades, various chemical modifications (fig. 1.4) have been developed for use in antisense technology, differing in sugar and backbone chemistry and having different biophysical, biochemical and biological properties. One of the earliest, and one of the most common, of these modifications, the 2'-*O*-methyl phosphorothioate (2OMePS) chemistry, has an increased affinity for RNA and improved cellular uptake. Disadvantages of the PS backbone are that the safety profile could be improved and some sequences elicit an immune response, although the latter is also observed with other backbone chemistries.[150] The tendency to induce an immune response is partly counteracted by the 2OMe modification. Furthermore 2OMePS AONs bind to serum proteins, which prevents renal clearance and thus improves bioavailability.[153]

Peptide nucleic acids (PNA) contain a flexible, uncharged, achiral *N*-(2-aminoethyl)glycine backbone to which nucleobases are attached via methylenecarbonyl linkages instead of the PO backbone and riboses of DNA oligos. PNAs have a high affinity for RNA, are nontoxic even at high concentrations, are peptidase- and nuclease-resistant and have a high sequence-specificity. PNAs are very difficult to transfect due to their uncharged nature and their insolubility caused by their hydrophobic nature. This can be solved by the attachment of carrier groups, which can easily be linked to the peptide backbone, or addition of cationic lysine residues. Another disadvantage is the rapid clearance of PNAs *in vivo*. Their mechanism of action is by steric hindrance.[154]

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 tar-

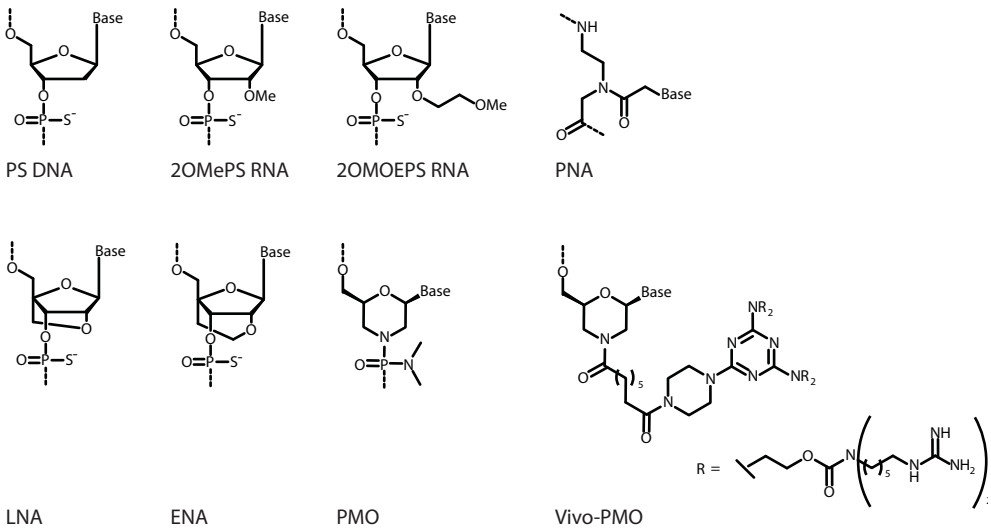


**Fig. 1.4:** Chemical structure of different backbones of antisense oligonucleotides
Phosphorothioate (PS) DNA; 2'-*O*-methyl phosphorothioate (2OMePS) RNA; 2'-*O*-(2-methoxy)ethyl phosphorothioate (2OMOEPS) RNA; peptide nucleic acid (PNA); locked nucleic acid (LNA); ethylene bridged nucleic acid (ENA); phosphorodiamidate morpholino (PMO) and dendrimeric octaguanidine conjugated phosphorodiamidate morpholino (Vivo-PMO).
*Picture kindly provided by Peter de Visser.*

get mRNA or DNA, thereby forming stable duplexes. This is an advantage, but also a disadvantage, since LNAs longer than 15 base pairs tend to self-anneal and are not very sequence-specific, which increases the chance of unwanted side effects.[155] However, currently mainly LNA/2'-*O*-methyl oligonucleotide mixmers are used, which show much more sequence-specificity.[156] 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'-*C* of the ribose. They have similar properties to LNAs, but have a higher affinity for RNA, are very stable and more nuclease-resistant.[157,158]

Phosphorodiamidate morpholino oligomers (PMO/morpholino) have a six-membered morpholino ring instead of the ribose and the PO bond is replaced by a phosphorodiamidate linkage. They do not activate RNase H, are very resistant to nucleases and are non-toxic. The fact that they are uncharged prevents undesired binding to proteins, limiting circulation time. However, this also results in limited nuclear uptake, where pre-mRNA splicing takes place. Their uncharged nature makes them hard to transfect in cell cultures, but *in vivo* PMOs were found to be taken up by tissues after local injection. This is probably due to the fact that the uncharged nature does not interact with other cellular components. In contrast to 2OMePS AONs, which bind serum proteins and are thus protected from renal clearance,[159] PMOs are efficiently filtered out by the kidney within minutes after injection.[160] In general, for use in DMD PMOs are often a bit longer than 2OMePS AONs (25 nucleotides or more compared to around 20 nucleotides for 2OMePS AONs). They primarily act by steric prevention of ribosomal assembly.[150,155,161] 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, though the latter has not yet been observed.[162] Conjugation of PMOs with an octaguanidine dendrimer (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,[163] though the polymer is toxic at higher concentrations as well.

### *1.3.2 AON design and targets*

Target sites for exon skipping AONs are splice sites, 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 five small nuclear ribonucleoproteins (snRNPs) 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 (serine- and arginine-rich (SR) proteins) can bind. AONs targeting ESEs have been proposed to sterically hinder the binding of SR proteins and thus prevent exon recognition.[164-167]

### *1.3.3 Antisense-mediated exon skipping* in vitro

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.[168,169] 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.[170,171] 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.[172]

By that time, exon skipping with 2OMePS AONs, unable to activate RNase H, had also been explored, both in DMD patient derived cells[173] and *mdx* myoblasts.[174,175] In two 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.[173] Subsequently, 2OMePS AONs against other exons were developed, which showed specific skipping of the particular exon in healthy control cells[167,176] or DMD patient-derived cells for a variety of exons and mutations.[177-180] In DMD patient-derived cells 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 DGC, as shown by increased membrane expression of DGC-associated proteins. Another interesting finding was the higher exon skipping levels observed in these patient cell lines, than previously seen in control cell lines. A possible explanation is that nonsense-mediated decay (NMD) reduces levels of the original out-of-frame transcripts, which are therefore less stable than the newly formed in-frame transcripts.[177]

2OMOE AONs containing a PO bond (2OMOEPO) were also able to induce exon skipping in *mdx* derived cell cultures. When the PO bond was replaced by a PS bond (2OMOEPS), increasing its stability and sequence-specificity, effectiveness was increased. Furthermore, whereas for 2OMePS AONs targeting mouse exon 23, 20 oligonucleotides appears to be the optimal length,[161] for 2OMOEPS AONs 25-mers were more efficient than 20-mers. When compared to 20-mer 2OMePS AONs, these 25-mer 2OMOEPS AONs induced higher exon skipping levels *in vitro* for skipping of exon 23, probably due to improved cellular uptake.[181]

*In vitro* testing of PMOs, the other chemistry currently mainly used for exon skipping in DMD, is far more difficult, since the neutral PMOs are, in contrast to the negatively charged 2OMePS AONs, not easily taken up by cells. However annealing of the PMOs to various complementary DNA/RNA molecules ('leashes') in combination with lipofectamine as a transfection reagent could overcome this problem and exon skipping and dystrophin restorations was shown in *mdx*-derived cell cultures after PMO transfection.[182] Efficiency of PMOs has also been shown in control cells and cells derived from DMD patients or animal models.[130,183,184]

AONs with a RNA/ENA chimeric backbone (a fusion of 2OMe RNA and ENA) have been used to induce exon skipping in DMD patient-derived cells requiring exon 19 skipping far more efficiently than the aforementioned PS AON against this exon.[158] These chimeras were also able to induce exon 45 or 46 skipping and restore dystrophin restoration in patient cells requiring exon 45 skipping.The same was shown for an RNA/ENA chimera targeting exon 41 in patient-derived cells with a nonsense mutation in this exon.[185]

Finally, 20-mer PNA AONs have been tested in *mdx*-derived myoblasts. PNAs against exon 23 were able to induce specific skipping of this particular exon.[186]

Comparison of AON analogues with different backbone chemistries against exon 46 in

cells from a patient with an exon 45 deletion showed highest skipping levels for LNAs, followed by 2OMePS, whereas PMOs induced low skipping efficiency and PNAs were ineffective. However, as mentioned before, LNAs were far less sequence-specific as 2OMePS AONs.[155] Sequence-specificity of 2OMePS AONs has further been tested by comparing exon skipping levels in cultured cells from patients with small mutations in in-frame exons versus control cells. Some AONs were equally efficient in both types of cells, whereas for others efficiency was largely reduced in the cells carrying small mutations, which indicates that for this kind of mutations mutation-specific AONs may be required.[180]

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 two or more exons for reading frame restoration.[187] 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 two 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%).[188] Subsequently, successful double exon skipping was tested in the GRMD and $CXMD_J$ canine models for DMD which need double skipping to bypass the mutation. Cells derived from these dogs have also been used to show double exon skipping *in vitro* (see paragraph 1.3.4.2).[189,190]

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 would largely increase its therapeutic applicability for a number of different mutations. Indeed the same result could be induced in patient cells with an exon 48-50 deletion.[188] Furthermore multi-exon skipping has been shown for exon 19-25 using 2OMePS AON cocktails in *mdx*-derived cell cultures.[191] Skipping of other larger stretches of exons however turned out to be technically challenging and has had limited success so far.[192] The use of several ratios of 45AON and 55AON in both control and patient cell lines resulted in undetectable to very low exon 45-55 skipping frequencies.[193] Recently Aoki *et al.* showed successful multiple skipping of exons 45-55 *in vivo* in the *mdx52* mouse, although these results raise some questions (see paragraph 1.3.4.1).[194]

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: the actin-binding parts, the cysteine-rich C-terminal part (binding to the DGC), 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.[187] 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, skipping a single exon 45 was indeed possible, but in other cases the skipping was so efficient that both exons were skipped, leading to an out-of-frame transcript.[178] 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.[195] Successful skipping of multiple

exon duplications has not yet been achieved.[178] In total 6% of patients could benefit from single or multiple skipping of exon duplications.

