Cover Page

Universiteit Leiden


The handle http://hdl.handle.net/1887/25807 holds various files of this Leiden University dissertation

**Author**: Verhaart, Ingrid
**Title**: Optimising antisense oligonucleotide-mediated exon skipping for Duchenne muscular dystrophy
**Issue Date**: 2014-05-20

# Optimising antisense oligonucleotide-mediated exon skipping for Duchenne muscular dystrophy

Proefschrift

ter verkrijging van

de graad van Doctor aan de Universiteit Leiden,

op gezag van Rector Magnificus prof.mr. C.J.J.M. Stolker,

volgens besluit van het College voor Promoties

te verdedigen op dinsdag 20 mei 2014

klokke 16.15 uur

door

Ingrid Esther Cynthia Verhaart

geboren te Eindhoven

in 1986

**Promotiecommissie**

| | |
|---|---|
| Promotor: | Prof. Dr. G.J.B. van Ommen |
| Co-promotor: | Dr. A. Aartsma-Rus |
| Overige leden: | Prof. Dr. M.H. Breuning |
| | Prof. Dr. P. ten Dijke |
| | Prof. Dr. A. De Luca[1] |
| | Dr. W.M.H. Hoogaars[2] |

[1] Department of Pharmacobiology, University of Bari, Bari, Italy
[2] Laboratory for Myology, MOVE Research Institute Amsterdam, Faculty of Human Movement Sciences, VU Medical Center, Amsterdam, the Netherlands

Cover: designed by Elisabeth Salmin (kleine hex)

Printed by: Uitgeverij BOXPress, 's-Hertogenbosch

ISBN 978-90-8891-859-9

A trade edition based on this thesis is in preparation (LAP LAMBERT Academic Publishing, ISBN 978-3-659-28683-4)

The studies described in this thesis have been performed at the department of Human Genetics, Leiden University Medical Center, Leiden. The work was financially supported by a grant from the Prinses Beatrix Spierfonds, the Netherlands (grant W.OR09-12).

Copyright© 2014 I.E.C. Verhaart. All rights reserved. No part of this thesis may be reproduced or transmitted in any form or by any means, without prior permission in writing of the author.

## Table of Contents

# General introduction

Partly adapted from:
Verhaart IEC, Aartsma-Rus A (2012), *AON-mediated exon skipping for Duchenne muscular dystrophy.* In: Zaher A, editor, Neuromuscular Disorders. InTech.

and

Verhaart IEC, Aartsma-Rus A (2012), *Gene therapy for Duchenne muscular dystrophy.* Curr Opin Neurol; 25: 588-596.

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

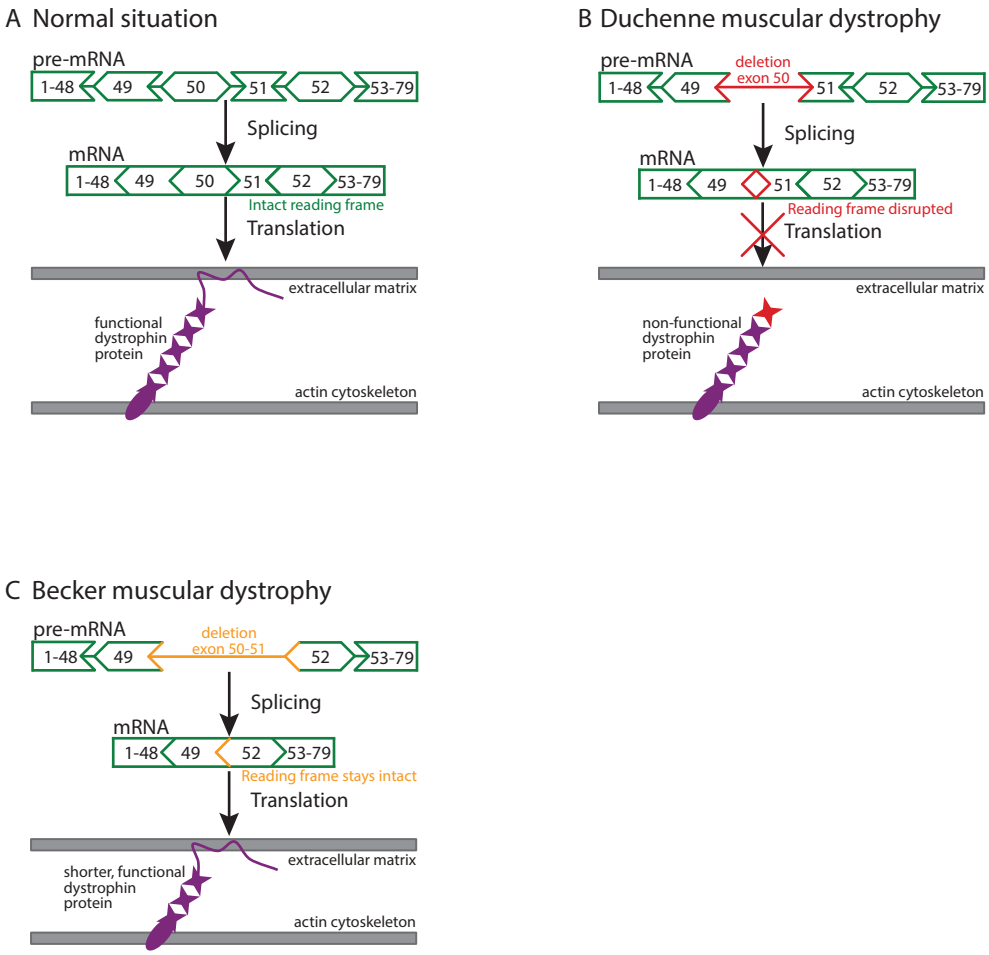


**Fig. 1.1:** The reading frame rule

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

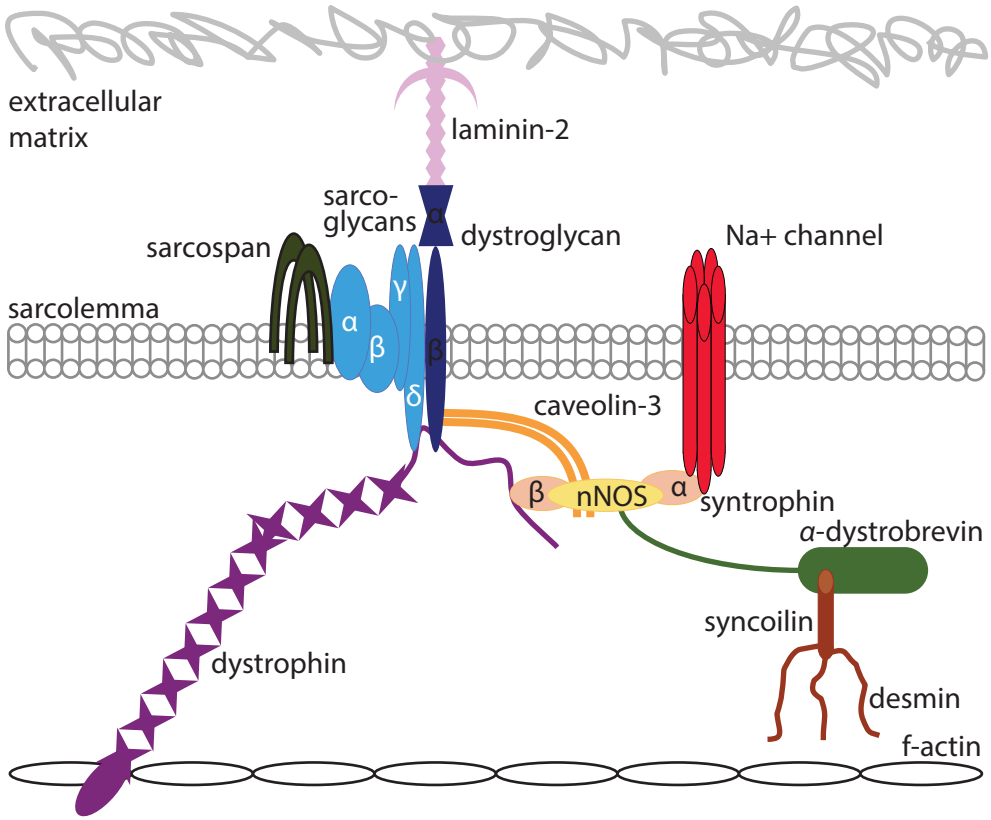


**Fig. 1.2:** The dystrophin-associated glycoprotein complex
The dystrophin-associated glycoprotein complex (DGC) is composed of *α*- and *β*-dystroglycan, a sarcoglycan-sarcospan complex and the dystrophin containing cytoplasmic complex. Dystrophin (purple) forms the link between the actin cytoskeleton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

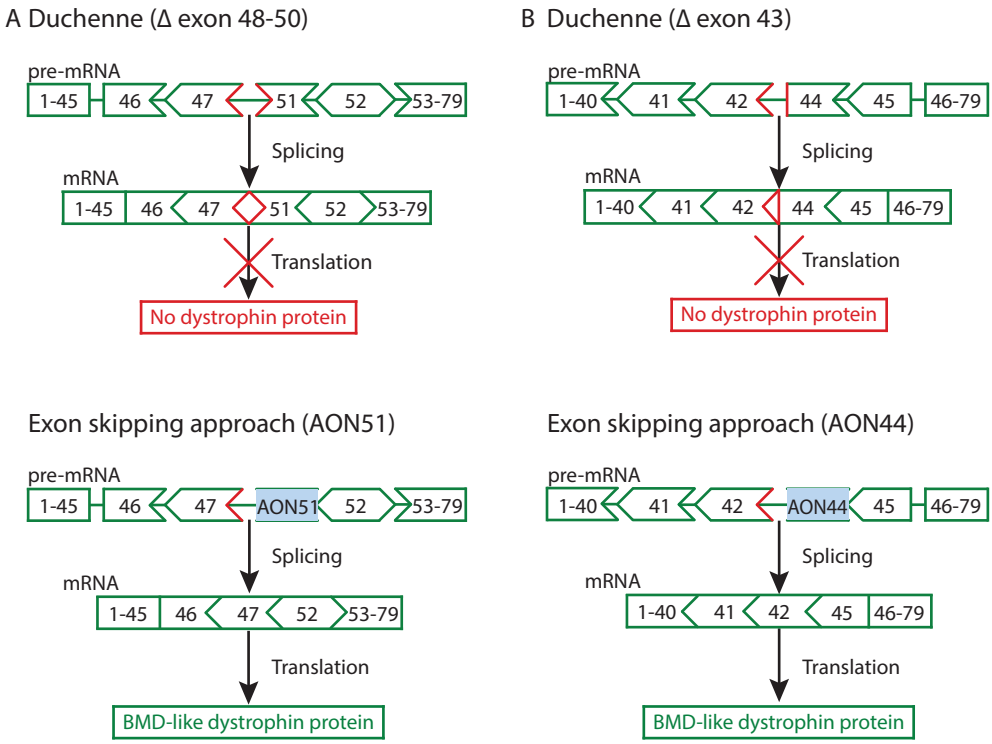


**Fig. 1.3:** The exon skipping approach
In Duchenne muscular dystrophy (DMD), mutations in the DMD gene cause a disruption of the open reading frame, causing translation into protein to stop prematurely and loss of functional dystrophin. The exon skipping approach aims to restore the open reading frame 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-

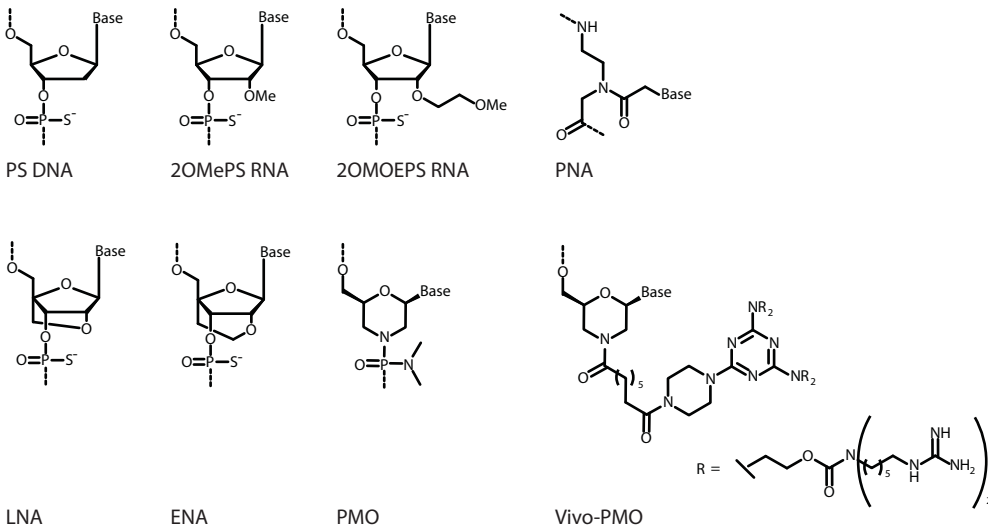


**Fig. 1.4:** Chemical structure of different backbones of antisense oligonucleotides
Phosphorothioate (PS) DNA; 2'-*O*-methyl phosphorothioate (2OMePS) RNA; 2'-*O*-(2-methoxy)ethyl phosphorothioate (2OMOEPS) RNA; peptide nucleic acid (PNA); locked nucleic acid (LNA); ethylene bridged nucleic acid (ENA); 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

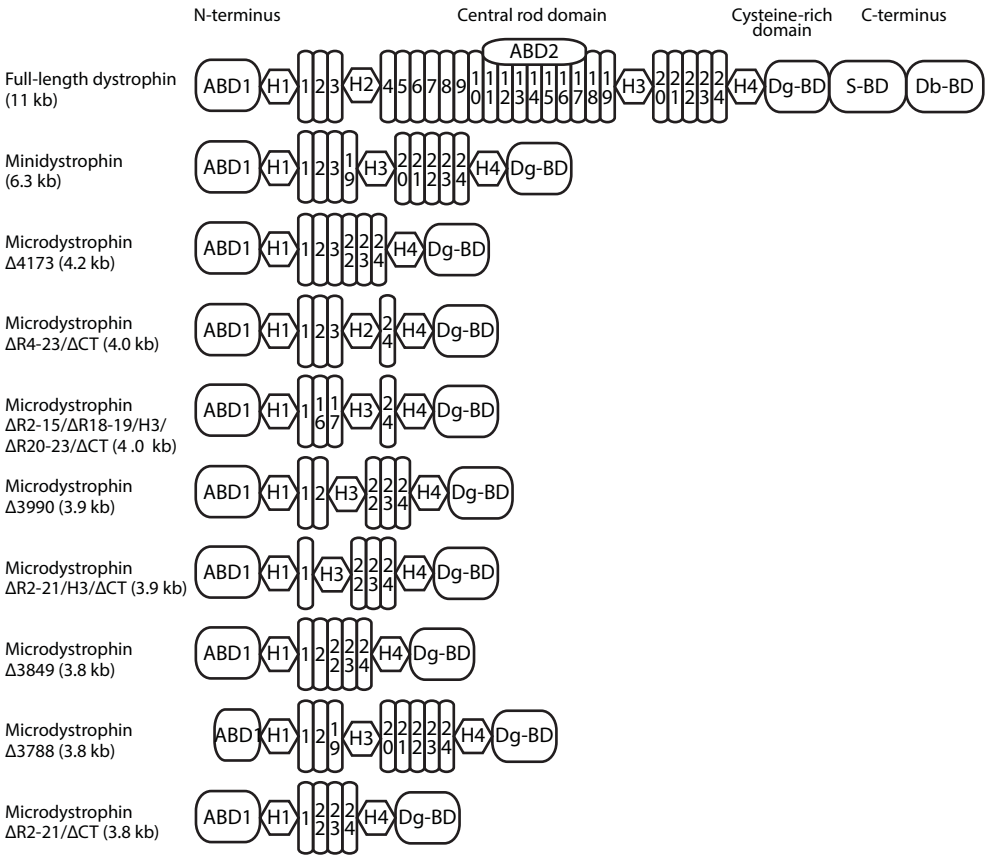


**Fig. 1.5:** Schematic overview of the dystrophin protein, the minidystrophin protein and various microdystrophin proteins

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.

# Targeting the secondary defects in Duchenne muscular dystrophy

Chapter 2

## 2 Other therapeutic approaches

Since at present there is no cure for DMD, there are other, mainly pharmacological, approaches that attempt to combat the symptoms caused by the underlying genetic defect. In this chapter an overview of the different players, and thereby targets, in the DMD pathology is given, together with compounds/approaches that act on those targets and have been used/tested in DMD patients or dystrophic animal models. A schematic overview of the pathways involved in the pathology and the main approaches acting on these parts is given in fig. 2.1. Table 2.1 gives an overview of all compounds, except from those discussed in chapter 1, currently in clinical trials for DMD and/or BMD. The overview in this chapter is certainly not an exhaustive list of all compounds ever tested in context of DMD or related diseases, which might relevant for DMD too, but aims to give a broad overview of possible approaches and their current status/results. In particular it aims to provide insight in which groups of compounds, with most promising outcome or mechanism of action, could be tested to try to enhance the therapeutic effect of AONs.