### *1.3.4 Antisense-mediated exon skipping* in vivo *in animal models*

#### *1.3.4.1 AONs in murine models for DMD*

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 25-mer 5' splice site targeting AON with a 2OMePS backbone (called 5'SS-25; later renamed to M23D(+12-13)), which was tested locally in the *mdx* mouse. This showed the first proof-of-concept for inducing exon 23 skipping *in vivo* with a 2OMePS AON. After two and four weeks, intramuscular treatment (weekly 1 μg of AON complexed with 2 μg of lipofectamine) induced low levels of dystrophin expression and restoration of *γ*-sarcoglycan at the cell membrane, suggesting at least partial functionality of the newly formed dystrophin protein. However this AON also induced skipping of additional exons (21 and/or 22), which resulted in in-frame Δ21-23 transcripts and out-of-frame Δ22-23 transcripts.[196] Thereafter the sequence and target site for this AON was further optimised *in vitro* in *mdx* myotube cultures, resulting in a 20-mer, named M23D(+02-18), which was effective in inducing exon skipping and dystrophin protein expression for concentrations as low as 5 nM.[197] A single intramuscular injection of 5 μg of this compound in combination with a copolymer F127 in the tibialis anterior of both young (two or four weeks old) and aged (six months old) mice resulted in marked dystrophin expression two weeks after injection, persisting up to three 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 tetanic force, of the treated muscles. Importantly no auto-immune response against the newly formed dystrophin protein was observed.[198]

Also a 25-mer 2OMOEPS induced exon skipping after intramuscular injections (5 μg) in the tibialis anterior of *mdx* mice. This was more effective than 20-mer 2OMePS AONs, whereas no differences between 25-mer 2OMOEPO, 20-mer MOE20PS and 20-mer 2OMePS AONs were observed.[181] However, direct comparison has only been tested for exon 23 skipping in the *mdx* mouse, so could also be a sequence-depended effect, as is seen with 2OMePS versus PMO AONs.[161]

PMO AONs have shown to be effective *in vivo* as well. Whereas transfection was difficult in cultured cells due to their neutral backbone, this is no problem *in vivo* were no transfection reagent is needed. Also for PMOs the AON design was first optimised in cultured *mdx* cells by complexing it to leashes and lipofectamine as described above. This resulted in a ψM23D(+07–18):leash 3 lipoplex, which 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 two weeks after a single intramuscular injection of 1 μg in the tibialis anterior. Efficiency was comparable in both young (three weeks old) and aged (six months old) *mdx* mice.[182]

Local injection of 5, 10 or 20 μg of PNAs or several PNA-peptide conjugates in the tibialis anterior of *mdx* mice resulted in dystrophin positive fibres two weeks after a single injection in both young (two months old) and older (six months old) mice in a dose-dependent manner.[186]

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. For M23D(+02-18) AONs with the 2OMePS backbone, three intravenous injections with 2 mg of AON combined with copolymer F127 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.[199]

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 three 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 repeated subcutaneous treatment (two weekly injections of 100 mg/kg for eight weeks) also a decrease in serum creatine kinase (CK) levels was observed. CK is an enzyme that leaks out of the muscles into the blood stream when muscles are damaged, so a decrease indicates an improvement of muscle integrity.[200]

Long term treatment for six months of *mdx* mice with 200 mg/kg/week of these AONs was well tolerated and the beneficial effect was maintained. Furthermore the same study showed that this treatment had higher therapeutic effects in the more severely affected *mdx*/*Utrn*$^{+/-}$ model.[201]

For PMOs repeated systemic (intravenous) injections with 2 mg of AON per week 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.[202] Wu *et al.* showed that dystrophin restoration in cardiac muscle could be achieved (up to 30% of healthy levels) by systemic PMO treatment, although extremely high doses (up to 3 g/kg body weight) were required.[203]

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 AONs targeting mouse exon 23. 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 (2.9 nmol AON on two consecutive days). Systemic (intravenous) comparison in the *mdx* mouse (three times 14.52 μmol/kg/week) 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. 2OMePS AONs bind to serum proteins while PMOs do not, probably explaining the high renal clearance of PMOs versus 2OMePS AONs (see paragraph 1.3.1). 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 showed higher levels in skeletal muscle compared to cardiac muscle too.[161]

To further optimise exon skipping efficiency multiple strategies can be used (see also paragraph 1.3.6). One approach is to conjugate the AON to a cell-penetrating and/or muscle-targeting peptide. A PMO conjugated to an arginine-rich 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 led to a decrease in CK levels.[204] Another study confirmed that the long term (12 weeks) systemic treatment with 30 mg/kg/biweekly of pPMOs was effective in restoring dystrophin expression in skeletal muscle, improving muscle function and preventing heart failure.[205] Therapeutically relevant effects were still observed after one year of treatment with 1.5 mg/kg/biweekly in *mdx* mice without serious adverse events.[206] These pPMOs were also able to rescue the severe *mdx*/*Utrn*$^{-/-}$ mouse model by systemic (intraperitoneal) treatment with 25 mg/kg/week for six weeks. Considerable improvement of survival and muscle function was observed, combined with dystrophin expression in almost all muscles, except for the heart.[207] Peptides might elicit an immune response, but no signs of such a response or toxicity were found in the murine models so far. The study of Wu *et al.* indicated that pPMOs have a high acute toxicity (an $LD_{50}$ of approximately 85 mg/kg). Nonetheless, effective doses for inducing exon skipping and dystrophin restoration in mice are much lower.[206] Unfortunately, when a pPMO compound was tested in primates, there were safety issues. In cynomolgus monkeys pPMO doses equivalent to the ones used in mice, were not toxic, but also induced only very limited exon skipping. Higher doses were effective, but also caused tubular degeneration in the kidneys, a sign of renal toxicity. By contrast naked PMO did not cause toxicological effects at much higher doses.[162,208]

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 (3 mg/kg/week for six weeks) in restoring high levels of dystrophin expression body-wide without obvious signs of toxicity.[209]

Novel cell-penetrating peptides have been discovered by inducing modifications to a *D. 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 of 5 µg in the tibialis anterior of *mdx* mice.[210] More and improved Pips have been developed. Pip5e fused with a PMO (Pip5e-PMO) showed high exon skipping efficiency after a single intravenous injection of 5 µg in the *mdx* mouse. Most importantly it also efficiently targeted the heart, leading to dystrophin levels of more than 50% of wild type levels.[211] Further characterisation of this Pip-peptide by using different derivatives of Pip5e-PMO (Pip6-PMOs) revealed that the central hydrophobic core and the hydrophobic/cationic balance is imperative for efficient targeting of the heart.[212] These results will be used to further optimise the Pip-PMO for testing in a clinical trial.

Another modification of the PMO is conjugation to a dendrimeric octaguanidine polymer (vivo-morpholino). This modification also significantly improved the delivery and dystro-

phin production in *mdx* mice after intravenous injection. Repeated treatment (five times 6 mg/kg/biweekly) 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.[163]

A third method to reduce the dose is through the use of biodegradable nanoparticles. Intraperitoneal injection of cationic polymethylmethacrylate (PMMA) nanoparticles (NP) loaded with a low dose of 2OMePS AON were able to induce body-wide exon skipping and dystrophin restoration in the *mdx* mouse far more efficiently than naked 2OMePS AONs at the same dose (0.9 mg/kg/week).[213] Further optimisation of the NPs resulted in PMMA/N-isopropilacrylamide+ NPs, called ZM2, which were, after seven weeks of injections with 7.5 mg/kg/week, able to restore dystrophin protein in both skeletal and cardiac muscle in up to 40% of the fibres albeit at very low levels.[214] They also demonstrated its longer term efficacy, since this effect was, in contrast to naked 2OMePS AONs, still detectable 90 days after the last injection, although the amount of dystrophin positive fibres had decreased till 7% at maximum.[215]

Both 2OMePS and PMO AONs have also been tested in the *mdx*$^{4cv}$ 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 *mdx*$^{4cv}$ mice. 2OMePS AONs induced exon skipping in these cell cultures, but no dystrophin protein was observed. Intramuscular injection of 100 µg of the AON cocktail complexed with F127 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 of 40 µg of cocktail tested in different ratios in the tibialis anterior) in exon skipping and restoration of dystrophin expression.[216]

The *mdx52* mouse has been used to test the feasibility of multi-exon skipping targeting the human mutation hotspot (exon 45-55) with vivo-morpholinos. First *in vitro* in cultured myotubes from these mice and after intramuscular injection with a mixture of ten AONs, skipping of all target exons could be demonstrated. Furthermore systemic injections of this AON cocktail resulted in dystrophin expression up to 15% of wild type levels, accompanied by improved functional performance and histopathology.[194] This is the first successful application of multi-exon skipping, which was shown to be difficult *in vitro* (see paragraph 1.3.3). However these results are controversial, since they needed a cocktail containing a large number of AONs to achieve skipping of this stretch of exons, while none of the anticipated intermediate products showing skipping of only one or a few exons were observed.

AONs are sequence-, and therefore species-, specific. So, to be able to test human-specific AONs *hDMD* mice can be used. 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 two or three mismatches, no detectable skipping was observed.[217] 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 after intramuscular injection. This indicated that the differences between PMO and 2OMePS observed for exon 23 targeting AONs are probably more due to sequence differences than to chemistry differences. Furthermore,

this paper also suggested important differences in sequence-specificity. 2OMePS AONs with two mismatches had a greatly reduced efficiency, whereas PMO AONs remained equally effective. This can increase the risk of off-target side effects.[161]

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 CK into the bloodstream. It has been proposed that the AONs migrate into the muscle fibres through these same holes.[218] 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. A pilot experiment with systemic (intravenous) injection of nine times 100 mg/kg 2OMePS AONs targeting exon 51 in the *hDMD* mouse resulted in very low or undetectable exon skipping in the muscles.[219] An alternative hypothesis is that the uptake of AONs depends on myotube formation, *e.g.* during development or active regeneration, and that AONs are therefore more easily taken up by immature myotubes than by mature myofibres. PMO uptake and PMO-induced exon skipping was greatly enhanced after cardiotoxin-induced injury in wild type mice.[220] Any or both hypotheses might also provide an explanation 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'. Furthermore in heart, in contrast to skeletal muscle, hardly any regeneration takes plays. Recently, vivo-morpholinos against exon 50 were shown to be able to achieve high levels of exon skipping after systemic (intravenous) injection (15 mg/kg) in the healthy skeletal muscles of the *hDMD* mouse and even low levels in the cardiac muscle. There were no overt signs of toxicity or adverse effects, only a small increase in serum CK levels, which could reflect a bit of membrane integrity disturbance.[221]

### *1.3.4.2 AONs in other animal models*

First AON experiments with the canine model have been performed *in vitro* in myoblast cell cultures of the GRMD dog. The nature of the mutation requires the skipping of two exons (exon six and exon eight) to restore the reading frame, thereby making it more challenging. *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.[189] Further testing of these AON cocktails *in vivo* by intramuscular injections, revealed that the AONs targeting exon eight were effective, but the AONs targeting exon six, which had shown effectiveness *in vitro*, were not.[222] Another small experiment (in a six months old and a five year 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 of 1.2 mg. The structure of the dystrophin-positive cells was reported to be improved.[223]

Systemic (intravenous) treatment of $CXMD_J$ dogs with a cocktail of three PMO AONs targeting exon six (two PMOs) and exon eight (one PMO), generated body-wide production of functional dystrophin. In the heart there was, as observed in mice, only modest production of dystrophin. 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 with doses up to 200 mg/kg/injection for five to 11 times.[190] Further optimisation of the cocktail and using a vivo-morpholino backbone resulted in a four AON cocktail (two against exon six and two against exon eight), inducing more efficient exon skipping and higher dystrophin expression after intramuscular injection with 30 µg of each AON in the cocktail.[224]

### *1.3.5 AONs in clinical trials*

After the promising preclinical results *in vitro* and *in vivo*, the first clinical trials were initiated. An overview of all clinical trials, completed and currently ongoing, is given in table 1.1. 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).

#### *1.3.5.1 Local treatment with AONs*

The first trials used local (intramuscular) injections to obtain proof-of-principle in humans and examine possible adverse effects. 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%).[187]

A single injection in the tibialis anterior with 0.8 mg of a 2OMePS AON (called PRO051) in four 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.[225]

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 and saline into the contralateral muscle. In two patients receiving the lowest dose (0.09 mg) this resulted in low levels of exon 51 skipping, but no detectable 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.[226] Since both studies studied different muscles and used different techniques for quantifying immunocytochemistry the results are not directly comparable.[227] However both studies showed unequivocal effectiveness of the used compound in the absence of side effects.

#### *1.3.5.2 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 one DMD patient

intravenously with a weekly dose of 0.5 mg/kg body weight of a PS AON against exon 19 for four weeks. Only very low levels of exon skipping and dystrophin protein were observed in a muscle biopsy.[228] This is not surprising, as the dose used was very low and the PS backbone chemistry is not ideal for exon skipping purposes (see paragraph 1.3). Furthermore, this was only one single patient, so no real, reliable conclusions can be drawn from this experiment.

More extensive, open-label, dose-escalation, phase I/IIa studies have been completed for both 2OMePS and PMO AONs. The first was a study with abdominal subcutaneous injections of PRO051 (2OMePS AON; now called GSK2402968 or drisapersen) in 12 patients testing five weekly doses (0.5, 2, 4 and 6 mg/kg body weight) in groups of three patients. Doses of 2 mg/kg body weight or higher resulted in specific exon 51 skipping. In ten 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 (six 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 six minute walk test. Since a placebo group is lacking, interpretation of this improvement must be done with caution. Nevertheless, the overall results were encouraging and only mild adverse events, like irritation at the injection side and mild proteinuria, were observed.[229] Furthermore the extension trial has been continued. Patients have now been treated for over three years. In the first 72 weeks they received weekly injections, followed by an intermittent dosing scheme (eight weeks on, four weeks off). Except from two patients who lost ambulation in between the initial trial and the extension trial and two patients who were already in decline, the remaining eight showed stabilisation of six minute walking distance [presented at the 18th international conference of the World Muscle Society, Asilomar, CA, USA, Oct 2013]. Considering the age of the patients and the natural history data, this is unlikely to be solely to be attributed to a placebo effect.

To determine the safety, tolerability and pharmacokinetics of drisapersen a study in non-ambulant patients with different AON doses has been performed. Patients received a single dose of drisapersen (or placebo) by subcutaneous administration. Initially doses of 3, 6, 9 and 12 mg/kg were planned, but since the study objectives were met with the 9 mg/kg dose, it was decided not to proceed with the 12 mg/kg dose. Only between the 3 and 6 mg/kg some dose proportionality was seen, but not in the whole range from 3 to 9 mg/kg. The two lowest doses did not raise large safety concerns, but in the 9 mg/kg group fever and transient elevation of inflammatory parameters was observed. It was concluded that 6 mg/kg is the maximum tolerated dose for drisapersen in non-ambulant DMD patients.[230]

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 body weight) 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 body weight, albeit variable between individual patients. The responding patients showed dystrophin levels of 8-16% of healthy controls by immunofluorescent staining. Notably, there were three 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 four other patients a more modest improvement in dystrophin levels was observed. 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.[231] Not all patients responded equally well, which may be explained by the short serum

| *Trial registration*[a] | *Exon* | *Compound*[b] | *Phase* | *Primary purpose* | *Patients* | *Delivery* | *Dose(s)* | *Location*[c] | *Status*[d] |
|---|---|---|---|---|---|---|---|---|---|
| NTR712 | 51 | Drisapersen/ GSK2402968/PRO051 (2OMePS) | I open-label | Safety | Non-ambulant | Intramuscular | 0.8 mg single dose | NL | Published[225] |
| NCT00159250 | 51 | Eteplirsen/AVI-4658 (PMO) | I/II placebo-controlled | Safety/efficacy | Non-ambulant | Intramuscular | 0.09 and 0.9 mg single dose | UK | Published[226] |
| NTR1241 | 51 | Drisapersen/ GSK2402968/PRO051 (2OMePS) | I/II open-label, escalating dose | Pharmacokinetics/ safety | Ambulant/ non-ambulant | Subcutaneous | 0.5, 2, 4, and 6 mg/ kg weekly (5 weeks) | Be, Se | Published[229] |
| NCT00844597 | 51 | Eteplirsen/AVI-4658 (PMO) | I/II open-label, escalating dose | Safety/efficacy | Ambulant | Intravenous | 0.5, 1, 2, 4, 10 and 20 mg/kg weekly (12 weeks) | UK | Published[231] |
| - | 51 | Drisapersen/ GSK2402968/PRO051 (2OMePS) | I/II open-label extension | Safety/efficacy | Previous study (NTR1241) | Subcutaneous | 6 mg/kg weekly (after 72 wks: 8 wks on; 4 wks off) | Be, Se | Ongoing (First 12 wks published[229]) |
| NCT01128855 | 51 | Drisapersen/ GSK2402968/PRO051 (2OMePS) | I placebo-controlled, escalating dose | Pharmacokinetics/ safety | Non-ambulant | Subcutaneous | 3, 6, 9 and 12 mg/kg single dose | USA, Fr | Published[230] |
| NCT01153932 | 51 | Drisapersen/ GSK2402968/PRO051 (2OMePS) | II double-blinded, placebo-controlled | Safety/efficacy | Ambulant | Subcutaneous | 6 mg/kg weekly or twice weekly | Au, Be, Fr, Ge, NL, Sp, Tu, UK | Completed (press release published[232]) |
| NCT01254019 | 51 | Drisapersen/ GSK2402968/PRO051 (2OMePS) | III pivotal study | Safety/efficacy | Ambulant | Subcutaneous | 6 mg/kg weekly | World-wide | Completed (press release published[233]) |
| NCT01462292 | 51 | Drisapersen/ GSK2402968/PRO051 (2OMePS) | II double-blinded, placebo-controlled | Safety/efficacy | Ambulant | Subcutaneous | 3 and 6 mg/kg weekly | USA | Ongoing |
| NCT01480245 | 51 | Drisapersen/ GSK2402968/PRO051 (2OMePS) | III open-label extension | Safety/efficacy | Previous studies (NCT01153932/ NCT01254019) | Subcutaneous | 6 mg/kg weekly continuous or inter-mittent dosing | World-wide | Ongoing |
| NCT01803412 | 51 | Drisapersen/ GSK2402968/PRO051 (2OMePS) | III open-label extension | Safety/efficacy | Previous studies (NCT01462292/ NCT01254019) | Subcutaneous | 6 mg/kg weekly continuous or 8 wks on; 4 wks off | USA | Ongoing |