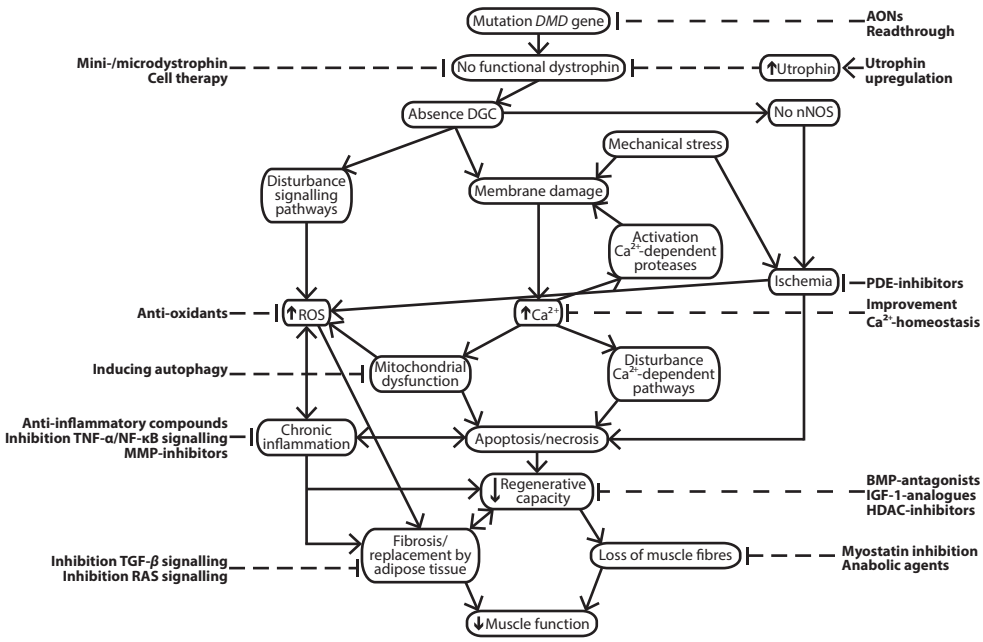


**Fig. 2.1:** Schematic overview of DMD pathology and therapeutic strategies

In DMD the primary defect, the absence of a functional dystrophin protein due to reading frame disruption or stop codon introducing mutations in the *DMD* gene, causes a cascade of downstream effects, including disturbance of calcium homeostasis, chronic inflammation and increase of reactive oxygen species (ROS). This in turn eventually leads to a loss of muscle fibres, which are replaced by fibrotic and adipose tissue, resulting in a loss of muscle function.

The therapeutic approaches described in this thesis and where they address the pathology are indicated.

| *Trial registration*[a] | *Compound* | *Paragraph*[b] | *Primary mechanism* | *Phase* | *Primary Purpose* | *Patients*[c] | *Status*[d] |
|---|---|---|---|---|---|---|---|
| NCT01603407 | Prednisone or deflazacort | 2.2 | Anti-inflammatory | III<br>double-blinded | Safety/efficacy (optimal regimen) | Age 4-7 years; ambulant | Ongoing |
| NCT01580501 | Tadalafil and sildenafil | 2.3 | PDE5-inhibitors | I<br>open-label | Safety/efficacy | Age 7-15 years; ambulant | Ongoing |
| NCT01359670 | Tadalafil or sildenafil | 2.3 | PDE5-inhibitors | ?<br>open-label | Safety/efficacy | Age 7-15 years; ambulant | Completed |
| NCT01350154 | Sildenafil | 2.3 | PDE5-inhibitor | IV<br>double-blinded, placebo-controlled | Efficacy | BMD<br>Age 18-80 years; reduced cardiac function | Completed |
| NCT01168908 | Sildenafil | 2.3 | PDE5-inhibitor | II<br>double-blinded, placebo-controlled | Efficacy | DMD/BMD<br>Age 18-50 years; reduced cardiac function | Suspended[e] |
| NCT01865084 | Tadalafil | 2.3 | PDE5-inhibitor | III<br>double-blinded, placebo-controlled | Efficacy | Age 7-14 years; ambulant | Ongoing |
| NCT01070511 | Tadalafil | 2.3 | PDE5-inhibitor | IV<br>double-blinded, placebo-controlled | Efficacy | BMD<br>Age 18-55 years | Completed |
| NCT00758225 | Idebenone | 2.4 | Anti-oxidant | II<br>open-label extension | Safety/efficacy | Previous studies[423,424] | Completed |
| NCT01027884 | Idebenone | 2.4 | Anti-oxidant | III<br>double-blinded, placebo-controlled | Safety/efficacy | Age 10-18 years | Ongoing |
| NCT01126697 | Coenzyme Q10 and/or lisinopril | 2.4/2.8 | Anti-oxidant/ACE-inhibitor | II/III<br>open-label | Safety/efficacy | DMD/BMD/LGMD<br>Age>8 years; preserved cardiac function | Ongoing |
| NCT01183767 | Epigallocatechin gallate | 2.5 | Anti-oxidant/blocking IKK activity | II/III<br>double-blinded, placebo-controlled | Safety/efficacy | Age 5-10 years; ambulant | Ongoing |
| NCT01856868 | (-)-Epicatechin | 2.5 | Anti-oxidant | I/II<br>open-label | Safety/efficacy | BMD<br>Age 18-60 years; ambulant | Ongoing |
| NCT01335295 | Flavocoxid | 2.5 | Anti-inflammatory/anti-oxidant/ NF-kB inhibition | I<br>open-label | Safety | Age 4-16 years; ambulant | Unknown[f] |
| NCT01388764 | L-arginine | 2.6 | Anti-inflammatory/anti-oxidant | I<br>open-label | Safety/efficacy | Age 7-11 years; ambulant | Completed |
| NCT01099761 | ACE-031 | 2.7.1 | Myostatin inhibition | II<br>double-blinded, placebo-controlled | Safety/efficacy | Age>4 years; ambulant | Terminated[e] |
| NCT01239758 | ACE-031 | 2.7.1 | Myostatin inhibition | II<br>open-label extension | Safety/efficacy | Previous study (NCT01099761) | Terminated[e] |

| *Trial registration*[a] | *Compound* | *Paragraph*[b] | *Primary mechanism* | *Phase* | *Primary Purpose* | *Patients*[c] | *Status*[d] |
|---|---|---|---|---|---|---|---|
| NCT01519349 | Follistatin344 (AAV-mediated) | 2.7.1 | Myostatin inhibition | I<br>open-label | Safety | BMD/SIBM<br>Age>18 years | Ongoing |
| NCT01847573 | HT-100 (halofuginone) | 2.7.2 | Inhibiting TGF-$\beta$ signalling | I/II<br>open-label | Safety | Age 6-20 years | Ongoing |
| NCT01978366 | HT-100 (halofuginone) | 2.7.2 | Inhibiting TGF-$\beta$ signalling | II<br>open-label extension | Safety | Previous study (NCT01847573) | Ongoing |
| NCT01982695 | Lisinopril or losartan | 2.8 | ACE-inhibitor or AT1-antagonist | ?<br>double-blinded | Efficacy | Impaired cardiac function (ejection fraction <55%) | Completed |
| NCT01207908 | IGF-1 | 2.9 | Increase IGF-1 | I/II<br>single-blinded (vs corticosteroids) | Safety/efficacy | Age>5 years; ambulant | Ongoing |
| NCT01761292 | Givinostat | 2.11 | HDAC-inhibitor | I/II<br>open-label | Safety/efficacy | Age 7-11 years; ambulant | Ongoing |
| NCT01521546 | Eplerenone | 2.15 | Aldosterone antagonist | ?<br>double-blinded, placebo-controlled | Efficacy | Age>7 years; preserved cardiac function | Ongoing |
| NCT01648634 | Nebivolol | 2.15 | $\beta$-blocker | III<br>double-blinded, placebo-controlled | Efficacy | Age 10-15 years; preserved cardiac function | Ongoing |
| NCT00606775 | Carvedilol | 2.15 | $\beta$-blocker | IV<br>open-label | Safety/efficacy | Age 8-45 years; preserved to moderate cardiac function | Unknown[f] |
| NCT00819845 | Ramipril or carvedilol | 2.15 | ACE-inhibitor or $\beta$-blocker | IV<br>open-label | Efficacy | DMD/BMD<br>Age 2-45 years; preserved cardiac function | Unknown[f] |
| NCT01540604 | CRD007 | - | Anti-inflammatory (vascular) | II<br>open-label | Safety/efficacy | DMD/BMD<br>Age 2-11 years | Completed |
| NCT01826422 | Docosahexaenoic fatty acid (DHA) and eicosapentaenoic fatty acid (EPA) | - | Dietary supplements (anti-inflammatory and beneficial effect on metabolic disorders) | II<br>double-blinded, placebo-controlled | Efficacy | DMD/BMD<br>Age 6-18 years | Ongoing |
| NCT01995032 | L-citrulline and metformin | - | Dietary supplement and insulin sensitizer | III<br>double-blinded, placebo-controlled | Safety/efficacy | Age 7-10 years; ambulant | Ongoing |

**Table 2.1:** Overview clinical trials, currently ongoing and unpublished, of all compounds targeting secondary defects in DMD

[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]Based on preliminary safety data

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

## 2.1 Utrophin upregulation

Instead of trying to bring back dystrophin into muscle, an alternative strategy is to increase the expression of the dystrophin homologue utrophin. In the *mdx* mouse model it has been shown that transgenic overexpression of truncated utrophin ameliorates the dystrophic phenotype.[346] Full-length utrophin is even more efficient and could prevent pathology in *mdx* mice and improve the phenotype in CXMD dogs.[347-349] This started off high-throughput screens for drugs that can increase utrophin expression. Expression of utrophin is controlled by two isoforms of the utrophin promoter. The utrophin A is active in skeletal muscle and the utrophin B promoter in heart, lungs and endothelial cells. Most research focuses on the utrophin A isoform.[350] A cell model stably expressing the utrophin A promoter linked to luciferase has been used to assess the potential of thousands of drugs. One of these drugs, SMT C1100 was one of the most efficient hits. Its effect has been validated in the *mdx* mouse model, where oral treatment with this compound increased utrophin expression up to twofold, resulting into improved muscle histology and function. Unfortunately, a phase I clinical trial in healthy volunteers revealed that the drug plasma levels needed to induce an effect could not be obtained in humans.[351] Current research focuses on reformulation of this compound. A new trial in healthy volunteers with SMT C1100 in a nanoparticle aqueous suspension formulation resulted in higher plasma levels that were sufficient to increase utrophin levels in animal models. There are plans for a dose-finding phase Ib study in DMD patients.[352] A concern with utrophin upregulation approaches is that utrophin, in contrast to dystrophin, turned out not to be able to bind nNOS and restore its expression at the sarcolemma, whereas disturbances in nNOS function are important in DMD pathology.[353]

Biglycan is an endogenous protein that is present during development outside skeletal muscle fibres and cardiac muscle. It plays an important role in the regulation of signalling pathways and structural proteins, among which proteins (*e.g.* sarcoglycans, dystrobrevins, syntrophins and nNOS) that are part of the DGC.[354] Biglycan injection (local and systemic) in *mdx* mice resulted in upregulation of utrophin, recruitment of nNOS and improved muscle function and resistance to exercise-induced damage.[355] A possibility why biglycan, in contrast to utrophin alone,[353] could restore nNOS expression at the sarcolemma, is that biglycan also restores other compounds of the DGC, creating a more complete utrophin-associated complex, including nNOS.

The expression levels of utrophin in skeletal muscle are also controlled by post-transcriptional mechanisms, *e.g.* the activation of p38, which enhances the stability of utrophin mRNA. An activator of p38, heparin, was able to increase utrophin A expression in the diaphragm of *mdx* mice (ten days treatment with 500 UI/kg).[356]

## 2.2 Corticosteroids and other anti-inflammatory compounds

In healthy persons inflammation and activation of immune cells are beneficial for removing necrotic or damaged tissue after minor muscle damage and actually enhance muscle repair. However in DMD patients muscle damage is continuous.[357] Therefore, chronic inflammation is a prominent feature in DMD pathology. It was first thought to be mainly a non-specific secondary feature: the continuous damage to muscle fibres in the absence of dystrophin, the increased $Ca2^{+}$-influx and production of reactive oxygen species (ROS) were thought to lead to immune cells infiltration and the production of pro-inflammatory cytokines and chemok-

ines. However, it has become more and more clear that inflammation also plays an important specific role in the onset and progression of the disease pathology. Many different types of inflammatory cells (*e.g.* lymphocytes, macrophages, neutrophils, eosinophils and mast cells) have shown to play a role in the increased inflammation in DMD and *mdx* muscles. Large infiltrates of immune cells (mainly macrophages and $CD4^+$ T-cells) in muscle fibres are already observed before onset and in early stages of the disease. The characterization of this population is also distinct from that of non-specific inflammation following muscle injury, indicating an auto-immune component in the disease pathology. Furthermore muscle fibres from DMD patients have a large increase in major histocompatibility complex (MHC) I expression, which is normally lowly expressed by skeletal muscle cells, suggesting they can serve as potential antigen presenting cells.[357,358]

Both $CD4^+$ (helper) and $CD8^+$ (cytotoxic) T-lymphocytes levels are elevated in DMD. This T-cell activation is probably muscle-specific, since the frequency of activated T-cells was not elevated in *mdx* lymph nodes. Their role in aggravating pathology is shown by *in vivo* antibody-mediated depletion of T-cells, which reduced muscle pathology by around 60% ($CD4^+$ cells) to 75% ($CD8^+$ cells) in *mdx* mice.[359] In early stages $CD4^+$ T helper cells, which can activate other immune cells, are present. $CD4^+$ T-cells consist of two subpopulations: $T_H1$ $CD4^+$ T-cells and $T_H2$ $CD4^+$ T-cells. $T_H1$ $CD4^+$ T-cells produce IFN-γ and IL-12, which promote inflammation, but may have an attenuating effect on fibrosis. $T_H2$ $CD4^+$ T-cells are associated with IL-4, IL-5 and IL-13 cytokines and are strongly linked to fibrogenesis.[360] In addition, T-lymphocytes are an important source of TGF-$\beta_1$ (see paragraph 2.7).

Macrophages are also a large source of TGF-$\beta_1$ and platelet-derived growth factor (PDGF), a fibrogenic cytokine.[361] Depletion of macrophages in neonatal *mdx* mice resulted in a large decrease in muscle necrosis, suggesting they play a role in the early stages of the disease.[362] Macrophages can have phenotypically and functionally distinct subtypes. Classically activated M1 macrophages are activated by $T_H1$ cytokines IFN-γ and IL-12, express high levels of inducible NOS (iNOS) and promote tissue inflammation. Those are found first after acute muscle injury, cleaning up necrotic tissue and presenting antigens. Alternatively-activated M2 macrophages are activated by $T_H2$ cytokines IL-4 and IL-13, express high levels of arginase and suppress the $T_H1$ response.[363] Different M2 subtypes exist, of which M2c macrophages are seen in early phases and de-activate M1 macrophages. M2b macrophages have been observed in regenerating muscle after injury and M2a macrophages are abundant in final phases of tissue repair.[357]

Eosinophils are increased in DMD biopsies and can secrete IL-4 and IL-10, which promote $T_H2$ $CD4^+$ T-cell responses and thereby fibrogenesis. They also produce MBP-1, which attenuates the cellular immune response and promotes fibrosis. Knockout of MBP-1 in *mdx* mice (*mdx*/*Mbp-1*$^{-/-}$) results in a reduction in diaphragm fibrosis, without changes in macrophage content or inflammatory cytokines as tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ).[364]

In addition mast cells and dendritic cells have been suggested to play a role in the pathophysiology of DMD, since the mast cell content is elevated in dystrophic humans, dogs and mice and activated dendritic cell infiltration is increased in dystrophic myofibres.[365,366] Furthermore *mdx* myofibres have been shown to be vulnerable to purified mast cell granule-induced necrosis.[367]

Considering the significance of inflammation in DMD pathology, several therapeutic strategies are based upon immune interventions.