| *Trial registration*[a] | *Exon* | *Compound*[b] | *Phase* | *Primary purpose* | *Patients* | *Delivery* | *Dose(s)* | *Location*[c] | *Status*[d] |
|---|---|---|---|---|---|---|---|---|---|
| NCT01890798 | 51 | Drisapersen/ GSK2402968/PRO051 (2OMePS) | III open-label extension | Safety/efficacy | Previous studies (NCT01462292/ NCT01254019) | Subcutaneous | 6 mg/kg weekly | USA | Ongoing |
| NCT01396239 | 51 | Eteplirsen/AVI-4658 (PMO) | II double-blinded, placebo-controlled | Safety/efficacy | Ambulant | Intravenous | 30 and 50 mg/kg weekly | USA | Published[234] |
| NCT01540409 | 51 | Eteplirsen/AVI-4658 (PMO) | II open-label, escalating dose | Safety/efficacy | Previous study (NCT01396239) | Intravenous | 30 and 50 mg/kg weekly | USA | Ongoing (First 24 wks[234]/ press release published[235]) |
| NCT01037309 | 44 | PRO044 (2OMePS) | I/II open-label, escalating dose | Safety/efficacy/ pharmacokinetics | Not specified | Subcutaneous/ Intravenous | 0.5, 1.5, 5, 8, 10 and 12 mg/kg weekly | Be, I, NL, Se | Ongoing |
| NCT01826474 | 45 | PRO045 (2OMePS) | I/II open-label, escalating dose | Safety/efficacy | Ambulant | Subcutaneous | 0.15, 1, 3, 6 and 9 mg/kg weekly | Be, Fr, I, NL, UK | Ongoing |
| | | | IIb Selected dose | Pharmacokinetics/ pharmacodynamics | | | Selected dose weekly | | |
| NCT01957059 | 53 | PRO053 (2OMePS) | I/II open-label, escalating dose | Safety/efficacy | Ambulant | Intravenous & Subcutaneous | 1& 6 and 3 & 9 mg/kg weekly | World-wide | Ongoing |
| | | | IIb Selected dose | Pharmacokinetics/ [harmacodynamics | | Subcutaneous | 6 and 9 mg/kg weekly | | |

**Table 1.1:** Overview clinical trials, completed and currently ongoing, of antisense oligonucleotide-mediated exon skipping for Duchenne muscular dystrophy

[a]NTR=registration in the Netherlands Trial Registration; NCT=registration on www.clinicaltrials.gov

[b]2OMePS: 2'-*O*-methoxyethyl phosphorothioate, PMO: Phosphorodiamidate morpholino oligomers

[c]Au: Australia, Be: Belgium, Fr: France, Ge: Germany, I: Italy, NL: the Netherlands, Sp: Spain, Se: Sweden, Tu: Turkey, UK: the United Kingdom, US: the United States of America

[d]Status on 1st of December 2013

half-life of PMOs. Since PMOs do not bind to plasma proteins (see paragraph 1.3.1), 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.

The next steps are larger randomised, placebo-controlled studies and targeting of other exons. For drisapersen a study in ambulant patients where different treatment regimens are compared (NCT01153932*), is conducted. Recently a press statement was released, stating that patients treated continuously with 6 mg/kg/wk showed increased walking distance in six minutes compared to placebo after 24 and 48 weeks of treatment and patients receiving intermittent dosing (10-week cycles of 9 doses at 6 mg/kg over 6 weeks and 4 weeks off drug) only after 48 weeks. Dystrophin analysis was performed on muscle biopsies obtained before and after 24 weeks of treatment. Dystrophin restoration was reported for 72% of patients on the continuous dose, 59% of the intermittent dose and 5% of placebo-treated patients.[233] In parallel, a large phase III study (pivotal study) was initiated in January 2011 (NCT01254019). Hundred and eighty ambulant patients received 6 mg/kg body weight AON once weekly for one year or placebo. This study was done to show whether long term treatment is safe and leads to functional improvement or slowing down of disease progression. Unfortunately the primary endpoint, *i.e.* a significant increase in six minute walk distance compared to placebo was not met.[232] The study was conducted in a broader patient group compared to previous studies (*e.g.* wider age range and less strict requirements in time rising up from the floor), which might explain the lack of differences between the treatment group and the placebo group. For example, if patients below and above the age of seven were analysed separately, walking distances compared to placebo of respectively 22 meter and 6 meter were observed [presented at the 18th international conference of the World Muscle Society, Asilomar, CA, USA, Oct 2013]. More detailed analysis on subsets and dystrophin restoration will be performed. Most ongoing and completed trials for drisapersen also have an open-label extension study (see table 1.1). However, due to the disappointing results of the phase III study dosing in those studies has been suspended, pending further analysis, but patients are still monitored.

In addition a placebo-controlled clinical trial for AVI-4658 with higher doses (30 mg/kg and 50 mg/kg body weight) for 24 weeks (four patients per group) has been completed (but not yet published) to assess its efficacy and safety. Thereafter these patients were enrolled in an open-label extension study. In this extension phase the placebo-treated patients were equally, *i.e.* 2:2, divided over the 30 and 50 mg/kg group (placebo/delayed). During the initial study significant increases in dystrophin positive fibres (as measured by immunofluorescent staining) were seen after 24 weeks in biopsies of the 30 mg/kg group compared to placebo, but not in the 50 mg/kg group after 12 weeks. Dystrophin restoration was also seen in the placebo/delayed group after 48 weeks (24 weeks after start treatment). This was accompanied by restoration of compounds of the DGC at the sarcolemma. Functionally, a stabilization in walking distance during the six minute walk test was observed in the groups treated from the start (combined), whereas the delayed treatment group (both combined) declined rapidly till 36 weeks (12 weeks after start treatment) and then also stabilised. However these results must be interpreted with caution, since two patients in the 30 mg/kg group, who lost ambulation during the trial are not included in the analysis. Furthermore, to explain the observed patterns the investigators suggest that it takes around 12 weeks before clinically meaningfully dystrophin levels are produced, accounting for the large drop in walking distance in the delayed treatment group until after ~12 weeks of treatment. However, this drop was not seen

* Registration on www.clinicaltrials.gov

in the first 12 weeks of the trial in the other two groups (when excluding the two patients who lost ambulation). Furthermore, dystrophin staining was not yet observed in the 12-week biopsies of the 50 mg/kg treated patients. Nonetheless, encouraging is that overall treatment was well tolerated in all groups for this long period.[234] Patients are currently still treated in the extension study (NCT01540409), showing continued stabilisation of both groups in functional performance as measured by the 6MWD up till 84 weeks, without adverse events.[235]

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%).[44] A phase I/IIa study with PRO044 (2OMePS AON against exon 44) with a similar set-up as the phase I/IIa study for PRO051, but also comparing subcutaneous and intravenous injection routes, is currently ongoing (NCT01037309). Also, a phase IIb trial with PRO045 (2OMePS AON against exon 45), first comparing different doses (0.15-9.0 mg/kg/week) by subcutaneous injection and thereafter 48 weeks of treatment with the selected, most optimal dose, has recently started (NCT01826474). Furthermore an open-label phase I/II trial for PRO053 (2OMePS AON against exon 53), assessing escalating doses of PRO053 via intravenous or subcutaneous injection (NCT01957059).

Furthermore, preclinical studies with other 2OMePS AONs (targeting exon 52 and 55) are performed by Prosensa Therapeutics and for PMOs targeting exon 45, 50 and 53 are performed by Sarepta. 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.

Next to all (pre-)clinical trials with AONs also a large trial (NCT01539772) is ongoing studying the natural history of disease in BMD patients. The phenotype will be characterised and specific abnormal dystrophin proteins will be correlated with the range of clinical outcomes in order to predict the clinical effect of therapeutic exon skipping for different AONs/mutations. In the Netherlands a similar study has been conducted. This revealed that BMD patients with out-of-frame mutations that could theoretically be corrected by the skipping of one exon (exons 22, 44, 45, 51, 53 or 55) had a relatively milder course of disease pathology, as reflected by age of wheelchair dependency, than BMD patients with other mutations and, most importantly, a milder phenotype compared to DMD patients. Compared to DMD patients, ambulation, respiratory and cardiac function was better. Although this might be an overestimation of possible therapeutic effects of AON treatment, since in BMD patients these in-frame-mutations are present from birth, this is encouraging for possible outcomes of the exon skipping therapy.[236]

For DMD the natural history and progression of the disease will be studied in two large clinical trials. A prospective study (NCT01753804) will also search for biomarkers that correlate with disease progression and could potentially be used as a surrogate outcome measure in future clinical trials. Another prospective study (NCT00468832) looks at the natural history by studying the preservation of functional capabilities, respiratory function and the progressive loss of muscle strength. It focuses mainly on the effect of glucocorticoids on these parameters and the first part (consisting of 340 participants) has recently been published.[237,238] Patients were divided in three groups: glucocorticoid naïve, past glucocorticoid users and current glucocorticoid users. Higher preservation of muscle function in glucocorticoid users was seen and this effect remains when treatment continues throughout teenage and adolescent years, although effects are largest when treatment starts early. Furthermore, outcome measures currently used in clinical trials show variation (either increasing, stable or decreasing) depending on the age of the patient and the disease stage.