Since at the moment no cure or therapy targeting the underlying genetic effect is available, most patients are treated with corticosteroids, most used are prednisone, prednisolone (prednisone is converted in the liver to its active form prednisolone) and deflazacort (an oxazolidine derivative of prednisone). Corticosteroids are 21 carbon steroid hormones, which have a wide range of actions, *e.g.* on carbohydrate and protein metabolism, lipid metabolism, electrolyte and water balance, the cardiovascular system, the neuromuscular system, lymphoid tissue and immune response.[368] Their exact mechanism of action in DMD is not known, but they probably reduce muscle necrosis and inflammation.[10,13,369] They might work partly via increasing $\alpha_7\beta_1$-integrin and laminin-2, thereby stabilising the DGC.[370] Prednisolone had a positive effect on muscle strength and histology (decrease in centrally located nuclei) in *mdx* mice.[371] Furthermore, in *mdx* mice it could reverse alterations in the brain. *Mdx* mouse brains show increased vascular permeability (impaired blood-brain barrier functionality) and reduced levels of several DGC-associated proteins and the Dp71isoform. These levels and the function of the blood-brain barrier were restored by prednisolone treatment.[372] In a dystrophin- and MyoD-deficient *C. elegans* model a reduction in the number of degenerating fibres was seen after prednisolone treatment.[369] As *C. elegans* only has a simple immune system, other mechanisms seem to be involved as well. One study in *mdx* mice suggests that deflazacort increases the calcineurin/NF-AT pathway. JNK1 activation in dystrophic muscle leads to an increased interaction and thereby nuclear restriction of the nuclear transcription factors of activated T-cells (NF-AT), which are regulatory proteins for hypertrophic growth of both cardiac and skeletal muscle. Deflazacort treatment leads to an increase of NF-AT target genes, among which the dystrophin homologue utrophin, thereby it decreases the dystrophic muscle fibre pathology.[373,374] An alternative possibility is that the anabolic effect of corticosteroids increases muscle regeneration and growth by enhancing proliferation of myogenic precursor stem cells or myoblasts.[10] Furthermore corticosteroids have a positive effect on $Ca^{2+}$-homeostasis, which is deregulated in DMD patients (see paragraph 2.12).[368] Unfortunately, corticosteroids also have deleterious side effects like osteoporosis, weight gain, growth inhibition, delayed puberty, mood swings and cataracts.[13,375-377] In unaffected individuals they have a catabolic effect on muscle, but in DMD this is generally abrogated by the positive effects.[378] Whilst in *mdx* mice prednisolone treatment is initially beneficial, it induces fibrosis and is detrimental to muscle function on the long term.[379,380] Notably, prednisolone induced fibrosis in the heart in mice, but this is not observed in DMD patients, where corticosteroid use prevents/delays ventricular dysfunction.[11,381-383] A comparative trial suggests that prednisone and deflazacort are equally effective at slowing disease progression, but that deflazacort causes less side effects than prednisone (less weight gain).[384] On average corticosteroids have shown to prolong ambulation for around three years, but, since they do not target the underlying cause, they cannot prevent the eventual loss of muscle fibres and function.[10] Therefore early start of treatment might be appropriate, since it cannot recover lost function.[385] In addition, not all patients tolerate long term use of corticosteroids, due to the numerous side effects. Several trials with prednisone have been conducted with altering treatment regimens, *e.g.* alternate day, weekend only or continuous low dose, to try to keep the positive effects, but reduce the side effects. However mostly less functional benefit was observed compared to the most widely recommended daily dose of 0.75 mg/kg/day or no reduction in side effects.[386-390] As there are many different dosing regimens for both prednisone and deflazacort, recently a large trial has been initiated comparing 0.75 mg/kg prednisolone daily and on an intermittent (ten days on, ten days off) dosing regimen and 0.9 mg/kg/day deflazacort (the dose equivalent to 0.75 mg/kg/day prednisolone) in

order to determine the most optimal balance between increasing efficacy and decreasing side effects and toxicity (FOR-DMD study, NCT01603407).[7]

Due to the side effects of corticosteroids after prolonged treatment, research has been conducted to find compounds with similar therapeutic actions, but fewer side effects. Serra *et al.* compared the efficiency of several non-steroidal anti-inflammatory drugs (NSAIDs) to methylprednisolone in *mdx* mice: two non-specific COX-inhibitors (aspirin and ibuprofen) and a selective COX-2-inhibitor (parecoxib). The anti-inflammatory effects of all drugs were largely comparable and resulted in amelioration of muscle morphology, with aspirin being slightly less effective and parecoxib slightly more.[391] In contrast, in another study, another specific COX-2-inhibitor (meloxicam) had little or no effect on exercise-induced muscle pathology in *mdx* mice.[392]

Another compound, HCT 1026, a NO-donating derivative of the NSAID flurbiprofen, was more potent than prednisolone in preventing muscle damage after one year treatment in *mdx* mice. Importantly HCT 1026 does not induce the side effects of corticosteroids.[393] It normalizes blood flow and thereby alleviated functional muscle ischemia.[394]

Doxycyline is an antibiotic member of the tetracycline family that, next to antimicrobial properties, also inhibits matrix metalloproteinases (MMPs; see paragraph 2.13) and reduces inflammation. Administration to *mdx* mice resulted in less inflammation, thereby protecting against the onset of myonecrosis on short term and reduction of pathology in both skeletal and cardiac muscle and improved muscle function in aged mice in the long run (nine months).[395] However, it must be noted that the dosage used was far higher than the human equivalent dosage used for its antimicrobial properties. Therefore additional studies are needed to test whether this dosage is applicable in humans.

Resveratol is a compound that can be found in food like grapes and red wine and has shown to have anti-inflammatory and oxidative metabolic enhancing properties. It acts through the Sirt1-PGC-1α pathway. A study treating nine weeks old *mdx* mice for eight months showed, via Sirt1 activation, a reduction of oxidative stress, loss of muscle mass and fibrotic tissue, but no reduction of inflammation.[396] The same treatment also resulted in amelioration of cardiomyopathy by Sirt1-mediated downregulation of p300, a pro-hypertrophic co-activator which is critical for cardiac development, but induces cardiomyocyte hypertrophy after overexpression.[397] Eight weeks treatment of younger (four weeks old) mice improved specific muscle force and short term (ten days) treatment of five weeks old *mdx* mice resulted in a decreased expression of inflammatory genes.[398,399] Utrophin is a downstream target of PGC-1α, but no increase in utrophin protein expression was observed in either study, although the last study did see a positive effect on utrophin mRNA levels.

Oxatomide (Tinset®), a histamine H1 antagonist used for the treatment of allergies, has anti-inflammatory effects by inhibiting the release of pro-inflammatory mediators from among others mast cells.[400] Furthermore, oxatomide has suppressive effects on antigen-presenting cells such as macrophages and dendritic cells.[365] In young, exercised *mdx* mice oxatomide improved whole body strength.[401] However in a pilot open-label trial in steroid-naïve DMD patients no improvement in muscle strength or function was observed after three or six months of treatment.[402]

## 2.3 NO-cGMP signalling pathway

NO is synthesized by nNOS and is an important regulator of contractile function and muscle

integrity in both skeletal and cardiac muscle.[403,404] In DMD and many other neuromuscular diseases the NO-cGMP signalling pathways are disturbed. Contracting muscle normally produces NO to ensure vasodilation and increased blood flow to fulfil the increased need of the working muscle for oxygen and nutrients. Lacking nNOS, contraction leads to a decrease in blood supply during contraction, sometimes resulting in micro-infarctions and thus increasing the amount of muscle damage. Hence a therapeutic strategy is to amplify NO-cGMP signalling by phosphodiesterase (PDE) inhibitors, mainly PDE5. These compounds are well known for their vasodilative properties in among others treatment of erectile dysfunction and heart failure.[405,406] A large drug screening in the dystrophin-deficient zebra fish *Sapje* identified the non-selective PDE-inhibitor aminophylline as a potential candidate to restore normal muscle histology. Aminophylline is known for its anti-inflammatory effects by inhibiting several inflammatory mediators. Thereafter also the PDE4-inhibitor rolipram and especially the PDE5-inhibitor sildenafil citrate (Viagra®/Revatio®) showed a positive effect.[407] Sildenafil citrate also improved respiratory and cardiac function in *mdx* mice.[408,409] The exact mechanism of the cytoprotective effects of sildenafil citrate is not known. Mitochondria are dysfunctional in *mdx* mice and DMD patients, resulting in reduced ATP production, thereby not meeting the energy demand of the muscle cells. Since the NO-cGMP pathway plays a regulatory role in mitochondria, it was thought sildenafil citrate-mediated PDE5 inhibition might have its beneficial effects by improving mitochondrial function. However, detailed analysis showed no effect of sildenafil citrate on mitochondrial function in *mdx* mice.[410] It probably exerts its effects by inhibiting $Ca^{2+}$-entry via transient receptor potential canonical channels (mainly TRPC6) and pro-inflammatory cytokines, like NF-κB and MAPKs.[411,412] An alternative hypothesis is that it has its positive effects by improving the blood supply to the muscles. Other PDE5-inhibitors were shown to be effective as well. The PDE5A-inhibitor tadalafil resulted in amelioration of muscle damage in *mdx* mice.[413] A subsequent clinical trial in BMD patients showed alleviation of muscle ischemia.[414] Several clinical trials with both compounds are currently ongoing: trials to determine if tadalafil and/or sildenafil citrate can improve muscle blood flow during exercise in DMD or BMD patients (NCT01359670, NCT01580501 and NCT01070511) and the effect of Revatio® (sildenafil citrate) on cardiac, muscular and cognitive function in BMD (NCT01350154). However a six months trial in DMD/BMD patients has recently been suspended (NCT01168908), due to negative results during an interim analysis. No benefit of Revatio was observed and, although group sizes were small, more subjects in the Revatio-treated group showed a worsening of cardiac function than in the control group, indicating it may even have adverse effects on cardiac function [presented at the Annual Connect Conference of the Parent Project Muscular Dystrophy, Baltimore, MA, USA, June 2013]. Tadalafil acts more specific, since it only stimulates cGMP, while sildenafil stimulates cGMP but also cAMP, which can result in putting too much stress on the heart. Furthermore, tadalafil has a better pharmacokinetical profile (longer acting) and a good safety profile. Pilot studies in DMD, mainly single dose-escalation studies, did not show serious adverse events and showed indications of protective effects to exercise induced damage. Therefore, a large phase III randomized, double-blind, placebo-controlled study in 306 DMD boys will start soon, examining the efficacy of 48 weeks of tadalafil treatment (NCT01865084).

Isosorbide dinitrate and MyoNovin (guaifenesin dinitrate) are NO-donating drugs. Short term treatment (18 days) of *mdx* mice had a positive effect on the stem cell population and resulted in accelerated fibre regeneration, especially in combination with prednisolone treatment.[415] A clinical trial in healthy volunteers to compare the pharmacokinetics of isosorbide

dinitrate and the NSAID ibuprofen and mainly to define possible drug interactions of a combined administration of both compounds is currently ongoing, in preparation for a trial in DMD patients (NCT01478022).

## 2.4 Anti-oxidants

As mentioned above oxidative stress plays a role in DMD pathology. One of the pathways mediating this response is via nNOS. nNOS produces NO, an important modulator involved in the regulation of numerous processes, *e.g.* muscle contractility, glucose uptake and repair. Normally nNOS interacts with the DGC, but it is dislocated in DMD (see paragraph 1.2.2). In itself the absence of nNOS does not lead to muscular dystrophy, but in dystrophin-deficient muscles it may aggravate the damage via, among others, increasing NAD(P)H oxidase activity and increasing inflammation and decreased blood flow to muscle due to impaired vasodilation. However oxidative stress itself probably also contributes to the dislocation of nNOS and the disruption of the DGC, by activating the NF-κB pathway and caveolin-3 signalling pathway.[416]

In *mdx* mice pathology differs between limb muscles and the diaphragm. Limb muscles undergo various cycles of necrosis, inflammation and degeneration around the age of two to eight weeks and show massive regeneration, which compensates for the loss of necrotic fibres, from three to 12 months, after which it starts to decline.[417,418] In contrast the diaphragm displays continuous and progressive degeneration throughout the life-span, combined with ineffective regeneration leading to muscle fibre loss, more resembling the human DMD pathology.[419] This difference in degeneration/regeneration in combination with the increase oxidant production observed in the *mdx* diaphragm compared to skeletal muscle, makes the diaphragm extra vulnerable for oxidative stress.[416]

Considering its role in DMD pathology, several strategies aim to target the oxidative stress. One class of compounds targets the NF-κB pathway (see paragraph 2.5). Furthermore several other anti-oxidants have been tested at various levels in DMD.

Coenzyme Q10 is essential for several enzymatic steps in the production of energy and functions as an anti-oxidant. In a pilot clinical trial addition of coenzyme Q10 to prednisolone treatment improved muscle strength.[420] A clinical trial with coenzyme Q10 alone or in combination with the ACE-inhibitor lisinopril is currently ongoing (NCT01126697; see paragraph 2.8). Another clinical trial comparing coenzyme Q10, prednisone or a combination in non-ambulant patients has been terminated, since the American Academy of Neurology now recommends that all patients with DMD should be offered treatment with corticosteroids.

Idebenone (2-(10-hydroxy-decyl)-5,6-dimethoxy-3-methyl-[1,4] benzoquinone; Catena®), a synthetic analogue of coenzyme Q10, is a compound that reduces oxidative stress by acting as a free radical scavenger and it improves mitochondrial function by increasing ATP-production and protecting the mitochondria against lipid peroxidation.[421] In the *mdx* mouse model idebenone treatment resulted in a reduction of inflammation and lower levels of fibrosis in heart, thereby normalizing heart function and improving voluntary running.[422] In a small phase I/II clinical trial in DMD patients no significant difference in heart function was observed. However, this study was not ideal due to the small number of patients and the fact that the idebenone-treated group was significantly older than the placebo-treated group. Nevertheless, the forced vital capacity of treated patients was better than those of

control patients, suggesting a protective effect on respiratory function.[423] In addition more in depth analysis of the results on respiratory function showed that effectiveness on respiratory parameters was only seen in the glucocorticosteroid-naïve patients receiving idebenone versus placebo and not in the patients receiving concomitant steroids. Importantly, respiratory parameters were higher at baseline in the steroid users compared to non-users, indicating positive effects on respiratory function by steroids itself. These analyses may indicate that the maximal treatment effect is already reached by steroids alone or that steroids suppress the effects of idebenone, although the latter seems unlikely, since there is no mechanistic explanation for this.[424] These patients have been followed for an additional two years to assess the long term safety and tolerability and the efficacy on skeletal muscle, respiratory and cardiac function, but no results have been published yet (NCT00758225). Idebenone is currently also tested in a larger group of DMD patients in two stages: first, in patients that have never been treated with corticosteroids and then in combination with corticosteroids (NCT01027884).

## 2.5 TNF-α and the IKK-NF-κB signalling pathway

Another hallmark of the chronic inflammation is a large increase in cytokine expression, *i.e.* TNF-α and IFN-γ.[358,425-427] TNF-α activates the IKK/NF-κB signalling pathway (fig. 2.2). NF-κB is present in all cells and is kept in an inactive form in the cytoplasm by the inhibitory protein IκB. When IκB is degraded by IκB kinase (IKK), NF-κB migrates to the nucleus where it can activate a cascade of inflammatory factors: activating pro-inflammatory macrophages and inhibiting myogenesis, thereby increasing necrosis and inflammation and reducing regeneration in muscle fibres.[428-430] Normally, anti-inflammatory factors deactivate NF-κB to reduce the inflammation. However, when NF-κB is not inactivated this can lead to chronic inflammation. In *mdx* mice NF-κB is not deactivated, but initiates a cascade of inflammatory molecules, which in turn further activate NF-κB (positive feedback loop).[431]

Since the TNF-α/NF-κB pathway is highly elevated in DMD, drugs that inhibit activation of this pathway may possibly prevent the muscle degeneration in DMD.

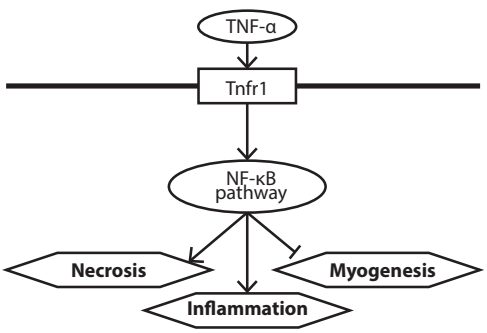


**Fig. 2.2:** Simplified scheme of the TNF-α/NF-κB signalling pathway
TNF-α activates the NF-κB signalling pathway, thereby increasing necrosis and inflammation and inhibiting myogenesis

Blocking TNF-α with a specific antibody (infliximab/Remicade® or etanercept/Enbrel®) has an anti-inflammatory effect and thereby reduces the breakdown of dystrophic muscle in *mdx* mice, without impairing the formation of new myotubes. This drug is already being used in other inflammatory diseases, such as rheumatoid arthritis and Crohn's disease.[392,432,433]

Pyrrolidine dithiocarbamate (PDTC) stabilises cytosolic IκB-α, thereby reducing NF-κB activity. In *mdx* mice systemic treatment with 50-75 mg/kg resulted in a decrease in muscle

fibre degradation and enhanced regeneration of skeletal muscle and diaphragm, accompanied by an improve in muscle function, and had a positive effect on the $Ca^{2+}$-homeostasis.[434-436] However PDTC can cause seizures at slightly higher doses (75-150 mg/kg) and brief periods of ocular discharge (excretion of fluids from the eyes) can occur already at a concentration of 50 mg/kg as used in the *mdx* experiments.[437]

NEMO-binding domain (NBD) peptide is a more selective inhibitor of NF-κB. It binds IκB-*β*, thereby preventing the formation of an IKK complex and inhibiting the activity of NF-κB. Via this pathway, NBD peptide selectively prevents inflammation-induced NF-κB activation without inhibiting basal activity required for cell survival. In *mdx* mice it greatly reduces myofibre necrosis and treatment with NBD peptide showed no dose-dependent toxicity.[428,430]

Flavocoxid, a mixed flavonoid extract with anti-inflammatory, anti-oxidant and NF-κB inhibiting properties, showed a reduced activity of NF-κB signalling pathways and so reduced muscle necrosis and enhanced regeneration in *mdx* mice.[438] A clinical trial to determine its safety in DMD patients after oral administration has been terminated for unknown reasons (NCT01335295).

Green tea extract is rich in anti-oxidants (polyphenols), the major one being epigallocatechin gallate (EGCG; a flavan-3-ol). EGCG can inhibit NF-κB activation by blocking IKK activity. Treatment of *mdx* or *mdx*$^{5cv}$ mice with green tea extract or EGCG resulted in improvement anti-oxidant capacity, thereby decreasing muscle pathology and improving functions.[439] Treatment with green tea extract reduced fibrosis and improved muscle function in *mdx* mice.[440] Oral administration via a diet supplemented with EGCG seemed the most effective route of administration compared to subcutaneous EGCG injection.[441] A double-blinded, placebo-controlled, randomized pilot clinical trial to investigate the safety and possible effects on pathology of EGCG in DMD patients is currently ongoing (NCT01183767) and a pilot study with (-)-epicatechin (another flavan-3-ol) in BMD-patients (NCT01856868).