### *1.3.6 Improvement of AON delivery and efficiency*

Next to the backbone-modification methods or choosing the most efficient delivery route described above (see paragraph 1.3.4) several other strategies can be applied to increase AON delivery and/or efficiency. First of all the dosing regimen can influence the exon skipping and dystrophin levels. For PMOs it has been shown that a dosing regimen of multiple low doses (four times per week 50 mg/kg) seems to be preferable above a few high doses (once 200 mg/kg/week) to reduce the risk of toxicity and increase the efficiency, since both AONs and dystrophin protein show an accumulation over time.[239] In this thesis this is also shown for 2OMePS AONs (chapter 3), where a clear increase in AON levels was seen after dividing the total dose of AONs per week in daily injections compared to administering it all at once or in two times, which was followed by increased exon skipping in some muscles. However, this effect on AON levels was also observed in non-target organs as the liver and the kidney, which can increase the risk of unwanted side effects.[240]

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 paragraph 1.3.4). For specifically enhancing nuclear uptake Moreno *et al.* synthesized a 5'-cap containing a nuclear localization signal (2,2,7-trimethylguanosine cap; $m_3$G-CAP) that is naturally found in snRNAs. Attaching this cap to 2OmePS AONs increased their efficacy, possibly due to improved nuclear delivery.[241]

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 less than 2.5 hours for PMOs. A way to allow a more prolonged effect is the use of viral vectors stably expressing modified snRNP genes. SnRNPs are small protein-RNA hybrids that are amongst others involved in pre-mRNA splicing and histone processing. The natural antisense sequence can be replaced by antisense sequences targeting dystrophin exons.[242-245] The U1 and U7 snRNPs have been used most in splicing modulation experiments.[246] Exon 51 targeting U1 snRNPs induced effective skipping of exon 51 and rescue of dystrophin synthesis in a patient-derived cell line.[242,247] SnRNPs are expressed under their own promoter, so there is less likelihood of eliciting an immune response, due to the presence of viral promoters. Since snRNPs are very small they easily fit within an adeno-associated viral (AAV) vector. AAVs are very efficient at transferring genes into skeletal muscles. Injection of AAV vectors expressing U1 or U7 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.[244,245] They could even rescue the severe phenotype of the *mdx*/*Utrn*$^{-/-}$ mouse.[248] However a serious problem with the use of AAV vectors is the possibility of an immune response against the viral capsid. Therefore maximising the efficiency of snRNP delivered per AAV vector is necessary to reduce the vector dose. Modifying an U7 snRNP with a muscle- and heart specific enhancer increased antisense delivery and exon skipping. However, it revealed that, although the amount of transcripts was increased, many of them were not appropriately processed, which is necessary for their functionality, and many non-functional by-products were formed. This indicates that the exon skipping capacity of these U7 snRNPs is limited by saturation of the processing capacity of the host cell.[249] Other problems are the difficulty to produce AAV particles on a large scale under good manufacturing practice, necessary for implementation in the clinic, and 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. U1 and U7 snRNP antisense constructs targeting multiple human exons are presently developed for use in clinical trials.[250] Furthermore there are concerns for an immune response against AAV vectors, as has been observed in dogs with AAVs used for gene replacement therapy (see paragraph 1.4.3). In a five year follow-up study in GRMD dogs, notably no immune response was observed. However, still a large decline in dystrophin positive fibres was observed.[251] Therefore repeated would be required, which would largely increase the chance of immune rejection. A study in *mdx* mice showed that the speed of decrease in AAV vectors correlated with loss of dystrophin restoration and was mainly due to regeneration. Therefore optimal dosing would be required to induce sufficient dystrophin levels to arrest the dystrophic process and maintain therapeutic value on the long term.[252] This will be challenging in DMD patients.

Another approach to induce or enhance exon skipping is the use of small chemical compounds. Several compounds that influence splicing have been reported for DMD and other diseases caused by incorrect splicing events. For example the cytokine kinetin to specifically correct the splice defect in familial dysautonomia and several compounds have been identified to enhance exon seven inclusion in spinal muscular atrophy.[253,254] For DMD, TG003 has been reported to induce exon 31 skipping and dystrophin restoration in patient cells harbouring a point mutation in this exon. TG003 is a specific inhibitor for Cdc-like kinases, proteins involved in the activation of splice regulator proteins, binding to ESEs. TG003 specifically inhibited exon 31 inclusion in mutated, but not in wild type cells.[255] However this compound is specific for this mutation and, since this mutation is very rare, its application is rather limited. Also one cannot exclude that the compound will disrupt splicing of regular exons in other genes.

In order to identify more general exon skipping enhancing compounds, large drug screening systems have been used. Thereby a guanine analogue, 6-thioguanine (6TG) was identified that increased PMO-induced exon 23 skipping levels *in vitro* in cultured *mdx* cells. Furthermore it enhanced exon skipping after local intramuscular injection in the tibialis anterior of both AONs and 6TG in *mdx* mice.[256] However repetition of these experiments *in vitro* and *in vivo* with both 2OMePS and PMO AONs, resulted in numerous splicing events induced by 6TG alone *in vitro*, only enhancement of exon skipping levels by suboptimal AON sequences/doses *in vitro* and no effect on treatment with AONs of either chemistry after local injection *in vivo* (this thesis; chapter 5).[257] Additionally, compounds should enhance AON-induced exon skipping rather than inducing skipping by themselves, as the latter involves the risk of aspecific exon skipping events.

Recently, dantrolene has been described as a sequence- or backbone-independent enhancer of AON-mediated exon skipping. It increased 2OMePS-mediated exon skipping for different exons *in vitro* and PMO-mediated exon skipping both locally and systemically *in vivo* in *mdx* mice, but did not induce exon skipping by itself. Dantrolene targets the RyR, regulating $Ca^{2+}$-signalling in the nucleus, which is influencing splicing.[258]

Rather than increasing AON delivery and/or activity itself, a strategy could be to increase the possible targets for these AONs. These targets are the *DMD* pre-mRNA, which is expressed only by muscle tissue. Since more and more muscle tissue gets replaced by fibrotic and adipose tissue over time in DMD patients, fewer targets remain for the AONs and the therapeutic effect will be lower in older patients. Improving muscle quality or mass can be attempted by various pharmacological approaches targeting different parts of the DMD pathology (see

chapter 2). Increasing muscle mass by myostatin inhibition, *i.e.* by inhibitory compounds or AON-mediated exon skipping, has been tested in combination with dystrophin AONs (see paragraph 2.7.1). Furthermore, in this thesis some pharmacological compounds have been investigated in combination with AONs. First of all, the corticosteroid prednisolone, the current standard of care for DMD patients has been tested (see paragraph 2.2). This had no effect on the exon skipping levels induced by AONs itself, but might result in a moderate increase in dystrophin protein expression *in vivo* after systemic AON-treatment of *mdx* mice (this thesis chapter 6).[260] Secondly, the angiotensin II type 1 receptor (AT1) antagonist losartan (see paragraph 2.8), described to improve pathology in *mdx* mice,[259] was tested. However these experiments were discontinued since no effect of losartan itself was seen in our hands (see chapter 7) and new literature could not or only partially reproduce published results.[261-264]

## 1.4 Other approaches targeting the underlying genetic defect

Next to antisense-mediated exon skipping several other potential therapies aim to (partially) correct the mutation in the DMD gene or replace the dysfunctional gene. An overview of the current trials for these therapies is given in table 1.2.

### *1.4.1 Stop codon readthrough*

A small subset of patients (around 14%) carries nonsense mutations due to single nucleotide substitutions.[187] It has long been known that certain antibiotics can force the cell to ignore premature stop codons, allowing the production of full-length proteins. Gentamicin was tested in clinical trials for stop codon readthrough decades ago. *In vitro* studies in cells derived from DMD/BMD patients variable readthrough efficiencies (from 1 to 10%) were observed and showed that this is dependent on the type of stop codon mutation (UGA, UAA or UAG) and the nucleotides flanking the stop codon.[265] Early studies with gentamicin in the *mdx* mouse resulted in dystrophin levels of up to 20%.[266]

While results in mice were very promising, initial results in humans were disappointing.[267,268] This turned out to be due to different gentamicin isomers. Only one isomer has high readthrough activity, and gentamicin batches contain a mix of different isomers of which the ratio varies between different batches.[269] After identifying the most efficient gentamicin isomer, a new clinical trial was performed. This indeed revealed that short term gentamicin treatment reduced serum CK levels in patients with premature stop codons, but not frame-shifting mutations. Treatment for six months resulted in a significant increase in dystrophin levels of ~15% in three out of 12 patients.[270] However, due to the risk of renal and ototoxicity, long term treatment with gentamicin is not a viable option.