Another dietary derived NF-κB-inhibitor is curcumin, obtained from the spice turmeric. In *mdx* mice mixed results were observed with curcumin. Intraperitoneal injections with curcumin caused reductions in NF-κB and TNF-α levels, followed by enhancement in muscle strength.[442] In contrast, in another study, dietary supplementation with curcumin had no effect on NF-κB activity.[443] These discrepancies might be due to the different routes of administration. Studies in rats have shown the limited bioavailability of curcumin after oral compared to intravenous administration.[444]

Several other dietary derived anti-oxidant supplements (*e.g.* beta-carotene, vitamin A, vitamin C, vitamin E, and selenium) have been tested in clinical trials in DMD and other diseases. However mostly no, limited or even detrimental effects were observed.[416,445] This is probably due to lack of specific targeting. A recent study suggests increased protein thiol oxidation in dystrophic muscles plays an important role in oxidative stress mediated damage.[446] The thiol reducing anti-oxidant N-acetylcysteine that naturally occurs in vegetables reduces TNF-α and thereby NF-κB activation. After systemic treatment lower TNF-α levels were observed in *mdx* mice as well as protection against myonecrosis.[447,448] N-acetylcysteine treatment also resulted in a reduction of ROS in muscle and a decrease in centrally located nuclei. Furthermore an increase in DGC-associated proteins and utrophin expression was observed.[449]

## 2.6 Nutritional intervention and dietary-derived compounds

Malnutrition is commonly observed in end stages of DMD, due to eating difficulties and weight loss as a consequence of muscle wasting. Approaches to manage this are food texture modification and supplementary feeding. Importantly, daily calcium and vitamin D supplementation is recommended in combination with corticosteroid therapy, since corticosteroids can enhance osteoporosis.[450] In addition, other nutritional supplements might be beneficial against muscle wasting as well. Some dietary-derived compounds, used for their anti-oxidant properties, have been described above (see paragraph 2.4). Next to these, amino acid supplementation (taurine, creatine, arginine and glutamine) is tested in DMD patients and *mdx* mice.

Taurine is a free amino acid abundant in skeletal muscle having anti-oxidant and anti-inflammatory effects and modulating $Ca^{2+}$-homeostasis. Taurine deficiency has been observed in dystrophic *mdx* muscle.[451] In *mdx* mice taurine supplementation in the food prevented exercise-induced damage in hind limb muscle and ameliorated measurements of degeneration/regeneration cycles. However no change in degeneration/regeneration was seen in the diaphragm.[452] In combination with prednisolone a synergistic effect was seen in the improvement of muscle strength and $Ca^{2+}$-homeostasis.[453]

Creatine monohydrate is a nutritional supplement used as an ergogenic aid by athletes. After cellular uptake CK phosphorylates creatine to phosphocreatine using ATP. Phosphocreatine serves as an energy buffer and transport vehicle. Upon energy demand, *i.e.* muscle contraction, CK reverses this phosphorylation by converting phosphocreatine to ADP to regenerate ATP. Ingestion of creatine can increase creatine and phosphocreatine levels inside muscles, thereby enhancing mainly short, intense exercise performance.[454] *In vitro* the provision of creatine to *mdx* myotube cell cultures, resulted in higher phosphocreatine concentrations, improved survival, and less $Ca^{2+}$-accumulation by stimulating sarcoplasmic reticulum $Ca^{2+}$-ATPase.[455] Total creatine levels are lower in *mdx* muscles compared to wild type. In *mdx* mice a creatine-enriched diet from birth reduced the first necrosis wave normally seen after four weeks in fast-twitch, but not in slow-twitch muscle fibres. It restored the mitochondrial respiration capacity to wild type levels.[456] Less effectiveness was observed when supplementation was started after this first wave (at three month of age), suggesting necrosis was already too advanced. Only a lowering of muscle mass towards wild type levels, but no significant increase in tetanic force, was observed. As expected it did not change other pathological features, such as centrally nucleated fibres and increased $Ca^{2+}$-content.[457] Several clinical trials with creatine supplementation in DMD, BMD or other muscular dystrophy patients have been conducted with moderate results.[458] Small improvements in muscle strength and daily-life activities were observed in patients with several types of muscular dystrophies.[459] However in myotonic dystrophy type 1 patients, an inherited muscle disorder in adults, no improvement was observed.[460] Three months administration to DMD and BMD patients increased voluntary muscle contractions, decreased muscle fatigue and lowered urinary collagen excretion.[461] A more extensive double-blinded, placebo-controlled, cross-over study in which DMD patients, of which half was using corticosteroids, received creatine or placebo for four months followed by a cross-over after a six week washout period, showed an improvement in motor function and improvement of body composition (an increase in fat free mass) during creatine treatment irrespective of corticosteroid use. However no improvement of daily activities was found.[462] Another trial determined phosphorus metabolite ratio (PCr/Pi), which is lowered in skeletal muscle of DMD patients and associated with impaired

muscle function. In the placebo group a reduction in PCr/Pi ratio was observed, whereas an increase was seen in creatine-treated patients. Furthermore therapeutic variability was seen with age. Before treatment, PCr/Pi ratios were lower in young patients (under seven years) compared to older patients (above seven years). Whereas young creatine-treated patients showed an increase in PCr/Pi ratio after supplementation, no difference was observed in older creatine-treated patients. Some improvement/preservation of muscle strength was observed by creatine treatment.[463]

Glutamine is the most abundant free amino acid in body and muscle protein synthesis. Studies on the mechanisms behind possible anti-oxidative effects of L-glutamine have been performed in *mdx* mice, which show an increase in muscle free glutamine and glutamate, probably due to increased muscle glutamine production during catabolic stress. Exogenous administration of L-glutamine reduced the glutamine levels and decreased the ratio of oxidized glutathione versus total, which is an inducer of oxidative stress via activation of ERK1/2 that was also decreased.[464] Oral administration of L-glutamine in DMD patients for two or ten days resulted in a decrease in whole body protein degradation.[465,466] However in a longer double-blinded, randomized, cross-over trial four months of L-glutamine administration did not result in improved muscle mass or function.[467] Furthermore, although acute L-glutamine transiently stimulates insulin secretion, no effect on glucose metabolism or insulin resistance was observed.[468]

In a randomized, placebo-controlled trial comparing six month treatment with either creatine or L-glutamine versus placebo in steroid-naïve DMD patients did not improve muscle strength or function. Only in younger patients a small improvement of both compounds and in older patients of creatine might have been observed, although the difference was not significant.[469]

The activation of satellite cells (muscle precursor cells) upon damage to initiate muscle regeneration is mediated by NO-production by NOS, normally present at the sarcolemma, but mislocalised in DMD due to disruption of the DGC. L-Arginine is a substrate for NOS. Short term studies showed beneficial effects of L-arginine supplementation in *mdx* mice. After three weeks treatment of adult mice an increase in utrophin at the sarcolemma was observed.[470] This was also seen after two weeks treatment of young mice. L-Arginine led to a decrease in pro-inflammatory cytokines (IL-6 and TNF-α) which led to a decreased activation of the NF-κB pathway and its downstream targets (*e.g.* MMP-2 and MMP-9). L-Arginine treatment led to a decrease in MMP-2/MMP-9-mediated *β*-dystroglycan cleavage, thereby increasing its level. It stabilised the utrophin/*β*-dystroglycan interaction and led to targeting of nNOS towards the sarcolemma via syntrophins.[471] Also here applies that the upregulation of utrophin cannot solely be responsible for nNOS restoration,[353] but that other mechanisms must also be involved. Combinational treatment with deflazacort, which increases nNOS expression, suggested an additional benefit of L-arginine since three weeks treatment resulted in a decrease of contraction-induced injury in the limb muscle.[472] However, long term treatment with L-arginine in six months old *mdx* mice for 15 months did not affect cardiac fibrosis, although a decrease in inflammatory cell density was observed.[473] Long term studies even raised concerns of detrimental effects. Seventeen months of treatment in *mdx* mice resulted in an increase in skeletal and cardiac fibrosis. The short term effects of a decrease in MMP-2 or MMP-9 and increase in utrophin were not observed.[474] The mechanism behind this is probably a shift in macrophage type. In four weeks old *mdx* mice, at the peak of necrosis, high levels of M1 (pro-inflammatory) and M2 (alternatively-activated) macrophages were observed in quadriceps with prominent fibrosis. M1 macrophages have been shown to lyse

muscle by an NO-mediated mechanism. M2 macrophages express arginase, an enzyme that catalyses arginine into L-proline, a precursor molecule for collagen synthesis. M2a macrophages reduce lysis by M1 cells via competition of arginase with iNOS in M1 cells for its substrate arginine. At twelve weeks of age, at the peak of regeneration, an increase in IL-4 and IL-10 was observed, which deactivates M1 cells and promotes $CD136^{+}$ M2c macrophages that can increase tissue repair.[363] The deactivation of M1 cells by M2c cells can increase the substrate availability for arginase, thereby shifting the arginine metabolism from iNOS to arginase, resulting in an increase in arginase-derived metabolites, which create a more pro-fibrotic environment. Therefore L-arginine supplementation might increase arginase activity, resulting in more fibrosis on the long term. Indeed arginase-2 null mutant *mdx* mice showed reduced skeletal fibrosis; although cardiac fibrosis and function was unaltered.[474] A clinical trial assessing the safety and efficacy of L-arginine administration for 30 days on muscles, assessed by MRI, in boys with DMD or BMD, has been completed, but no results have been published yet (NCT01388764).

Long term (six months) treatment of three months old *mdx* mice with arginine butyrate did result in increased grip strength and reduced fibrosis. However no effect on skeletal muscle or cardiac histology, cardiac function and behaviour of the mice was observed.[379] Another study administering arginine butyrate for only six weeks to eight weeks old *mdx* mice also revealed an improvement in histology of the diaphragm and hind limb muscles. In addition an increase in utrophin was observed.[475] The beneficial effects might be due to the butyrate moiety that has HDAC-inhibitory activity. In the arginine butyrate-treated mice changes in HDAC-related gene expression were seen: an increase in growth promoting pathways, *e.g.* IGF-1, and a decrease in genes associated with fibrotic pathways.[379]

Payne *et al.* tested several compounds alone or as a cocktail in exercised *mdx* mice: creatine monohydrate, conjugated linoleic acid, α-lipoic acid and *β*-hydroxy-*β*-methylbutyrate. Conjugated linoleic acid consists of a number of different isomers of the essential fatty acid linoleic acid conjugated by double bonds. It can decrease body fat accretion, improve immune function and act as a free radical scavenger. A-lipoic acid is a disulfide mitochondrial coenzyme and a potent anti-oxidant that can improve mitochondrial function and enhance creatine monohydrate uptake. B-hydroxy-*β*-methylbutyrate is a leucine metabolite that is used by athletes to enhance strength and muscle mass gain by strength exercise and reduce exercise-related damage and muscle protein breakdown. Each compound separately showed some benefit and the combination of all four components showed the most prominent effect. Especially in combination with prednisolone treatment therapeutic effects were seen by increasing grip strength performance and improving muscle histology, as reflected by a decrease in centrally located nuclei and retroperitoneal fat pad stores.[476] B-hydroxy-*β*-methylbutyrate has also shown in an *in vitro* screen to improve tetanic force in dystrophic myoblasts.[477]

## 2.7 The TGF-*β* superfamily

The transforming growth factor-*β* (TGF-*β*) superfamily consists of proteins (myostatin, TGF-*β*, activin and BMP) involved in the regulation of many cellular processes, like cell growth, differentiation and maintenance of homeostasis. Most members are synthesized as propeptides, which become activated by proteolytic cleavage and form functional dimers. They signal by inducing and binding to receptor complexes consisting of a type II and a type I

receptor, leading to activation of downstream, intracellular signalling pathways. The affinity for type I and/or type II receptors in different cell types is affected by the presence or absence of coreceptors.[478] These ligands can signal both via a canonical Smad-dependent pathway and via non-canonical Smad-independent pathways. The Smad-dependent pathway involves the phosphorylation of Smad-proteins, which are transported to the nucleus where they regulate transcription. Myostatin, TGF-$\beta$ and activin commonly use pSmad2/3 and BMP uses pSmad1/5/8.[479] Non-canonical pathways are, among others, the PI3K/Akt/mTOR, Raf/MEK/ERK and p38/MAPK pathways.[480]

Myostatin (growth and differentiation factor 8) negatively regulates muscle growth. It is present in skeletal muscle cells and in adult animals myostatin has an inhibitory effect on satellite cells. It is encoded by the *MSTN* gene located on chromosome 2q32.2 and consists of three exons. It is transcribed as a precursor protein and acquires its active form of 12.5 kDa after several posttranscriptional modifications, among which proteolytic cleavage from its propeptide.[481] Myostatin inhibits myogenesis and increases fibrosis via several pathways (fig. 2.3a). It binds mainly to the activin receptor type IIb (Acvr2b) and to a lesser extent to Acvr2a. Thereafter this complex combines with activin type I receptors Acvr1b (ALK4) or

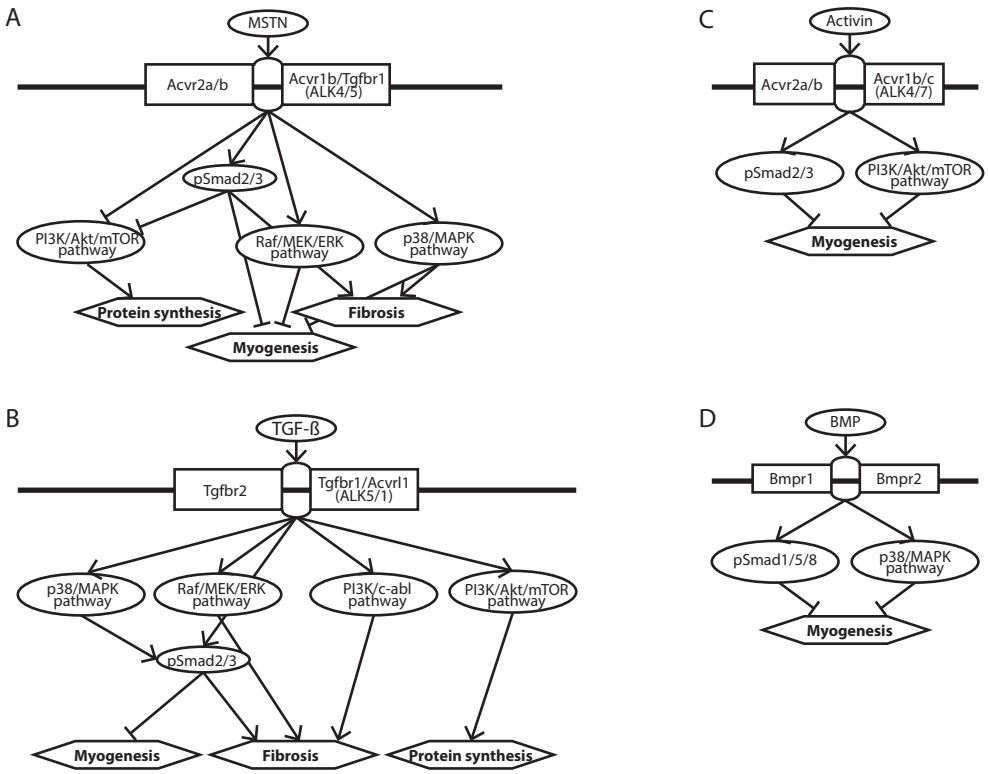


**Fig. 2.3:** Simplified scheme of signalling by members of the TGF-$\beta$ superfamily
All members bind to type II-type I receptor combinations, leading to activation or inhibition of several intracellular pathways. They signal both via canonical pSmad-dependent and non-canonical pSmad-indepedent pathways. Myostatin (a) by signalling via pSmad2/3 and several non-canonical pathways mainly inhibits muscle growth. TGF-$\beta$ (b) also signals via pSmad2/3 and various non-canonical pathways and is a potent inducer of fibrosis. Activin (c) is involved in the regulation of muscle mass via the canonical pSmad2/3 pathway and uses, amongst others, PI3K for non-canonical signalling. BMP (d) uses the pSmad1/5/8 and p38/MAPK pathway to inhibit myogenesis.