High throughput screening using a cell line containing a luciferase gene with a premature stop codon identified PTC124 (ataluren), a compound with a much better safety profile. This compound restored dystrophin in the *mdx* mouse up to levels of 25%, was well tolerated by healthy volunteers and DMD patients in short dose-escalation studies were in the latter a modest increase in dystrophin levels was seen.[271-273] The final results of the trial in DMD patients or its extension study have not yet been published. These trials were followed by a large, multicentre double-blinded placebo-controlled Phase II/III trial, where patients received high or low doses of ataluren or placebo for 48 weeks and then all were treated with the high dose in an open-label extension study. Unfortunately, the primary outcome (30 meter

| *Trial registration*[a] | *Compound* | *Paragraph*[b] | *Primary mechanism* | *Phase* | *Primary Purpose* | *Patients*[c] | *Status*[d] |
|---|---|---|---|---|---|---|---|
| NCT00759876 | PTC124 (ataluren) | 1.4.1 | Readthrough | II<br>open-label extension | Safety | Previous study[271] | Terminated[g] |
| NCT00592553 | PTC124 (ataluren) | 1.4.1 | Readthrough | II/III<br>double-blinded, placebo-controlled | Safety/efficacy | DMD/BMD<br>Age>5 years; nonsense point mutation; non-ambulant | Completed |
| NCT00847379 | PTC124 (ataluren) | 1.4.1 | Readthrough | II/II<br>open-label extension | Safety/efficacy | Previous study<br>(NCT00592553) | Terminated[g] |
| NCT01009294 | PTC124 (ataluren) | 1.4.1 | Readthrough | II<br>open-label | Safety | DMD/BMD<br>Age>7 years; nonsense point mutation; ambulant | Terminated[g] |
| NCT01247207 | PTC124 (ataluren) | 1.4.1 | Readthrough | III<br>open-label extension | Safety | Previously terminated/ suspended trials[e] | Unknown[h] |
| NCT01557400 | PTC124 (ataluren) | 1.4.1 | Readthrough | III<br>open-label extension | Safety | Previously terminated/ suspended trials[f] | Ongoing |
| NCT01826487 | PTC124 (ataluren) | 1.4.1 | Readthrough | III<br>double-blinded, placebo-controlled | Efficacy | Age 7-16 years; nonsense point mutations; ambulant | Ongoing |
| NCT01610440 | Mesenchymal stem cells | 1.4.4 | Cell therapy | I/II<br>open-label | Safety/efficacy | Age 5-12 years | Ongoing |
| NCT01834040 | Mesenchymal stem cells | 1.4.4 | Cell therapy | I/II<br>open-label | Safety/efficacy | Age 4-20 years | Ongoing |
| NCT01834066 | Bone marrow derived stem cells | 1.4.4 | Cell therapy | I/II<br>open-label | Safety/efficacy | Age 6-25 years | Ongoing |
| NCT01918384 | NPC-14 (Arbekacin sulfate) | - | Readthrough | II<br>double-blinded, placebo-controlled | Safety/efficacy | Age>4 years; nonsense mutation; ambulant | Ongoing |

**Table 1.2:** Overview clinical trials, currently ongoing and unpublished, of all compounds, apart from antisense oligonucleotides, that target the primary defect for Duchenne and/or Becker muscular dystrophy

[a]Registration on www.clinicaltrials.gov

[b]Discussed in chapter in this thesis

[c]DMD patients unless otherwise stated; BMD=Becker muscular dystrophy; DMD=Duchenne muscular dystrophy; LGMD=Limb girdle muscular dystrophy; SIBM= Sporadic inclusion body myositis

[d]Status on 1st of December 2013

[e]Patients from the US, who participated in previously terminated/suspended trials NCT00759876, NCT00592553/NCT00847379 and NCT01009294

[f]Patients from Europe, Israel, Australia, or Canada, who participated in previously terminated/suspended trials NCT00759876, NCT00592553/NCT00847379 and NCT01009294

[g]Due to unexpected results

[h]Status has not been verified in more than two years

improvement in the six minute walk test) was not reached and the extension study was put on hold. Interestingly, patients receiving the low dose showed an increase in the distance walked in six minutes compared to the placebo group, although this was not significant. This counterintuitive result (low dose patients doing better than high dose patients) is due to the apparent bell shaped dose-response curve for ataluren.[274,275] The final results of this trial (NCT00592553) and its extension study (NCT00847379) have not yet been published. Due to these results, ongoing (extension) clinical trials have been suspended/terminated to first investigate the results in more depth and optimise dosing. Recently the extension study open for all patients treated in previous trials has been reopened (NCT01247207/NCT01557400). In addition, a new phase III double-blinded, placebo-controlled study comparing different lower doses of PTC-124 has recently started (NCT01826487). Furthermore, it turned out that the results of the luciferase screening assay may have been biased due to stabilisation of the luciferase enzyme by PTC124 derivatives resulting in enhanced luciferase efficiency.[276,277] Nevertheless, additional assays showed that PTC124 has at least some readthrough potential.[273,278]

The mixed results with PTC124 and the discovery of the luciferase assay bias, led to new luciferase independent high-throughput screening of possible, more potent readthrough compounds. Hereby RTC13 was identified, which resulted in partial dystrophin restoration in several skeletal muscles, diaphragm and heart, leading to improvement of muscle strength after four weeks of systemic (intraperitoneal) in *mdx* mice, without signs of toxicity.[279]

Another factor that can influence the outcome of stop codon readthrough is the aforementioned NMD, resulting in fewer targets for the readthrough compounds. Its efficiency varies between individuals, for different stop codons, location within the mRNA and sequence context. Studies *in vitro*, in cystic fibrosis patient-derived cells, carrying a premature stop codon in their *CTGF* gene, revealed that the response to gentamicin treatment was dependent on the activity of NMD in these cells. Indeed, blocking of NMD in cells from non-responders enhanced responsiveness to gentamicin treatment.[280]

### *1.4.2* Trans-*splicing*

Another approach to repair the mutated gene at mRNA level is *trans*-splicing by which a mutated exon can be replaced with its normal version, missing exons can be introduced or duplications corrected. It is based on a naturally observed mechanism mainly in lower eukaryotes called *trans*-splicing, *i.e.* the splicing between two independently transcribed pre-mRNAs. For mRNA repair purposes trans-splicing means the splicing *in trans* of the endogenous mutated pre-mRNA and an exogenous engineered pre-*trans*-splicing molecule (PTM). In addition to the sequence to be introduced, this PTM contains also antisense sequences for intron-specific annealing and sequences to be recognized by the splicing machinery. *Trans*-splicing will only take place when the target pre-mRNA is expressed, rendering it tissue-specific and preserving expression level of the repaired transcript.[281] Its feasibility has mainly been shown for 3' replacement, but 5' replacement and exon replacement by double *trans*-splicing *in vitro* using minigenes has been reported as well.[282-284] For DMD proof-of-principle has been shown *in vitro* in patient cells and *in vivo* in animal models. In cultured cells derived from a DMD patient with a nonsense mutation in exon 71 lentiviral transfection with PTMs containing human dystrophin cDNA from exon 59 to 79 induced correctly *trans*-spliced transcripts. A single intramuscular injection of AAV2/1 vectors expressing the PTM with the murine wild type exon 23 linked to exon 59-70 in *mdx* or

*mdx*$^{4cv}$ mice resulted in the expression of *trans*-spliced transcripts harbouring the exogenous sequence, showing one PTM can be used for multiple mutations. However in both cases, despite efficient viral transfection, repair mRNA levels were low (up to 30% *in vitro* and ~1% *in vitro*), resulting in undetectable levels of (micro)dystrophin by western blot. By immunofluorescent staining sarcolemmal localization of microdystrophin was observed *in vivo*. A risk of this *trans*-splicing approaches with PTMs is that translation of the PTM itself could resulted in a truncated protein acting as a dominant negative form or eliciting an immune response.[285]

### *1.4.3 Gene replacement*

In contrast to exon skipping or stop codon readthrough, replacing the mutated gene (gene replacement) is not mutation-specific. However, gene delivery to muscle is challenging due to its abundance (30-40% of the body) and accessibility (post-mitotic fibres surrounded by layers of connective tissue). This reduces the efficiency of gene delivery by plasmids.[286] Also, most viral vectors do not efficiently transduce muscle, except for AAV. However, AAV has a cloning capacity of ~4.5 kb, while the dystrophin cDNA is 14 kb.[287] Therefore the attention has verged to the use of shortened dystrophin constructs.