Tgfbr1 (ALK5) to activate downstream targets. Myostatin shows cell-type specific utilization of type I receptors: in myoblasts mainly the Acvr1b receptor is used, whereas in fibroblasts mainly the Tgfbr1. This is due to the presence of the coreceptor Cripto in myogenic cells, which is absent in non-myogenic cells. Cripto inhibits activin activity, which also uses the Acvr1b receptor. In non-myogenic cells myostatin uses betaglycan as a coreceptor.[482] Via the canonical pSmad2/3 pathway myostatin signalling leads to a downregulation of myogenesis transcription factors, decreasing muscle progenitor cells proliferation and differentiation. It also acts on non-canonical p38 MAPK and ERK1/2 signalling to inhibit expression of factors, *e.g.* Pax7, which leads to a decrease in satellite cell self-renewal, thereby reducing regeneration. In addition, myostatin inhibits the PI3K/Akt/mTOR pathway, both directly and via pSmad2/3, thereby reducing protein synthesis.[481,483,484] Finally, via among others pSmad2/3 and p38 MAPK it increases fibroblast proliferation.[485]

TGF-$\beta$s are another subfamily, which consists of three mammalian isoforms: TGF-$\beta_1$, TGF-$\beta_2$ and TGF-$\beta_3$. Their expression is induced in damaged and regenerating skeletal muscle and TGF-$\beta_1$ is capable of inducing fibrosis. TGF-$\beta$s bind to the type II receptor Tgfbr2, which then combines with a type I receptor, mainly Tgfbr1 or otherwise Acvrl1 (ALK1), and play a role in regulation of muscle growth, regulation and can stimulate fibrogenesis.[478,486,487] Signalling occurs both via canonical Smad2/3-dependent signalling pathways and non-canonical Smad-independent pathways (fig. 2.3b). Non-canonical TGF-$\beta_1$ signalling is mediated via pathways such as PI3K/Akt/mTOR and Raf/MEK/ERK, which all have, amongst others, pro-fibrotic effects upon activation.[480]

Activins are homo- or heterodimers of two subunits, A (inhibin-βA), B (inhibin-βB), C (inhibin-βC) or E (inhibin–βE).[487,488] They initiate signalling by binding to Acvr2a or Acvr2b, in combination with type I receptor Acvr1b or Acvr1c (ALK7) and also use pSmad2/3 for downstream signalling via the canonical pathway (fig. 2.3c). Activin A is also involved in regulation of muscle mass, since a mouse model carrying targeted deletion of inhibin-βA subunits, the constituents of activin A, displays increased muscle mass.[488] Inhibins are dimers of an inhibin-α subunit with an inhibin-βA subunit (inhibin A) or an inhibin-βB subunit (inhibin B). They inhibit activin signalling by binding to its receptors.[488]

Bone morphogenic proteins (BMPs) are known to inhibit myogenic differentiation via activating pSmad1/5/8 and p38 MAPK signalling pathways, which in turn decrease the expression of myogenic regulators like MyoD and Myog (fig. 2.3d).[489] Interestingly, expression of several BMPs was found to be increased in *mdx* muscle.[490] In DMD patient cells BMP4 was increased, which showed to inhibit myogenic differentiation.[491] The implication of induced BMP signalling and involvement in DMD pathology is currently unknown. However, recent studies showed the importance of BMPs in the regulation of myogenic differentiation. BMP type I receptor BMPR1A (ALK3) is expressed in activated satellite cells, which enhances proliferation by inhibiting genes associated with differentiation. Knockdown of the BMP antagonist Noggin enhanced proliferation, while overexpression induced premature differentiation.[492] Also in foetal muscle Noggin overexpression caused a reduced number of satellite cells and smaller muscle size.[493] After muscle injury BMP is activated and Noggin overexpression perturbed the regeneration process, leading to smaller regenerated fibres.[492,493] Recently two papers were published, which highlighted the importance of BMP signalling in stimulating muscle growth and maintaining muscle mass and the effects of interfering with BMP signalling in models for denervation-induced atrophy.[494,495] Winbanks *et al.* showed that by increasing BMP ligands or using a constantly active receptor skeletal muscle hypertrophy was promoted via Smad1/5/8-mediated activation of mTOR

signalling. Furthermore increasing BMP signalling protects muscle from atrophy during denervation, while inhibition worsens atrophy.[495] The same was shown by Sartori *et al.*, who also saw muscle atrophy after BMP inhibition. In addition BMP inhibition neutralizes the hypertrophic phenotype in mice lacking myostatin, suggesting that increased BMP signalling is responsible for the increase in muscle mass in myostatin knockout mice.[494] Therefore, BMPs are likely important regulators of myogenic differentiation, regeneration and muscle fibre growth, although the role of individual BMP ligands and receptors and their potential involvement in DMD pathology remains unknown.

Since various members of the TGF-$\beta$ family and/or their receptors have shown to be differentially expressed in DMD and are involved in regulation of muscle growth and fibrosis, they serve as a target for several therapeutic approaches.

### *2.7.1 Myostatin inhibition*

Mutations that lead to complete loss of myostatin are viable and have been described for several animals (*e.g.* Belgium Blue cattle, Texel sheep and dogs) and one human boy.[496-503] In each case lack of myostatin results in a vast increase in muscle mass. In whippet dogs that are heterozygous for a *MSTN* mutation (*i.e.* have only one functional copy) a lesser degree of muscle hypertrophy is observed, but they are more muscular and faster than wild type whippet dogs, indicating the mutation is quantitative and loss of one allele can improve skeletal muscle performance.[504] *Mdx* mice that also lack myostatin (*mdx*/*Mstn*$^{-/-}$) are more muscular and stronger than normal *mdx* mice. The muscle hypertrophy is due to an increase in muscle fibre number (hyperplasia) and in cytoplasmic volume (hypertrophy) rather than a change in the total number of myonuclei per fibre.[481] The fibrosis in the diaphragm, which increases with age, is reduced in these mice as well.[505] Furthermore, the effect of myostatin is only seen in skeletal muscle; the heart of myostatin knockout mice (*Mstn*$^{-/-}$) is similar to that of wild type mice. As anticipated, the absence of myostatin has no beneficial effects on cardiac muscle mass or cardiac fibrosis in the double knockout *mdx*/*Mstn*$^{-/-}$ mice.[506] Furthermore studies on *Mstn*$^{-/-}$ mice revealed that the increase in muscle mass and fibre size does not result in an increase in specific force, but even impairs it.[507] To study the effect of *Mstn* knockout after muscle maturation, *Mstn*$^{lf/lf}$ mice with a tamoxifen-inducible transgene have been generated to achieve muscle specific knockout in adult stages. This also leads to an increase in muscle mass, although to a lesser extent than in *Mstn*$^{-/-}$ mice.[502] So, prenatally myostatin acts predominantly on muscle progenitors and postnatally on differentiated muscle.[481] The increased muscle mass observed in all myostatin null animals served as the rationale to try to inhibit myostatin and thereby improve muscle mass in order to compensate for the loss of muscle tissue in DMD.

Treatment of C2C12-cells with pharmacological antibodies that block myostatin caused a decrease in the phosphorylation of Smad2/3, which in turn affects the formation of Smad-complexes, involved in the transcriptional regulation of genes associated with muscle cell progenitors. *In vivo* systemic (intraperitoneal) treatment of *mdx* mice with these myostatin blocking antibodies induced an increase in muscle mass and strength.[509] Importantly, a subsequent study observed improvement in diaphragm pathology by anti-myostatin antibodies in young *mdx* mice, but not in adult *mdx* mice, which already show marked disease progression.[510] Intravenous anti-myostatin antibody (MYO-029) treatment in adult neuromuscular disorder patients (including BMD patients) did not lead to a significant increase in muscle

mass in a first clinical trial. It should be noted that this was mainly meant as a safety trial: patients were treated for only one month and due to a lack of power of the study, no reliable conclusions could be drawn of the therapeutic effects. Overall the antibody was well tolerated and safe. Only at higher concentrations of ten and 30 mg/kg it caused hypersensitivity of the skin, but it had no adverse side effects on muscles. However, the hypersensitivity might limit the dose that can be used and therefore limit the therapeutic efficacy.[511]

Meanwhile, a new fusion protein joining a human antibody Fc-receptor and the ligand binding domain of type II receptor Acvr2b has been generated (sAcvr2b-Fc; ACE-031). This receptor can bind myostatin, but also its family member activin, also involved in the regulation of muscle mass and implicated in fibrosis formation in many diseases, including DMD. Thus, ACE-031 may act through separate mechanisms simultaneously, both increasing muscle mass and inhibiting fibrosis. In wild type mice it caused an increase in muscle mass, without altering the fibre type profile of the muscle, which is changed in *e.g.* myostatin deficiency.[512] In *mdx* mice similar compounds (a soluble form of Acvr2b linked to a murine Fc-receptors; RAP-031 or sAcvr2b) resulted in a normalisation of muscle force.[513,508] In a phase I clinical trial in healthy volunteers (postmenopausal women) treatment was well tolerated for both single and multiple doses and resulted in a dose-dependent increase in muscle mass at the cost of fatty tissue. Recently, a dose-escalation safety trial and its extension study in DMD patients have been terminated due to unexplained nose and gum bleeding and dilated small blood vessels observed in some treated patients (NCT01099761/ NCT01239758). The mechanisms behind these unexpected side effects are currently under investigation.[515] Combination of this sAcvr2b-Fc with AAV-U7-mediated dystrophin exon skipping resulted in increased muscle growth in *mdx* mice, as was also seen by sAcvr2b-Fc alone, and improved specific force and resistance to contraction induced injury, also seen by AAV-U7 treatment alone. No clear synergistic effect of the combination was observed.[516] In an earlier study in which the Acvr2b was knocked down by using shRNA, this knockdown strongly enhanced the force increase by AAV-U7-mediated dystrophin exon skipping. On the other hand no increase in muscle growth was observed.[517] This discrepancy might be due to the different ways of Acvr2b blockade.

As mentioned above, myostatin is synthesized as a precursor protein that is cleaved by BMP-1/tolloid metalloproteinases to generate the active C-terminal part and an N-terminal myostatin propeptide. This propeptide can inhibit myostatin activity by preventing its binding to its receptor. Myostatin propeptide fused to IgG-Fc to prevent cleavage by BMP-1/tolloid proteases resulted in increased body and muscle mass and improved function in *mdx* mice.[518] For long term expression, this fusion propeptide was delivered with an AAV8 vector, resulting in improved muscle histology, decreased CK levels and improvement of muscle force (grip strength); however it reduced exercise endurance (treadmill running).[519] To get stable transgene expression, which may not be feasible with skeletal muscle delivery, Morine *et al.* used a AAV-mediated expression of a dominant negative myostatin propeptide paired with a liver-specific promoter, thereby getting long term secretion by the liver resulting in body-wide expression. In *mdx* mice this approach resulted in increased skeletal muscle mass and function; however in 11 months old mice no improvement in diaphragm pathology was seen.[520]Administration of this same liver-targeting myostatin inhibiting compound in GRMD dogs resulted in increased muscle mass over a period of 13 months and a reduction of CK levels and fibrosis. Cardiac morphology was not assessed.[521]

Follistatin is a myostatin-ligand binding protein that can inhibit its activity. Transgenic overexpression of follistatin in mice results in a massive increase in muscle mass and increases

muscle regeneration.[522,523] Conversely, mice heterozygous for follistatin (*follistatin*$^{+/-}$) show a reduced muscle mass and regeneration.[488] An additional effect of follistatin knockout is seen in *Mstn*$^{-/-}$ mice and the effect of heterozygosity for follistatin is maintained by crossing with *Mstn*$^{-/-}$ mice, indicating that also other factors play a role in the control of muscle growth in parallel with myostatin and that follistatin also affects other ligands apart from myostatin.[488,524] Indeed, follistatin can also antagonise activin. Mice mutated for activin A also show a (more moderate) increase in muscle mass, suggesting both myostatin and activin A are involved in muscle mass regulation.[488] Administration of follistatin overexpressing muscle progenitor cells to immunodeficient *mdx* mice resulted in enhanced muscle regeneration.[522] A single injection with an AAV vector carrying a follistatin isoform that mainly affects skeletal muscle (FS-334) resulted in improvement of muscle mass and strength up to over two years in both young and aged *mdx* mice.[525] Yet, it is not selective for myostatin, but also binds to activins and inhibits their activity. Since activins have numerous functions in other tissues than skeletal muscle, increasing myostatin by using follistatin is likely to have many side effects. In *mdx* mice a transgenic follistatin-derived myostatin inhibitor FS I-I, which is much more selective for myostatin, increased skeletal muscle size and strength and reduced the infiltration of immune cells, without affecting activin activity.[526] The safety and efficacy of the FS-334 isoform expressed by AAV1 has been tested in nonhuman primates (cynomolgus macaque monkeys), resulting in increased muscle mass and strength, without immune response or toxic side effects on other organs in the long run (over one year).[527] A clinical trial where the follistatin gene (FS-334) in an AAV is injected in quadriceps muscles of BMD and sporadic inclusion body myositis (an inflammatory, progressive muscle wasting disease) patients is currently ongoing for testing safety and assessing its effect on muscle mass and function (NCT01519349). Recently combinational treatment with this FS-334 isoform and microdystrophin (ΔR4-23/ΔCT) delivery by AAV vectors was tested in aged *mdx* mice. Whereas both treatments by itself were not capable of fully protecting muscle against contraction-induced injury in this advanced disease state, the combination improved this to near normal levels as seen in wild type mice.[528]

Myostatin knockdown has also been achieved by exon skipping. 2OMePS against exon two, resulting in a premature stop codon, resulted in good exon skipping *in vitro* and lowering of myostatin expression and its downstream targets Myf5 and Myog. Also in *mdx* mice exon skipping was observed after intramuscular injection, albeit at low levels. Furthermore, in contrast to *DMD* exon skipping, which was homogenous throughout the muscle, variation in skipping levels throughout the muscle was observed. *In vitro* the feasibility of combined exon skipping of dystrophin and myostatin was shown in DMD patient-derived cells. However this has not been tested *in vivo*.[529] High levels of exon skipping were observed with PMO AONs against exon two of myostatin. Systemic injection of vivo-morpholinos induced myostatin exon skipping resulting in an increase in muscle mass and myofibre size in wild type mice.[530] Also pPMOs showed high efficiency and intramuscular treatment of *mdx* mice resulted in an increase of tibialis anterior muscle weight. When combined with a pPMO against dystrophin exon 23, both this increase in muscle mass and dystrophin exon skipping was observed, showing no detrimental effects of combining both AONs. Interestingly the increase in muscle mass after myostatin skipping only or after combined treatment was observed only in female mice, not in male mice, showing gender-specific results.[531]

Next to downregulation of myostatin signalling by exon skipping of myostatin itself, AON-mediated exon skipping has been tested for its type I receptors (Acvr1b/Tgfbr1), which

also influences other members of the TGF-$\beta$ family (activin and TGF-$\beta$). Acvr1b is a receptor for myostatin and activin, and Tgfbr1 is a receptor for myostatin and TGF-$\beta$. They commonly use the pSmad2/3 pathway as one of their downstream signalling pathways. Upregulation of some of these receptors has been found in DMD patients and animal models. The mRNA coding structure of these receptors is similar. Exon two encodes the ligand binding domain and exon six part of the kinase domain; skipping of exon two will result in a non-functional receptor, while skipping of exon 6 will lead to an out-of-frame transcript. Attempts to develop AONs to skip these exons resulted in effective AONs, both *in vitro* and *in vivo*, against exon six of Acvr1b and exon two of Tgfbr1, which showed specific skipping in the targeted receptor. 2OMePS AONs against Acvr1b, but not against Tgfbr1, showed *in vitro* downregulation of myostatin induced pSmad3 levels only in myogenic cells and not in non-myogenic cells; whereas in non-myogenic cells this was only observed after Tgfbr1 skipping. This is in line with previous results showing cell-type specific utilization of type I receptors by myostatin.[482] As expected, TGF-$\beta$ signalling was only affected by Tgfbr1 skipping. Enhanced myoblast and delayed fibroblast differentiation was observed after skipping with either AON. Intramuscular injection in *mdx* mice of 2OMePS AONs confirmed target specificity and showed no interference with *DMD* exon 23 skipping after co-injection. Gene expression and protein analysis also showed the different roles of Acvr1b and Tgfbr1 in disease pathology. Skipping in either receptor resulted in an increase in Myog expression, indicative of enhanced muscle differentiation. In contrast, Tgfbr1 skipping resulted in a decrease in all fibrotic markers (Collagen1a1, Serpine1 and CTGF); whereas Acvr1b skipping resulted in a decrease in Collagen1a1, but an increase in Serpine1 and CTGF. In general a reduction in pSmad2 levels was seen, suggesting an overall reduction of myostatin/activin/TGF-$\beta$ signalling [Kemaladewi *et al.*, unpublished results].

A potential risk for using myostatin signalling inhibition strategies is to induce/increase DMD cardiomyopathy. Several studies have been performed on the effect of myostatin knockout/inhibition on the heart showing inconsistent results. Results in *Mstn*$^{-/-}$ mice suggest it might induce cardiac hypertrophy and thereby be beneficial for the repair of damaged cardiac muscle, although others found no cardiac phenotype.[506,532] In *mdx* mice also variable results were seen: whereas no cardiac changes were observed in *mdx*/*Mstn*$^{-/-}$ mice and mice treated with soluble Acvr2b treatment for four months, cardiac hypertrophy resulting in reduced cardiac function was observed by liver-targeted myostatin propeptide administration after eleven months.[506,519,520]

### *2.7.2 Targeting TGF-β signalling*

Treatment of *mdx* mice with a TGF-$\beta_1$ neutralizing antibody for six weeks resulted in a decrease in TGF-$\beta_1$ expression and reduced fibrosis in the diaphragm; however no effect on muscle degeneration/regeneration was observed. Furthermore, an increase in inflammatory CD4$^+$ lymphocytes was detected. It is known that TGF-$\beta_1$ also plays a role in inflammation and immunomodulation. The overall effect of TGF-$\beta_1$-inhibition on fibrosis and inflammation could have implications for long term treatment with TGF-$\beta_1$-inhibitors and therefore combination with immunosuppressive agents might be a good strategy.[533]

Halofuginone (7-bromo-6-chloro-3-[3-(3-hydroxy-2-piperidinyl)-2-oxopropyl]-4(3H)-quinazolinone) has anti-fibrotic activity by inhibiting Smad3 phosphorylation downstream of TGF-$\beta$ signalling, thereby attenuating collagen type I gene expression by fibroblasts. In *mdx*

mice it could prevent an age-dependent increase in fibrosis in young mice as well as induce a decrease in fibrosis in older mice (eight to nine months old). Thereby it improved proliferation of muscle cells and skeletal muscle, diaphragm and, importantly, cardiac function.[534,535] More specifically, in *mdx* mice and muscle biopsies of DMD patients the expression of the gene collagen triple helix repeat containing 1 (*CTHCRI*) was shown to be elevated, correlating with disease severity. Large infiltrates of myofibroblasts were the source of Cthcr1 and halofuginone inhibited both these infiltrates and lowered Ctchr1 expression in skeletal and cardiac muscle of *mdx* mice.[536] A first, open-label clinical trial assessing the safety and pharmacokinetics of HT-100 (halofuginone hydrobromide delayed-release tablet) by comparing several single and multiple doses is currently ongoing (NCT018475730).

Decorin, a small leucine-rich proteoglycan, is a component of the ECM of all collagen-containing tissues. It can bind TGF-$\beta$, thereby blocking its activity. It is present in the ECM surrounding muscle fibres. Studies on DMD biopsies and cultured dystrophic myotubes found a decrease in decorin mRNA expression, mainly localised in fibroblasts, accompanied by an increase in TGF-$\beta_1$ expression.[537,538] Puzzling, another study found contradictory results, whereby decorin expression was increased in DMD biopsies, hypothesizing an attempt of the muscles to combat fibrosis.[539] Patients of the studies were in the same age range; however the last study only looked at two patients. Intraperitoneal treatment of *mdx* mice with decorin resulted in a 40% decrease of collagen I mRNA expression in the diaphragm.[540] In injured skeletal muscle decorin could prevent TGF-$\beta_1$-induced differentiation of myogenic cells into fibrotic cells.[541] Further studies on overexpression of decorin by gene transfer *in vitro* in cultured murine muscle cells showed an accelerated differentiation into myotubes and an increased expression of myogenic genes together with a decrease in TGF-$\beta_1$ and myostatin expression. *In vivo* an enhanced regeneration and healing of muscle after injury was observed by decorin gene transfer. The mechanisms behind this effect of decorin on muscle healing are not exactly known. This study suggest that it acts on various pathways, both by inhibiting fibrosis by lowering TGF-$\beta_1$ and myostatin expression and it may also upregulate regeneration, as they showed an increase in regeneration-stimulating genes like follistatin and MyoD.[542]

Suramin, a polysulfonated naphthylurea, is a TGF-$\beta_1$ blocker that was able to attenuate exercise-induced fibrosis and elevation of CK in six months old *mdx* mice after seven weeks of treatment. Only in cardiac muscle no improvement was observed.[543] This was also observed in another study treating eight month old *mdx* mice for three months. Hereby solely the heart was analysed by electrocardiogram and histology.[544] To protect the *mdx* diaphragm against muscle fatigue, it acted by decreasing MMP-9, involved in degradation of the ECM, activity and increasing $\beta$-dystroglycan, thereby having positive effects on the maintenance of the DGC.[545] Suramin is used for the treatment of other disorders (*e.g.* prostate cancer) however it may have side effects like polyneuropathy.[546]

Fibrinogen is a soluble acute phase protein, which is released from inflammatory sites and converted to fibrin, playing a role in fibrogenesis. In *mdx* mice it was shown that fibrinogen can bind the $\alpha_M\beta_2$-(MAC-1) receptor on macrophages, thereby inducing IL-1β expression, which in turn increases TGF-$\beta$ expression and signalling. Fibrinogen can also induce collagen synthesis directly by binding to the $\alpha_V\beta_3$-integrin receptor on fibroblasts. In fibrotic areas in DMD patients and *mdx* mice fibrinogen expression is increased, indicating it as a possible therapeutic target. Indeed genetic loss of fibrinogen in *mdx* mice (*mdx/Fibrinogen*$^{-/-}$) resulted in a reduction in fibrosis and slowed down disease progression. The same could be achieved by pharmacological inhibition of fibrinogen in *mdx* mice with ancrod, a defibrino-

genating agent. A reduction in TGF-$\beta$, pSmad2 and collagen I expression was observed, as well as a decrease in pro-inflammatory cytokines (*e.g.* TNF-α).[547] Fibrinogen also plays an important role in blood clotting.[548] Interference with this function could increase the chance of bleeding. Since different domains of the protein are involved in the different functions, mutants could be made in which a single domain is disabled. In *Fib*$\gamma^{390\text{-}396A}$ mice expressing a mutant form of fibrinogen with normal clotting function, but lacking the $\alpha_M\beta_2$ binding motif, or blocking $\alpha_M\beta_2$-binding by administration of a fibrinogen/$\alpha_M\beta_2$-binding peptide, an amelioration of dystrophic pathology was observed, which was reversed in the *Fib*$\gamma^{390\text{-}396A}$ mice by injecting fibrinogen. These results underline the specific interaction of fibrinogen with the $\alpha_M\beta_2$-integrin receptor on macrophages. Indeed, also in DMD patient biopsies a co-localization of fibrinogen and macrophages ($\alpha_M\beta_2$-integrin) was observed in degenerating areas. Next to a decrease in $\alpha_M\beta_2$-mediated signalling, ancrod treatment resulted in a decreased inhibition of satellite cells, thereby enhancing regeneration.[549]

Another compound that has an effect on amongst others TGF-$\beta$ signalling is imatinib mesylate, a 2-phenylaminopyrimidine derivative, which is already approved for the treatment of leukaemia. Imatinib selectively and competitively blocks the ATP binding sites of several tyrosine kinases, including c-Abl, c-Kit, and PDGF-receptors. The PI3K/c-Abl signalling route is also a non-Smad pathway mediating the TGF-$\beta$ effects stimulating fibroblast proliferation. c-Abl can be activated by both TGF-β and PDGF. Two studies in *mdx* mice showed a decrease in fibrosis/necrosis and inflammation, thereby improving hind limb strength, after imatinib treatment. This was mediated by an inhibition of both fibrotic signalling mediators as c-Abl and PDGF and inflammatory markers TNF-α and IL-1β.[550,551] It also attenuated dystrophy in a more severe *mdx* model, the DBA/2-*mdx* mouse, by decreasing fibrosis.[552]

Bowman-Birk Inhibitor concentrate is a soy-derived serine protease inhibitor, which resulted in improvement in muscle pathology and function in *mdx* mice after oral administration. It did not act on proteasome activity but reduced calpain activity. A decreased expression of TGF-$\beta_1$ and pSmad2/3 was seen and a reduced myostatin activation, resulting in increased muscle hypertrophy and decreased fibrosis. Bowman-Birk Inhibitor concentrate inhibits several proteases, but no changes in other growth signalling pathways (p38 MAPK, Akt or NF-κB) were seen.[553]

### *2.7.3 BMP antagonists*

BMP antagonists, *e.g.* Noggin, can enhance muscle differentiation and regeneration. Indeed, inhibition of BMP signalling with Noggin resulted in increased expression of regeneration markers both *in vitro* in C2C12-cells and *in vivo* in *mdx*/*Utrn*$^{+/-}$ mice. Muscle histology was improved in these mice. However these are non-selective BMP antagonists.[489] Furthermore, as described in paragraph 2.7 opposite effects were observed after denervation-induced atrophy. Hereby Noggin overexpression worsened pathology instead of ameliorating it.[494,495] These different observations demonstrate that BMP signalling has different effects on satellite cells/myoblasts and muscle fibres and that the effect of inhibition or stimulation is context dependent. In DMD a lot of muscle fibre damage and regeneration is observed, while in denervation-induced atrophy a lot of muscle mass is lost, leading to smaller and weaker fibres. However it indicates the large probability of side-effects while interfering with BMP signalling pathways.

## 2.8 The Renin-Angiotensin System

Activation of the renin-angiotensin(-aldosterone) system (RA(A)S; fig. 2.4) is known to play a role in the pathogenesis of, among others, cardiac fibrosis. This system has also been shown to be upregulated in muscular dystrophy. Angiotensin I is converted by ACE to angiotensin II, which in turn binds to its receptors angiotensin II receptor type 1 (AT1) and angiotensin II receptor type 2 (AT2). Via AT1 it asserts its main effects, among others increasing inflammation, fibrosis and stimulating cell proliferation.[554,555] It has pro-inflammatory actions by increasing the production of ROS which activate the NF-κB pathway.[556,557] Next to increasing inflammation, RAS also increases fibrosis via both TGF-$\beta_1$-dependent and TGF-$\beta_1$-independent mechanisms (fig. 2.3b/4). Firstly, it can upregulate the expression of TGF-$\beta_1$ directly via the NADPH oxidase/p38/MAPK pathway. Secondly, by enhancing downstream targets of TGF-$\beta_1$ signalling.[558] The RAS system is thought to act upon both the Smad-dependent and Smad-independent pathways. *In vitro* angiotensin II induces a late (24 hours) TGF-$\beta$-dependent pSmad2/3 activation. In cells lacking the *TGFB* gene or where endogenous TGF-$\beta$ was blocked, angiotensin II induced rapid phosphorylation of pSmad2/3 and increased expression of the pro-fibrotic markers CTGF and collagen I. This was blocked by a specific AT1 antagonist. Furthermore, inhibition of p38 MAPK also diminished this phosphorylation indicating that angiotensin II activates the Smad pathway via AT1 and MAPK activation.[559,560] AT1 signalling is thought to also stimulate the non-canonical pathways PI3K/Akt/mTOR and Raf/MEK/ERK TGF-$\beta$ independently.[561,562] Signalling via AT2 is thought to have opposite effects to AT1 signalling, *e.g.* anti-fibrotic and inhibiting growth, and there is negative crosstalk between both receptors. Therefore activation of AT2 during specific AT1 blockade may contribute to the beneficial effects on cardiac dysfunction.[262,563]

Considering the upregulation of RAS in DMD and its known pro-inflammatory and pro-fibrotic effects, several therapeutic strategies try to target this system at different levels.[555]

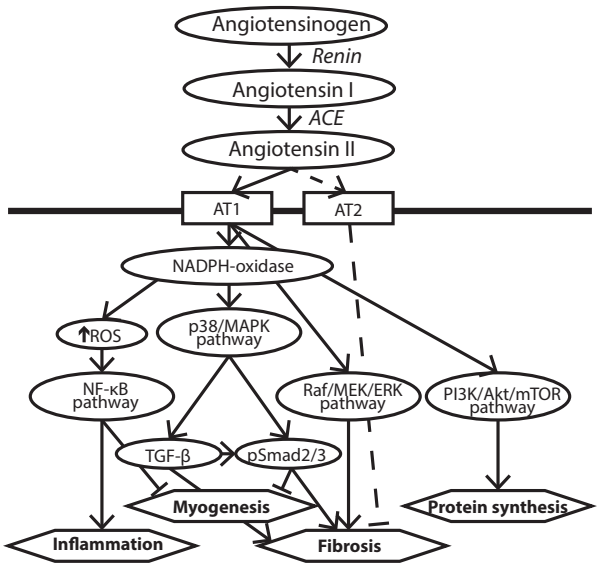


**Fig. 2.4:** Simplified scheme of the renin-angiotensin system
Angiotensinogen is converted by renin and angiotensin-converting enzyme (ACE) to its active form angiotensin II, which signals mainly via the AT1 receptor to increase inflammation and fibrosis. It has pro-inflammatory effects by activating the NF-κB pathway. It increases fibrosis/inhibits myogenesis both directly by activating several pSmad-dependent and independent pathways and indirectly by increasing TGF-$\beta$ expression. Signalling via AT2 is thought to have opposing effects and to be induced during AT1 blockade.

The first group of compounds are ACE-inhibitors, which are already widely used in the treatment of cardiomyopathies, where they have shown to reduce morbidity and mortality. They inhibit the conversion of angiotensin I to angiotensin II by ACE and can be divided in three groups based on their molecular structure. The first group are sulphydril-containing agents (*e.g.* captopril (the first ACE-inhibitor)), the second group are dicarboxylate-containing agents (*e.g.* enalapril, perindopril and lisinopril) and the third group (phosphonate-containing agents) only includes fosinopril.[8] However the clinical relevant differences between the different groups and/or compounds are marginal.[564] Nowadays it is recommended to start ACE-inhibitor treatment, possibly combined with a $\beta$-blocker, in DMD at the first signs of cardiomyopathy.[565]

Several ACE-inhibitors have been tested *in vitro* and *in vivo* in animal models with mixed results. Cozzoli *et al.* treated young *mdx* mice systemically with enalapril and observed an improvement in muscle function, accompanied by a decrease in necrosis. More detailed analysis of biomarkers, showed an effect on expression of genes related to inflammation and oxidation (*i.e.* decrease in NF-κB and ROS), but no change in fibrotic markers (TGF-$\beta_1$ and pSmad2/3), whereupon they hypothesised that pro-inflammatory and pro-oxidative events precede the pro-fibrotic events.[554] On the contrary, Nelson *et al.* treated young *mdx* mice for a short period with enalapril and did not observe an improvement in forelimb grip strength. However these results are rather limited, since they did not measure anything else.[263] The contradicting results could also be due to the fact that mice in the first study underwent an exercise protocol to aggravate muscle inflammation and pathology, making it easier to induce improvement. Furthermore the RAS potentially plays a role in impairing exercise performance. However, recently another group observed an improvement in muscle strength and decrease in CTGF-induced pro-fibrotic activity both in sedentary and exercised *mdx* mice. Again no change in TGF-$\beta_1$ expression.[566]

Lisinopril was identified in a large *in vitro* drug screening assay in dystrophic muscle to increase tetanic force.[477] When combined with an aldosterone antagonist, spironolactone, it was able to preserve cardiac function, reflected by a decrease in cardiomyocyte damage and fibrotic markers (MMPs), in *mdx*/*Utrn*$^{+/-}$ mice. Therapeutic effects at 20 weeks of age were larger when treatment was started at four weeks of age compared to start of treatment at eight weeks of age, indicating the importance of early intervention.[567]

Captopril showed its effectiveness *in vitro* by partially counteracting TGF-$\beta_1$-induced myoblast differentiation inhibition in C2C12-cells.[568] *In vivo* captopril was able to improve cardiac function in older *mdx* mice after starting treatment at two months of age.[381]

Studies in patients have shown positive effects of several ACE-inhibitors on cardiac function. Enalapril showed normalization of left ventricular function in half of the patients. The other half of the patients did not respond to enalapril treatment, but no association with type of mutation or age of onset of cardiac problems was found.[15] ACE-inhibitor therapy is often combined with $\beta$-blockers (see paragraph 2.15). Several ACE-inhibitors (often enalapril or lisinopril) showed to delay the progression of cardiomyopathy or even improvement of heart function and improvement of long term survival, when used alone or in combination with a β-blocker in DMD patients with heart failure.[569-571] In a randomized double-blinded clinical trial with perindopril versus placebo no difference between left ventricular ejection fractions (LVEFs) was observed after 36 months of treatment. Thereafter all patients (also the placebo) received in an open-label extension study perindopril for an additional two years. After this period a higher LVEF was seen in the patients who had received perindopril for five years compared to those who only received it during the last two years. Possible reasons for the

absence of an effect after the first phase, could have been the low *a priori* risk of developing heart failure (patients had normal LVEF at baseline) and the small study population. Since a placebo group is lacking in the second phase no conclusion can be drawn for the effect of perindopril in the group that started treatment later.[572] A ten year follow-up of the same patients showed a higher survival rate in the patients who received perindopril from the start of the trial (26 out of 28 versus 19 out of 29).[573] Overall all studies indicate the importance of early initiation of treatment.[565]

A clinical trial with the anti-oxidant coenzyme Q10 (see paragraph 2.4) and lisinopril is currently ongoing (NCT01126697). This open-label trial will compare the safety and the effects on cardiac function of coenzyme Q10 alone, lisinopril alone or combined treatment in DMD, BMD and limb girdle muscular dystrophy (LGMD) patients.

A second group of compounds targeting the RAS system are selective AT1 antagonists, to which losartan belongs. Losartan is used as a vasodilator to treat hypertension and is known to decrease TGF-$\beta$ signalling.

Cohn *et al.* first investigated the effect of losartan in *mdx* mice. Long term losartan treatment (from six weeks of age till six to nine months) resulted in decreased disease progression and fibrosis and improved muscle function. In addition, they showed that after muscle injury in aged *mdx* mice, losartan improved the regenerative capacity and reduced fibrosis by antagonism of TGF-$\beta$ signalling, reflected by lower levels of its downstream targets pSmad2 and TSP-1.[260] However a few years later the same group published another study indicating the protective effects were not mediated via TGF-$\beta$ signalling, but via the IGF-1/Akt/mTOR pathway.[262] They showed in a mouse model for sarcopenia (loss of skeletal mass and function during ageing), that losartan improved muscle architecture and functional recovery after cardiotoxin injection via blocking both the canonical (*i.e.* pSmad2) and non-canonical (*i.e.* pERK) TGF-$\beta$ signalling pathway. However, experiments in disuse atrophy during ageing, showed no change in either pSmad or pERK, but positive effects by activation of the IGF-1/Akt/mTOR pathway.[561] This pathway plays a role in increasing protein synthesis and preventing muscle regeneration.[574,575] In relation to these partly contradicting results, we did not see any change in *mdx* mice in the expression of downstream targets of TGF-$\beta$ signalling in fibrotic pathways, after losartan treatment. In addition no effect of losartan on pSmad2 levels after TGF-$\beta$ stimulation was observed in an *in vitro* assay. Only a modest increase in pERK levels might be seen (this thesis chapter 6). In addition, in the meantime other groups published less convincing results on the positive of losartan in muscular dystrophy. Nelson *et al.* indeed observed improvement of forelimb grip strength in *mdx* mice by losartan treatment, but only at two months of age and not at nine months of age. At two months, a decrease in TGF-$\beta$ activity was observed, but at nine months there was no change in fibrotic markers. However they did observe an improvement in respiratory function in these aged mice.[263] In two other studies long term (six months resp. two years) losartan treatment in *mdx* mice could only preserve cardiac function, but not skeletal muscle function. Probably the angiotensin II pathway plays a major role in the development of cardiac fibrosis, whereas in skeletal muscles other fibrotic pathways dominate.[261,264] This is in line with the results for ACE-inhibitors, whose main beneficial effects are also observed in cardiac tissue. A clinical trial comparing the efficacy of losartan to the ACE-inhibitor lisinopril in DMD patients with impaired cardiac function (ejection fraction <55%) has been conducted, but not yet published (NCT01982695).