As described above, dystrophin contains repetitive, partly redundant domains. This allowed the generation of mini- and microdystrophins containing only domains minimally required for functionality (fig. 1.5).[287-289] The 6.3 kb minidystrophin gene is based on a very mild BMD patient with a deletion of exon 17 to 48, thereby lacking 46% of the coding region.[290] This minidystrophin was able to almost completely prevent dystrophic symptoms when expressed in transgenic *mdx* mice.[116] Promising results with microdystrophins were first shown in the *mdx* mouse model. Local administration of several of these microdystrophins, delivered by AAV under a muscle-specific promoter, resulted in high and stable recovery of dystrophin expression and improvement of myofibre morphology.[115,288,291-294] Results on the effect of skeletal-specific expression of microdystrophin on cardiomyopathy are inconsistent. Whereas in young *mdx*$^{4cv}$ mice (4-5 months old) skeletal muscle repair by specific transgenic expression of a minidystrophin aggravates dilated cardiomyopathy due to increased exercise,[295] in six to nine months old *mdx*/*Utrn*$^{-/-}$ mice diaphragm rescue by pPMO treatment prevented heart failure[296] and in aged *mdx* mice skeletal-specific microdystrophin expression had no effect (neither positive nor negative) on cardiomyopathy.[297] Systemic (intravenous) treatment of *mdx* mice with ΔR4-23/ΔCT in an rAAV6 vector resulted in body-wide expression of these microdystrophins in skeletal muscle and heart after a single injection in both young and old *mdx* mice.[298,299]

Subsequent studies in dogs raised the issue of a cytotoxic immune response against viral capsid proteins and/or transgene product.[300] Intramuscular injection of AAV1, AAV2 and AAV6 serotypes in wild type dogs induced a robust cellular immune response to the viral capsid proteins itself.[301,302] The same response was observed after local injection of AAV9 in adult, but not in neonatal dogs.[303] Ohshima *et al.* demonstrated that immune responses were less for AAV8 compared to AAV2, however still present and rAAV8-mediated microdystrophin expression in dystrophic dogs lasted for eight weeks, but showed a decline over time.[304] Systemic (intravenous) administration of rAAV6 in healthy dogs also resulted in an immune response; however gene expression could be ameliorated with transient immunosuppression.[305] Furthermore in neonatal dogs AAV9 carrying a microdystrophin (Δ3990) eluted a marked early inflammatory response too, although widespread dystrophin expression was

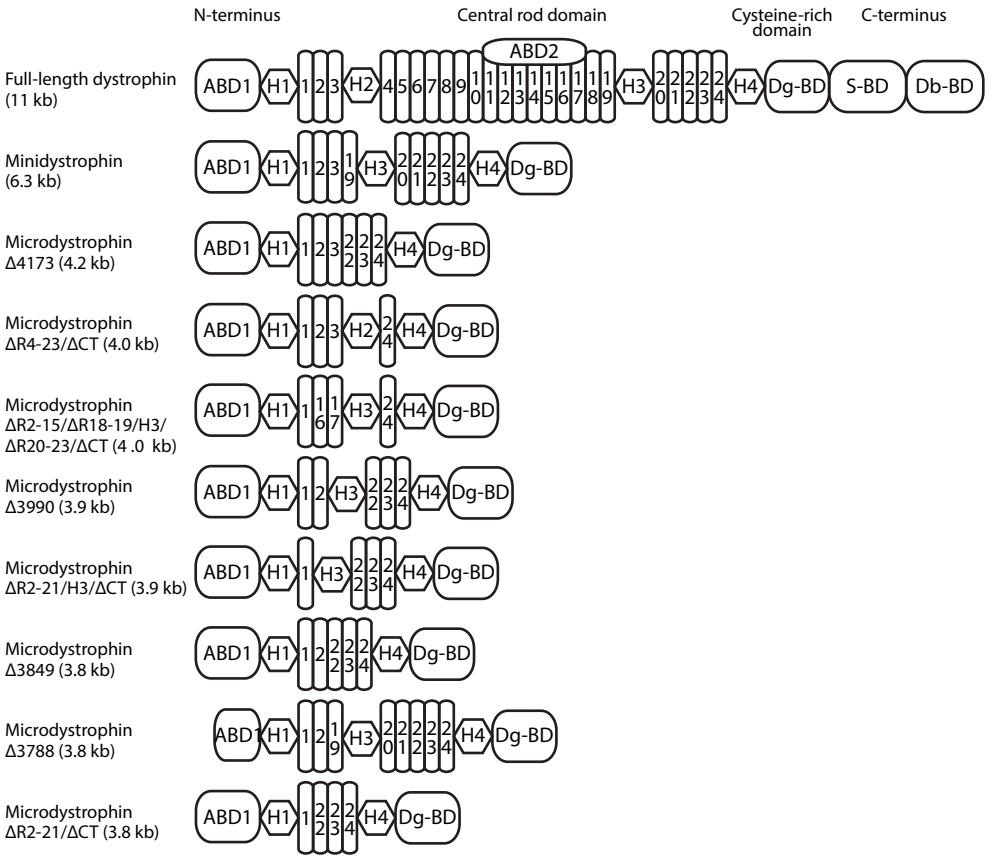


**Fig. 1.5:** Schematic overview of the dystrophin protein, the minidystrophin protein and various microdystrophin proteins

The dystrophin protein consists of four domains: first, an N-terminal actin-binding domain (ABD1); second, a central rod domain, consisting of 24 spectrin-like repeats, interrupted by four proline-rich hinge regions (H1–H4). A second actin-binding domain is present between repeat 11 and 17 (ABD2); third, a cysteine-rich domain, containing a $\beta$-dystroglycan-binding domain (Dg-BD), which is part of the dystrophin-associated glycoprotein complex; and finally, a C-terminal domain, containing an $\alpha$-sarcoglycan and an $\alpha$-dystrobrevin binding domain (S-BD and Db-BD). The 6.3 kb minidystrophin based on a very mild BMD patient with a deletion of exon 17-48 contains the N-terminal ABD1, nine of the 24 central rod repeats (1-3 and 19-24), three of the hinge regions (H1, H3 and H4) and the cysteine-rich Dg-BD. Several microdystrophins, as described in the text, are based on this minidystrophin by deleting extra parts of the protein to fit in AAV vectors.

detectable after 16 weeks in the two surviving dogs.[306]

In non-human primates immune responses were observed to AAV1 and AAV8 vectors after intramuscular, but not after regional intravenous injection.[307] Rodino-Klapac *et al.* did not observe a T-cell response after systemic injection AAV8-microdystrophin (ΔR4-23/ΔCT) in non-human primates and observed microdystrophin expression levels up to 80%. However this percentage dropped to 40% in macaques in which antibodies were present before injection.[308]

This work culminated in a clinical trial where patients were injected intramuscularly with different doses of AAVs expressing microdystrophins Δ3990 under a CMV promoter.[309,310] Unfortunately, only a few dystrophin positive fibres were observed in some patients, while viral genomes were detected in all muscle biopsies. Unexpectedly, T-cells targeting dystro-

phin epitopes were detected in blood of several patients using the ELISPOT assay.[310] In one patient the epitope was present in the microdystrophin, while it was deleted in the patient. This is likely a true "auto-immune" response to a foreign epitope. However, for other patients, T-cells specific for epitopes expressed in dystrophin-positive revertant fibres in patients' muscle were found also before AAV-microdystrophin injection. It is unclear whether the "immunisation" with microdystrophin exacerbated the anti-dystrophin immune response and what the consequences will be for approaches aiming to restore dystrophin. It is reassuring that dystrophin was expressed in the revertant fibres, despite anti-dystrophin T-cells in the circulation, and that in exon skipping trials the number of T-cells infiltrated into the muscle appeared to go down rather than up after dystrophin restoration.[231]

To allow delivery of larger, more functional minidystrophins it has been attempted to split the dystrophin cDNA over two AAV cassettes, relying on homologous recombination or trans-splicing to join the 5' and 3' ends.[311-313] This has been successful in animal models, albeit at lower efficiencies than when delivering a single transgene. Alternatively, intraperitoneal delivery of helper-dependent adenoviral vectors allowed delivery of the complete dystrophin cDNA to diaphragms of *mdx*/*Utrn*$^{-/-}$ mice, which improved respiratory function and diaphragm histology.[314]

The two main hurdles for AAV-mediated gene therapy are overcoming immunity and delivery. As described above an immune response can occur both to AAV and the transgene. However, long term transgene expression could be achieved in dogs without immunosuppression using AAV2/8-mediated delivery of a species-specific microdystrophin (ΔR4-23/ΔCT) in combination with a tissue-specific promoter.[315] Furthermore, transient immunosuppression was sufficient to allow long term transgene expression in dog after AAV6-mediated ΔR2-15/ΔR18-19/H3/ΔR20-23/ΔCT microdystrophin delivery.[316] The same immunosuppression scheme was also used in a larger study in eight normal and 14 dystrophic dogs in which in total six dystrophic dogs were injected in eight muscles with AAV9-ΔR2-15/ΔR18-19/H3/ΔR20-23/ΔCT. This resulted in robust dystrophin expression two months later, despite large T-cell infiltration, and restoration of DGC-components. Also some parts of muscle physiology were improved (protection against eccentric contraction-induced force decline), however others were not changed (specific tetanic muscle force), indicating that microdystrophin treatment in dogs still needs optimisation.[317]

Muscle and heart delivery can be improved by choosing optimal AAV serotypes, *e.g.* AAV6, AAV8 and AAV9.[318-320] Alternatively, AAV2 transduction could be improved up to tenfold in heart and diaphragm by pre-treatment with synthetic polylysines.[321] Suggested mechanisms for this are an altered AAV surface charge and/or a reduced humeral response against AAVs. Polylysine pre-treatment would allow using lower doses of viral particles. However, it did result in death of some of the mice,[321] so the clinical applicability of this approach remains to be seen.