## 2.9 Insulin-like growth factor 1 stimulation

Other ways to influence myogenic regeneration and differentiation are via insulin-like growth factor 1 (IGF-1) signalling. IGF-1 is a growth factor that plays a role in the maintenance of muscle mass and muscle growth. It stimulates satellite cell proliferation and differentiation during muscle regeneration via activating the PI3K/Akt/mTOR pathway, which stimulates protein synthesis and blocks apoptosis and via the Raf/MEK/ERK pathway, which increase cell growth and differentiation (fig. 2.5).[574,576,577] Therefore compounds that increase IGF-1 signalling may promote muscle hypertrophy and increase regeneration in muscle wasting disorders like DMD.

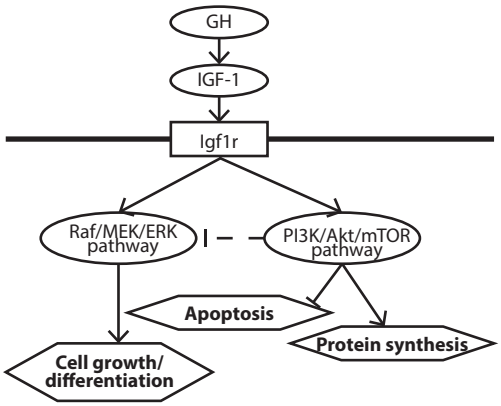


**Fig. 2.5:** Simplified scheme of the IGF-1 signalling pathway
Growth hormone (GH) stimulates IGF-1 production, which binds to the Igf1 receptor, thereby activating the PI3K/Akt/mTOR pathway to stimulate protein synthesis/block apoptosis and the Raf/MEK/ERK pathway to increase cell growth/differentiation.

Various IGF-1 analogues have been tested in *mdx* mice. A recombinant human IGF-1 improved contractile function of the diaphragm and fatigue resistance of hind limb muscles.[578,579] Whereas IGF-1 overexpression causes hypertrophy at a high dose (for example by transgenic overexpression in *mdx*/*Mglf*$^{+/+}$ mice),[580] at a low dose (1.0-1.5 mg/kg/day) it causes a shift of muscle fibre type towards a more oxidative and fatigue-resistance type, without changing the muscle mass. Thereby it reduces functional muscle deficits in *mdx* mice. It is known that in DMD patients fast-twitch muscles are more susceptible to damage than slow-twitch muscle.[581] A compound that releases IGF-1 from its binding peptides (IGF-1 aptamer; NBI-31772), thereby increasing the level of free IGF-1, also protected leg muscle and diaphragm to contraction-induced injury by shifting towards a slower muscle type when administered via continuous infusion (6 mg/kg/day) to *mdx* mice. However it increased muscle fatigability during repeated maximal contractions, but these are unlikely to be made by patients in practice.[582] An improved IGF-1 variant (PEG-IGF-1) showed that the benefit of treatment depended on the severity of pathology. In young (mildly affected) *mdx* mice it indeed protected skeletal and diaphragm muscles; yet these effects were less pronounced in more severely affected older *mdx* mice and absent in very severely affected *mdx*/*Utrn*$^{-/-}$ mice.[583] In a randomized clinical trial in DMD and BMD patients growth hormone, which stimulates IGF-1 production, induced a slight improvement in systolic heart function after three months, but no changes in skeletal muscle function.[584] A longer study to test the safety and efficacy of IGF-1 in DMD patients is currently ongoing (NCT01207908).

## 2.10 Anabolic agents

In contrast to catabolic agents, like corticosteroids, which reduce muscle fibre size, anabolic agents aim to increase fibre size. The rationale behind the first approach is that smaller fibres might be less susceptible to contraction-induced injury, thereby reducing the degeneration/regeneration cycles, whereas the idea behind the second approach is that increasing fibre size will increase muscle strength and enhance muscle repair. However, the latter might also make the muscles more susceptible to injury, thereby accelerating degeneration.[585]

B-adrenoreceptor agonists ($\beta$-agonists) are hormone-like substances that bind to the $\beta$-adrenoreceptors on the cell membrane. These receptors play a regulatory role in cardiovascular, respiratory, metabolic and reproductive functions. Skeletal muscles contain mainly $\beta_2$-adrenoreceptors. B-agonists bind to these receptors and activate a cAMP-protein kinase A signalling pathway, which is involved in protein synthesis and protein degradation. Via this pathway they have anabolic effects on skeletal muscles and can increase muscle growth and repair after injury.[586]

$B_2$-agonists like clenbuterol and albuterol were shown to have anabolic properties in *mdx* mice by increasing skeletal muscle mass.[587,588] Results on muscle histology and function are less consistent. It decreased muscle degeneration in both younger (20 weeks old) and old (87 weeks old) *mdx* mice and also prevented exercise-induced fibrosis after ten weeks of treatment.[588] However, 20 weeks treatment of six months old mice had no impact on force production.[587] Importantly no improvement of diaphragm muscle, either on fibrosis or force production, could be observed.[589]

Another $\beta$-agonist, albuterol, has been tested in clinical trials with DMD and BMD patients. In a pilot study 12 weeks of treatment with 8 mg/day showed only a small increase in muscle strength in DMD or BMD patients, without side effects.[590] In a longer double-blinded, placebo-control trial where patients received 12 weeks of albuterol treatment and 12 weeks of placebo treatment with 12 weeks in between, or the other way around, also mainly an increase in muscle mass was seen, which might be accompanied by an improvement in functional performance, but no increase in strength of several muscle groups.[591] The same was observed for clenbuterol in trials for facioscapulohumeral dystrophy, where mainly increases in muscle mass were observed, but only moderate or no improvements in muscle strength or function.[592-594] A small study in a few adult muscular dystrophy patients, among which one Becker patients, suggested that clenbuterol might be beneficial in early stages of disease, since it could improve better preserved muscles, but had no effect on more atrophic muscles.[595]

Clenbuterol and albuterol are older generation $\beta$-agonists. Formoterol is a new generation, more powerful $\beta_2$-agonist, which is already used for other diseases. In ten weeks old *mdx* mice, after four weeks of daily treatment with a low dose, it increased muscle mass, diameter and force producing capacity in peripheral muscle, without increasing muscle fatigue. Higher doses have generally shown to increase muscle fatigue. However also formoterol could not improve diaphragm muscle function.[596] A concern could be that increasing muscle size might increase their susceptibility to contraction-induced injury, since it has been observed that large, fast-twitch type II fibres, the most affected fibre type in DMD, are especially vulnerable to lengthening contractions. However, on the contrary, inducing muscle hypertrophy in these type of fibres by formoterol treatment in *mdx* mice did improve their force producing capacity and made them less susceptible to contraction-induced injury.[597]

A problem with the use of $\beta$-agonists can be that prolonged treatment leads to a downregulation of $\beta$-receptors on the muscle cell membrane and chronic administration is toxic for the heart and causes muscle tremor.[586] Since $\beta_1$-adrenoreceptors predominate in the heart, this might be prevented by the use of a highly selective $\beta_2$-adrenoreceptor agonists, like formoterol. Unfortunately these still lead to mitochondrial dysfunction, as reflected by a decrease in mitochondrial protein synthesis and oxidative capacity after chronic treatment of rats or mice, by reducing SR $Ca^{2+}$-ATPase activity in the heart and impairing cardiac relaxation.[598,599]

Anabolic androgenic steroids (AAS) are derivatives of testosterone. They have both anabolic (increasing protein synthesis and muscle growth) and androgenic (stimulating primary and secondary sexual development in males) effects. They are well known for their (ab)use by athletes because of (supposed) effects on increasing muscle mass and strength. Their anabolic effects occur via both direct and indirect mechanisms. Directly, they bind to the androgen receptor, thereby stimulating MHC protein synthesis. Indirectly, they competitively bind to glucocorticosteroid receptors, thereby blocking those signalling pathways and decreasing protein catabolism. Furthermore testosterone has shown in men to increase IGF-1 expression thereby increasing muscle protein synthesis.[600]

The AAS oxandrolone, an oral synthetic analogue of testosterone, has been tested in clinical trials in DMD patients. In a first pilot experiment an increase in overall functional muscle score was seen after three months of treatment.[601] Thereafter a larger randomized, double-blinded, placebo-controlled trial for six months was performed, with moderate results. Although some quantitative muscle tests showed an improvement, overall there was no significant improvement in muscle strength compared to the controls. Only, it may be that the oxandrolone-treated patients did not get worse, whereas the controls showed deterioration, but this was not significant.[602] A subsequent study on the mechanisms behind a possible muscle strength increasing effect of oxandrolone, showed, after three months of treatment, an increase in synthesis of MHC proteins and gene expression data suggested a decrease in muscle regeneration.[603]

Although in the oxandrolone trials no negative effects on muscle were observed or possibly even positive effects, an earlier study with another AAS, nandrolone decanoate, in *mdx* mice showed worsening of pathology. Three weeks treatment of young mice resulted in an increase in myofibre damage and CK levels.[604] The dose used in these mice (1.5 mg/kg/day) was comparable to those used in the clinical trials (0.1 mg/kg/day) after applying a correction factor based on normalisation to body surface area when translating doses between small and larger animals (1.5 mg/kg in mice≡0.12 mg/kg in humans).[605]

A possible explanation for the varying results with AAS could be due to the effects they exert on non-muscle tissues. Selective androgen receptor modulators such as GLPG0492 are non-steroidal hormones that are more selective for skeletal muscle and bone. In a comparative trial for four weeks in exercised *mdx* a high dose (30 mg/kg, 6 days/wk) resulted in increased fatigue resistance, diaphragm force and less fibrosis, whereas nandrolone (5 mg/kg) and α-methylprednisolone (1 mg/kg) only had a positive effects on some of these functions. No negative effects were observed with nandrolone. In a 12-weeks study the effect of GLPG0492 was maintained, also with lower doses (3 mg/kg or 0.3 mg/kg).[606]

Estrogens are mostly seen as female sex hormones, but skeletal muscles are a major source of estrogen production in both men and women. In muscle, estrogens can increase force output and protect them against injury. Tamoxifen is a selective estrogen receptor modulator with

anti-estrogenic effects that is used in the treatment of breast cancer. It has numerous actions, including scavenging of free radicals, inhibiting fibrosis and acting on $Ca^{2+}$-homeostasis; all playing a role in DMD pathology. Oral treatment of *mdx*$^{5cv}$ mice resulted in a large amelioration of dystrophic pathology, reflected by an increase in force production and histology. Importantly, also diaphragm and cardiac fibrosis was decreased.[607,608]

## 2.11 HDAC-inhibitors

Histone deacetylases (HDACs) play a role in the control of signalling networks involved in among others muscle growth, de- and regeneration. NO signalling regulates activity of HDAC2, a class I HDAC, by S-nytrosylation, which inhibits HDAC2-mediated gene repression, among which the myostatin inhibitor, follistatin. NO is produced by nNOS, which activity is disturbed in DMD (see paragraph 1.2.2), consequently HDAC signalling is also likely to be deregulated. Therefore HDAC-inhibitors (small molecule drugs) may be beneficial to DMD patients, since they can promote muscle hypertrophy and regeneration.[609]

The HDAC-inhibitors trichostatin A, valproic acid and phenyl butyrate decreased CK levels in 12 weeks old *mdx* mice. Trichostatin A was shown to be most efficient, also in inducing myotube formation and increasing regeneration marker expression in cells derived from treated mice. Furthermore increased mortality was seen amongst valproic acid- and phenyl butyrate-treated animals, whereas no side effects were observed due to trichostatin A treatment. Trichostatin A treatment resulted in an improved muscle cell integrity and recovery to exercise performance by increasing myofibre size, mediated by an upregulation of follistatin, reducing fibrosis and inflammatory cell infiltrates.[610]

Another HDAC-inhibitor, givinostat, was tested long term (3.5 months) in six weeks old *mdx* mice, leading to improvement in muscle formation, histology and function.[611] Givinostat is currently tested in DMD patients in an open-label phase I/II study (NCT01761292). First the safety and tolerability of escalating doses will be assessed. If this is well tolerated, patients will be treated for a year to determine the effects on histology and functionality.

As described in paragraph 1.2.2, in addition to the DGC, the $\alpha_7\beta_1$-integrin complex links laminin in the ECM to the actin cytoskeleton. After observing that transgenic overexpression of the $\alpha_7$BX2 chain extended the life span of *mdx*/*Utrn*$^{-/-}$ mice by threefold and ameliorated the pathophysiology,[612] Gurpur *et al.* searched for compounds that could increase $\alpha_7$-integrin levels. Hereby they also identified valproic acid, an FDA-approved branched chain fatty acid. Next to HDAC-inhibiting activity, this is also an activator of Akt. *In vitro* valproic acid increased $\alpha_7$-integrin thereby increasing hypertrophy and decreasing apoptosis via activating the Akt/mTOR/p70S6K pathway. Five weeks treatment of three months old *mdx*/*Utrn*$^{-/-}$ mice resulted in decreased fibrosis and inflammation and improvement of hind limb contractures. Detailed analysis showed an increased expression of Akt and a decreased expression of ERK. Here no increased mortality was reported although the used dosage (240 mg/kg twice daily) was higher than in the previous study by Minetti *et al.* (160 mg/kg/day).[613] In myotubes these pathways work antagonistic, whereby the Raf/MEK/ERK pathway promotes differentiation, thereby reducing hypertrophy, and the PI3K/Akt/mTOR pathway inhibits differentiation, thereby inducing hypertrophy. Both pathways are affected by valproic acid. Furthermore Akt can inhibit the Raf/MEK/ERK pathway in differentiated myoblasts, but not in their myoblast precursors.[614] Valproic acid was able to activate Akt by $\alpha_7$-integrin independently, since, in

contrast to the cultured muscle cells, no change in $\alpha_7$-integrin levels was observed in these mice. This discrepancy is probably due to the short serum half-life of valproic acid, therefore dosing was not optimal to have an effect on $\alpha_7$-integrin.[613]

In an *in vitro* reporter gene assay using HEK293 cells, a cell line derived from human embryonic kidneys, these HDAC-inhibitors, trichostatin A and valproic acid, were shown to enhance exon skipping by several 2OMePS AONs. A small pilot study by us in cultured human control myoblasts, did not show differences in AON-induced exon skip efficiency between valproic acid treated cells and control cells [Verhaart *et al.*, unpublished results].

## 2.12 Improvement of calcium homeostasis

As mentioned before $Ca^{2+}$-homeostasis is disturbed in DMD. Controversy exists whether this is a secondary, passive process due to the increased membrane permeability or a direct effect of defects in $Ca^{2+}$-regulating mechanisms.[615] Both hypotheses do not have to be mutually exclusive. The absence of dystrophin and thereby disruption of the DGC leads to damage of the sarcolemma, increasing its permeability. Hereby the influx of calcium is increased. At first, compensatory mechanisms can account for this increase influx, but eventually this capacity is exhausted and $Ca^{2+}$-homeostasis is lost. The high $Ca^{2+}$-concentration leads to activation of calpains ($Ca^{2+}$-dependent proteases), causing cell and membrane proteolysis, in turn increasing membrane damage and $Ca^{2+}$-influx, eventually leading to cell death.[616] On the other hand, it might also be a direct effect of a higher activity of $Ca^{2+}$-channels and defects in proteins involved in the removal of intracellular calcium.