Improved delivery of viral vectors and plasmids can also be achieved by hydrodynamic delivery to individual muscles or muscle groups. This has been reported for rodents and primates[322-325] and high pressure transvenous delivery using saline has been tested in the lower legs of adult muscular dystrophy patients.[326] Delivery of saline up to 20% of limb volume is feasible and tolerable. However, patients receiving 20% showed elevations in compartment pressure, suggesting that this might be the maximum tolerable volume. Currently only regional delivery is feasible, implying that multiple treatments are needed for whole body treatment. This poses obvious challenges due to the immunogenicity issues described above. Nevertheless, AAV gene delivery to heart is efficient and resulted in improved heart quality

and function in mouse and dog models.[327-330]

### *1.4.4 Cell therapy*

Cell therapy for DMD is in fact a form of gene therapy, as cells from a healthy donor will contain the functional *DMD* gene. The added benefit is that these cells will also contribute to the regeneration of damaged muscle in patients. In theory cell therapy thus seems very appealing. However, again the abundance and accessibility of muscle tissue is impeding this approach. Furthermore, muscle tissue is primarily post-mitotic. Upon muscle damage, satellite cells are activated and proliferate and repair the damage. These satellite cells can be isolated from healthy donors and expanded *ex vivo* and the resulting cells (myoblasts) can be transplanted into patients. Unfortunately, early clinical trials using this approach revealed that the majority of myoblasts died quickly and that none of them was able to leave the blood stream to migrate into muscle tissue.[331] Even upon direct injection into muscle the migration of myoblasts was poor. To overcome this, multiple injections have been used and dystrophin restoration has been obtained by high-density injections of many (25-250) injections per cubic centimetre.[332,333] While this may be feasible for some small superficial muscles, it is not for larger and/or more difficult to reach muscles such as the diaphragm and for whole body treatment.

It has become clear that there are other stem cells that are able to migrate from the blood stream into muscle tissue and participate in muscle fibre regeneration. These include cells from the immune system, from blood vessel walls (mesangioblasts), fat stem cells (pericytes) and bone cells.[331] For most of these cells the efficiency is very low and often dystrophin-positive fibres in treated animal models are below 5%.

Satellite cells are skeletal muscle progenitor cells that are normally in a quiescent state, but can be activated upon muscle damage to form new muscle fibres or fuse with existing fibres for repair. These Pax3- and Pax7-expressing cells could be isolated from wild type mice and contribute both to fibre repair and the muscle satellite cell compartment after injection into muscles of *mdx* nu/nu mice (immunodeficient *mdx* mice). Unfortunately it turned out that the myogenic capacity and proliferation potential of satellite cells, which is normally great, are rapidly reduced after expansion *in vitro*.[334] Myoblast transplantation was less successful in immunocompetent *mdx* mice. Although both short and long term dystrophin expression was observed in injected muscle, the number of dystrophin-positive fibres rapidly decreased over time. This turned out to be due to limited migration of the myoblasts, poor survival of the cells and immune rejection. In *NSG-mdx*$^{4cv}$ mice a low number (900) of Pax7-ZsGreen donor cells (satellite cells) could regenerate into functional muscle cells and increase maximal force generation capacity of transplanted muscle.[108]

Muscle-derived stem cells (MDSCs) were found to show better engraftment. MDSCs are isolated from skeletal muscle and have *in vitro* shown the ability to generate multiple cell lineages and to generate for example myofibres when transplanted into skeletal muscle or osteoblasts when transplanted into bone.[335] Wild type MDSCs implanted into the injured gastrocnemius of aged *mdx* mice resulted in improved myofibre repair and reduced fat deposition and fibrosis.[336]

Mesangioblasts are the most promising cell type so far. These cells express early, epithelial markers and can migrate from blood vessels into tissues. Furthermore they have the potential to differentiate into muscle. In the GRMD model, mesangioblast transplantation of donor or autologous corrected stem cells by intra-arterial injection resulted in up

to 10% of dystrophin-positive fibres and functional improvement in these dogs. However some dogs died of pneumonia, which could be the result of accumulation of the cells in the lungs.[337] The first clinical trials with mesenchymal stem cell transplantation either autologous (NCT01834040) or derived from healthy HLA-matched family members into DMD patients are currently ongoing (NCT01610440).

Pericytes have various similarities to mesangioblasts (and may in fact be the same cells) and can be derived from skeletal muscle, but also from other tissues, *e.g.* blood vessels. *In vitro* and *in vivo* these cells have shown to be able to differentiate into myoblasts and injection in SCID/*mdx* mice contributed to muscle repair.[338]

Next to the difficulty to achieve high efficiency necessary due to the abundance of muscle, another hurdle is the need for constant immunosuppression if donor stem cells are used to avoid an immune response against the newly formed myofibres. However a recent article describes the use of human adipose-derived stromal cells (hASCs) in four GRMD dogs without immunosuppression, although these results (no immune reaction to human cells in dogs) are controversial. These cells are able to differentiate into muscle cells after engraftment. Local injections did not result in engraftment of the hASCs, but was seen after systemic (in the cephalic vein) and dystrophin expression lasted until six months after the last injection.[339] Another alternative is to use autologous cells, which are corrected *in vitro*. However, as described above, it is difficult to maintain the myogenic potential during expansion *in vitro* and furthermore this may change the behaviour of the cells upon re-injection.

The effect of knockout of myostatin (see paragraph 2.7.1) on the success of myoblast transplantation has also been tested, with opposing results. *In vitro* myostatin knockout in MDSCs resulted in silencing of genes critical for early myogenesis and led to poor myotube formation. However after transplantation *in vivo* in aged *mdx* mice their myogenic capacity was restored upon myostatin knockout, probably due to myostatin in the host environment. These cells did have repair capacity after injection in injured *mdx* muscle, although they were not more effective than wild type MDSCs and were, in contrast to the wild type MDSCs, not able to reduce lipofibrotic degeneration.[336] On the contrary, another study using myoblasts in which myostatin signalling was blocked with a dominant negative Acvr2b using a lentiviral vector (see paragraph 2.7.1), resulted *in vitro* in increased proliferation and fusion. *In vivo* it improved transplantation success in immunodeficient *mdx* mice after intramuscular injection compared to control myoblast transplantation, by increasing myoblast proliferation and fusion and acting on the expression of myogenic regulatory factors.[340] This discrepancy could be due to the different ways, and thereby timing, of achieving myostatin blocking, which in the first study was achieved by genetic inactivation and in the second study by blocking signalling in expanded myoblast cultures.

### *1.4.5 Genome editing*

Direct modification of the gene itself at the DNA level (genome editing or genome surgery) has shown promising results for a number of other genetic diseases and is the newest therapeutic approach currently explored for DMD. For this purpose endonucleases (meganucleases, zinc finger nucleases, transcription activator-like effector nucleases (TALENs) or RNA-guided nucleases) can be used. These cut the DNA at specific places, causing double strand breaks that are subsequently repaired either by homologous recombination with a provided donor DNA template, which has the ability to correct point mutations and other small

mutations, or non-homologous end joining, resulting in the insertion or deletions of a few base pairs (indels).[341] Proof-of-principle was shown *in vitro* by co-transfection of a microdystrophin plasmid containing a premature stopcodon and a meganuclease plasmid. Meganuclease activity resulted in small deletions or insertions via non-homologous end joining resulting in in frame transcripts in some cells, thereby restoring microdystrophin expression. The same was shown *in vivo* intramuscularly in immunodeficient *mdx* mice.[342] A disadvantage of meganucleases is that they are too large to enter myoblasts; therefore electroporation is required, limiting the feasibility of the technique for systemic treatment. Furthermore, although these endonucleases are able to cut at a specific spot, *in vitro* experiments showed that the size of the deletions was not uniformly distributed throughout the gene.[343] First proof-of-principle for correcting human dystrophin mutations with meganucleases was shown using a lentiviral packed repair matrix carrying exons 45-52. Co-transfection in immortalized patients cells carrying a deletion of exons 45-52 with lentiviral vector packed meganucleases targeting exon 44 and a donor sequence containing exon 45-52, resulted in insertion of exons 45 to 52 by homologous recombination and full-length dystrophin expression.[344] The more recently developed endonucleases TALENs are more efficient and have shown a higher success rate for other diseases. Transfection via electroporation with TALENs specifically inducing indels in exon 51 via non-homologous end joining in DMD patient-derived myoblasts, resulted in reading frame restoration and in re-expression of dystrophin protein.[345] These are promising first steps, but many hurdles have to be overcome will this approach prove to be successful. The main challenge at the moment is that the correction efficiency is very low (~1 in 100 000 to 1 000 000).

## Outline of this thesis

In **chapter 2** a broad overview of secondary defects in DMD caused by the absence of a functional dystrophin protein is given and different pathways involved in pathogenesis are described. Furthermore therapeutic approaches targeting disease symptoms and disturbed signalling pathways are discussed.

In **chapter 3 and 4** more detailed studies into the pharmacokinetic and pharmacodynamic properties of AONs in *mdx* mice are described. **Chapter 3** discusses the effects of different dosage and maintenance schemes in *mdx* mice, while **chapter 4** provides a more detailed analysis of the pharmacokinetic and pharmacodynamic profile of AONs in *mdx* mice, by studying the turnover of the compound, skipped transcripts and newly formed dystrophin protein.

In the next three chapters the use of different compounds in combination with AONs to enhance the therapeutic effects of the AONs, is tested. In **chapter 5** a compound described in the literature to enhance antisense-mediated exon skipping itself, 6-thioguanine (6TG), is tested in cultured cells and locally in the *mdx* mouse. In **chapter 6 and 7** pharmaceutical compounds are used that could improve muscle quality, thereby potentially increasing the targets, *i.e.* dystrophin transcripts, for the AONs and indirectly enhancing exon skipping effects. In **chapter 6** prednisolone, a corticosteroid used by the majority of DMD patients, is used, and in **chapter 7** the angiotensin II type 1 receptor blocker losartan, which targets the renin-angiotensin system, known to be upregulated in DMD.

This is followed by a short summary in **chapter 8** of the results obtained in the preceding chapters and a general discussion putting these results in a broader context.