Stretch-activated channels for non-specific cations ($SAC_{NSC}$) allow the entry of calcium and sodium into cells under normal conditions. Studies have shown that these channels are more active in *mdx* mice, where both resting and stretch-induced intracellular $Ca^{2+}$-concentrations are elevated compared to wild type mice. This was prevented by blocking of these $SAC_{NSC}$, which also decreased membrane permeability, suggesting this to be a secondary effect of increased $Ca^{2+}$-influx. As described above oxidative stress occurs in DMD and ROS are increased. Studies suggest that ROS contribute to the activation of kinases that open these $Ca^{2+}$-channels.[617] Next to $SAC_{NSC}$, also others channels have shown to be deregulated in DMD and/or *mdx* muscle fibres. TRPV2, a cationic channel with mechanosensitivity, is present in elevated levels at the sarcolemma of dystrophin-deficient muscle fibres. Transgenic expression of a dominant-negative TRPV2 mutant in *mdx* mice improved muscle histology and performance.[618] Furthermore, store-operated calcium entry (SOCE) is increased, which is influenced by the $Ca^{2+}$/PLC/PKC pathway and regulated by the scaffolding protein α1-syntrophin, which is associated with the DGC.[619]

Next to an increase in $Ca^{2+}$-influx, an impaired removal might also contribute to the elevated intracellular levels. A protein involved in this process, sarcoplasmic/endoplasmic reticulum $Ca^{2+}$-ATPase was found to be dysfunctional in *mdx* and *mdx*/*Utrn*$^{-/-}$ mice.[620] In addition to the activation of proteases, the disturbed $Ca^{2+}$-homeostasis also contributes to the pathophysiology by increasing mitochondrial permeability, causing swelling of the mitochondria and downregulation of mitochondrial genes (mitochondrial permeability transition). Over time this causes rupture of the mitochondria, resulting in a metabolic crisis and contributing to apoptosis/necrosis of the cells. These processes might be regulated by cyclophilin D, a mitochondrial matrix prolyl cis-trans isomerase, encoded by the gene *Pfif*. The *Scgd*$^{-/-}$ mouse (lacking $\delta$-sarcoglycan) is a model for severe dystrophy in skeletal mus-

cle and heart. Knockout of the *Pfif* gene in these mice prevents mitochondrial swelling and thereby myofibre necrosis.[621] In addition, $Ca^{2+}$-dependent signalling transduction pathways are stimulated, which, through negative feedback, lead to a downregulation of these pathways, thereby contributing to the metabolic crisis.[622]

Pentoxifylline, the 1-5-oxohexyl analogue of the methylxanthine theobromine, is a compound with anti-inflammatory, anti-oxidant properties and effects on $Ca^{2+}$-homeostasis. It inhibits human dermal fibroblast proliferation and synthesis of, among others, collagen by these cells by selectively blocking the induction of collagen synthesis by TNF-α.[623,624] Furthermore it increases cAMP levels by inhibiting phosphodiesterase, thereby reducing the activity of $Ca^{2+}$-channels, which improves $Ca^{2+}$-homeostasis.[625] Studies of effects of pentoxifylline in *mdx* show mixed results. Pentoxifylline treatment (50-100 mg/kg) resulted in an improvement of muscle histology and muscle strength.[401,625] Another study showed a small delaying effect of pentoxifylline (150 mg/kg) on necrosis and increased mechanical function and resistance to fatigue of skeletal muscle.[439] However, Gosselin *et al.* reported that a lower dose of pentoxifylline (16 mg/kg) had no effect on collagen in the diaphragm muscle and it also did not improve its contractile function.[626] Subsequent clinical trials in DMD patients showed disappointing results. In an open-label safety study, pentoxifylline treatment for one year via an orally administered immediately release formulation was so poorly tolerated (65% of the patients experienced intolerable gastrointestinal side effects) that half of the patients withdrew from the trial and no conclusions of its efficacy could be made.[627] To circumvent these gastrointestinal side effects a subsequent randomized, double-blinded trial used a slow-release formulation. Yet, mild to moderate gastrointestinal and hematologic adverse events were still observed. In addition, no improvement or prevention of deterioration of muscle strength or function was found.[628] Importantly an *in vitro* assay on the contractile function of dystrophic myoblast showed a complete inhibition by pentoxifylline on the positive effect seen after treatment with prednisolone and creatine, highlighting the risk of deleterious interactions of cocktails of medicines used by some patients.[477]

BGP-15 (an [*O*-(3-piperidino-2-hydroxy-1-propyl)-nicotinic acid] amidoxime derivative), a compound that increases the expression of Hsp72, was able to preserve muscle strength and decrease fibrosis in both mild *mdx* mice and severely affected *mdx*/*Utrn*$^{-/-}$ mice. Hsp72 is a protein that inhibits pro-inflammatory cytokines like TNF-α and the NF-κB pathway. However the beneficial effects of BGP-15 were likely mediated by improvement in $Ca^{2+}$-homeostasis and not by a decrease in inflammation, since no differences in these inflammatory markers between *mdx* mice overexpressing Hsp72 (*mdx*$^{TG(+)}$) and normal *mdx* mice were found. Further analysis showed that sarcoplasmic/endoplasmic reticulum $Ca^{2+}$-ATPase activity was increased in these *mdx*$^{TG(+)}$ mice and that BGP-15 also stimulated its activity. BGP-15 is already used in clinical trials for diabetes.[620]

The L-type voltage-dependent $Ca^{2+}$-channel blockers verapamil and diltiazem were able to significantly decrease the $Ca^{2+}$-levels in heart muscle of *mdx* mice and there was a trend observed in the diaphragm. Degeneration of the diaphragm was decreased and thereby the dystrophic phenotype improved. Both drugs have a cardiovascular-targeted effect, so mainly act on the heart and the diaphragm. They did not decrease the $Ca^{2+}$-content of the skeletal muscle or even increased it.[629] A double-blinded clinical trial showed that, although after one year of treatment with diltiazem the number of $Ca^{2+}$-positive fibres was lower in the treated group versus placebo, after three years no obvious clinical benefits were observed.[630] Chronic treatment in another double-blinded trial suggested small beneficial effects by improving

some skeletal muscle functions and cardiac parameters, although all results were not significant.[631] Overall, several trials with these and related $Ca^{2+}$-channel blockers showed no beneficial effect in DMD.[632]

In *mdx* mice a structural and functional defect in RyR1, a sarcoplasmic reticulum $Ca^{2+}$-release channel, has been found too, which leads to 'leaky' channels.[59] NO-mediated hypernitrosylation of this channel has also been found in BMD patients with deletions in the nNOS binding region (exons 42 to 45), which resulted in increased cytosolic NO production, thereby causing disturbance of the RyR1/calsatabin-1 complex, important for $Ca^{2+}$-signalling. This is probably also the case in DMD, since there also nNOS is mislocalised in and NO-production disturbed.[633] Furthermore activation of RyRs has been shown to be able to evoke SOCE.[619,634] S107, a compound that inhibits these RyR1 channels, prevented $Ca^{2+}$-leak and thereby improved muscle function and exercise performance in *mdx* mice.[59]

Debio-025 is a drug that inhibits cyclophilin D, which is probably involved in mitochondrial dependent necrosis of myofibres. Subcutaneous treatment of both *mdx* and *Scgd*$^{-/-}$ mice (50 mg/kg) with debio-025 reduced swelling of the mitochondria and reduced fibrosis in the skeletal muscle and the diaphragm. Another cyclophilin D inhibitor is cyclosporine. However, cyclosporine is not selective for cyclophilins, but also inhibits calcineurin. Calcineurin is an important signalling protein for skeletal muscle regeneration after injury and differentiation of skeletal muscle cells and also increases the expression of utrophin (which can functionally compensate for loss of dystrophin). Thus, cyclosporine will have both a positive and a negative effect, which are likely to negate each other. By contrast, debio-025 has no inhibitory effect on calcineurin activity and is more potent in inhibiting cyclophilin D than cyclosporine.[621]

Skeletal muscle primarily expresses three isoforms of calpain called μ-calpain (calpain 1), m-calpain (calpain 2) and p94 (calpain 3). As calpain activity is increased in dystrophic muscle, calpain inhibitors may be beneficial. On the other hand, mutations in calpain 3 are the cause of LGMD type 2A (LGMD2A), indicating its important role in normal muscle functioning.[635] Therefore caution might also be required with this approach. Experiments with several calpain inhibitors showed mixed results. Calpastatin is an endogenous specific inhibitor of μ- and m-calpains. Transgenic overexpression of calpastatin in *mdx* mice resulted in decreased proteolysis by calpains. Histological benefit was seen or not, depending on which promoter was used.[636,637] Leupeptin (*n*-acetyl-L-leucyl-L-leucyl-L-argininal) is a non-specific calpain inhibitor. A short term study with intramuscular injections in *mdx* mice, showed improvement of muscle histology, reflected by decreased muscle degeneration, less centrally located nuclei and increased myofibre diameter.[638] In contrast, long term systemic treatment (six months) showed no improvements. A novel, more specific compound in which the inhibitory portion of leupeptin is linked to carnitine to improve muscle uptake (C101), also failed to improve muscle function and histology in both *mdx* mice and GRMD dogs. This is probably due to compensatory mechanisms, whereas activation of endogenous m-calpain and potentially also μ-calpain is increased in the presence of an exogenous calpain inhibitor.[608,639]

BN 82270 is a membrane-permeable prodrug of a chimeric compound BN 82204 that acts dually, both as a calpain-inhibitor and anti-oxidant, thereby targeting both damage due to disrupted $Ca^{2+}$-homeostasis and ROS-induced damage. In *mdx* mice it prevented calpain-overactivity in the diaphragm, thereby reducing fibrosis. Longer treatment (4-6 weeks) resulted in improved muscle function and lowering of TGF-$\beta_1$ levels in skeletal muscle and diaphragm. However, almost no improvement in histopathology of the gastrocnemius was observed.[640]

## 2.13 Matrix metalloproteinases

MMPs are zinc-dependent endopeptidases that play an important role in ECM degradation, inflammation and fibrosis in various pathologies. Differential expression of several MMPs has been found in DMD as well. MMP-2 and MMP-9 are downstream targets of NF-κB, which cleave the extracellular domain of *β*-dystroglycan, thereby disrupting the interaction with *α*-dystroglycan. An upregulation of MMP-2 and the natural inhibitors of MMPs, TIMP-1 and TIMP-2, was found in DMD muscle.[641] The same accounts for MMP-9, the expression of which has been shown to correlate with disease progression, *i.e.* to be higher in older DMD patients and to increase over time.[642] In *mdx* mice also an upregulation of MMPs was found, but a downregulation of TIMPs.[643] Inhibition of MMP-9 in *mdx* mice has shown to have a beneficial effect by decreasing inflammation and fibrosis. These effects are mediated by inhibition of both the NF-κB pathway and the caveolin-3 pathway.[644]

Since inhibition of MMP-9 has shown to have therapeutic effects in *mdx* mice and other MMPs that play a role in among others fibrosis, are upregulated in DMD as well, they might also serve as a therapeutic target.

Batimastat (BB-94) is an inhibitor of a broad spectrum of MMPs, including MMP-1, MMP-2 and MMP-9 and has reported to be effective in cancer models. Short term treatment of *mdx* mice resulted in a decrease in necrosis and inflammation, leading to an improved muscle function. Furthermore an improvement in components of the DGC and nNOS levels was seen.[643]

Different MMPs often activate each other, however the exact roles of different MMPs in skeletal muscle are not known. Furthermore, individual MMPs have shown to play different roles depending on the disease stage, therefore inhibition of MMPs can both be advantageous as well as disadvantageous. Although MMP-2 was shown to be upregulated in DMD, genetic knockout of MMP-2 in *mdx* mice (*mdx*/*MMP-2*$^{-/-}$) worsened pathology by impairing the growth of regenerating muscle fibres through reduction of VEGF-mediated angiogenesis.[645] In addition, although MMP-inhibitors as batimastat and marimastat have been found to be beneficial in several types of cancers and are used in cancer clinical trials, no successful results have so far been shown in these trials due to side effects. Therefore global inhibition of MMPs could be deleterious on the long term and targeting of specific MMPs during disease progression will probably be necessary to have a potential therapeutic effect.[643]

## 2.14 Autophagy

A more recently emerging target is autophagy. Autophagy is an important process for clearing dysfunctional organelles, *e.g.* defective mitochondria, augmenting energy production and preventing tissue damage. Autophagy was shown to be dysfunctional in DMD patient biopsies and *mdx* muscle. A persistent activation of Akt inhibits autophagy via mTOR and its downstream targets, like ribosomal protein S6 and 4E-BP1 (fig. 2.6). mTOR inhibition by itself has been shown to be insufficient to rescue autophagosome formation, indicating also other (pro-autophagy) pathways are involved. In DMD and *mdx* a decreased expression of its downstream targets LC3 II and increase in p62 (LC3 II reduces p62 expression) was observed.[646] pAkt inhibits the pro-autophagy FOXO3 pathway.[647] FOXO3 stimulates autophagy by both increasing LC3 and inhibiting mTOR.[648]

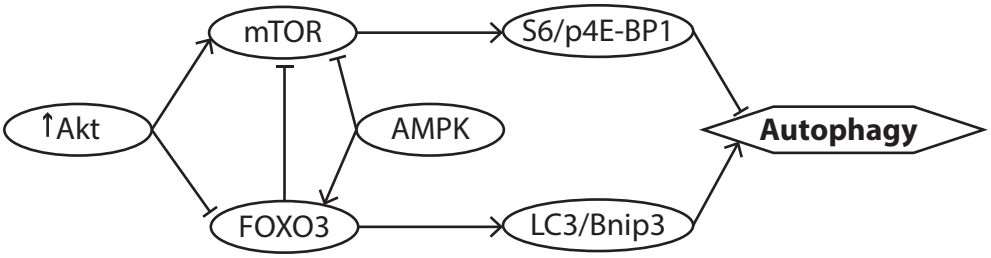


**Fig. 2.6:** Autophagy
Chronic activation of Akt in Duchenne muscular dystrophy inhibits autophagy by activating the S6/p4E-BP1 pathway through mTOR, which in turn inhibits autophagy, and by blocking FOXO3-mediated LC3/Bnip3 signalling. AMPK can switch on the autophagic pathway by inhibiting mTOR and stimulating FOXO3.

AMP-activated protein kinase (AMPK) is a major metabolic sensor of the energy status inside cells and can switch on the autophagy pathway. The AMPK agonist AICAR (5-aminoimidazole-4-carboxamide-1-$\beta$-D-ribofuranoside) was a potent trigger of autophagy in *mdx* diaphragm and caused improvement of mitochondrial function, thereby ameliorating histology and function.[649]

In *mdx* mice a long term low-protein diet, a potent autophagy-reactivating treatment, resulted in a decrease in pAkt/mTOR and an increase in LC3 II expression, accompanied by a decrease in p62. This reduced inflammation, fibrosis and myofibre damage and improved functionality.[646]

## 2.15 Treatment of cardiomyopathy

In most DMD patients cardiomyopathy develops at latter stages of disease. Signs of cardiomyopathy include left ventricular dilation, decreased ejection fraction and increased plasma levels of neuroendocrines. Neuroendocrines (*e.g.* atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and norepinephrine) are cardiac hormones thought to be secreted in response to increasing atrial and ventricular pressure. Cardiomyopathy is nowadays frequently the cause of death, especially since the introduction of assisted ventilation. Therefore treatment of cardiomyopathy is becoming more and more important; however most therapies described above do not or only modestly improve cardiac pathology and function. Several treatments used in cardiomyopathies with other causes, might also be effective in DMD and some of these have been tested in dystrophic models or DMD patients. ACE-inhibitors have already been discussed (see paragraph 2.8). Many patients are nowadays treated with ACE-inhibitors, often combined with $\beta$-blockers, when signs of cardiomyopathy start to develop.[565]

In the RA(A)S system (see paragraph 2.8), next to binding to and signalling via the AT receptors, Angiotensin II also stimulates the synthesis of the mineral corticoid aldosterone and emerging data indicate that aldosterone plays an independent role in vascular toxicity and fibrosis. Therefore aldosterone antagonists are widely used and have shown effectiveness in the treatment of cardiomyopathies. Aldosterone binds to aldosterone receptors (*e.g.* mineral corticoid receptors) and sexual hormone receptors. Activation of these mineral corticoid receptors by aldosterone and cortisol plays a role in patients with cardiovascular disease and addition of an aldosterone antagonist to ACE-inhibitor treatment has shown to have an

additional benefit on lowering mortality.[650,651] As described above the competitive antagonist of the aldosterone receptor spironolactone, a diuretic and antihypertensive agent, has been tested in *mdx* mice. However, since this was only in combination with lisinopril, no conclusions can be drawn about the effect of spironolactone itself.[567] Eplerenone, a spironolactone derivative, is a more selective compound, with a higher affinity for mineral corticoid receptors and lower for sexual hormone receptors, thereby reducing the risk of side effects. It has so far only been tested in DMD for treatment of muscle oedema, which is caused by an increase in cytoplasmic $Na^+$-content and probably contributes to muscle degeneration. In a small study, eplerenone decreased $Na^+$-concentration in muscles of DMD patients, thereby decreasing oedema and improving muscle strength.[652] Another pilot in one female DMD patient showed the same result of decreasing cytoplasmic sodium and water overload and increasing muscle strength and mobility.[653] A clinical trial to assess the effects of early treatment with eplerenone on cardiomyopathy in DMD patients is currently ongoing (NCT01521546). Eplerenone is thought to have fewer side effects than older aldosterone antagonists, *e.g.* spironolactone and its active metabolite canrenone, due to higher specificity for mineral corticoid receptors. However, a meta-analysis of randomized controlled trials with aldosterone antagonists in systolic heart failure concluded less reduction in mortality for eplerenone compared to spironolactone and canrenone. In addition, it did not have a better side effects profile and is much more expensive.[650]

B-blockers bind to $\beta$-adrenergic receptors to inhibit sympathetic effects of binding of (nor)epinephrine to these receptors, thereby reducing the work load on the heart. They are widely used in the treatment of cardiac dysfunction and hypertension. First generation $\beta$-adrenoreceptor antagonists are non-selective for $\beta_1$- and $\beta_2$-adrenergic receptors; whereas second generation $\beta$-blockers are relatively selective for $\beta_1$-adrenergic receptors, the predominant receptor in the heart.[8] Carvedilol (racemic lipophilic aryloxypropanolamine) causes vasodilatation by non-selective blockage of $\beta$-adrenoreceptors and $\alpha_1$-adrenoreceptors. It has proven therapeutic value as an adjunctive therapy in combination with diuretics and/or ACE-inhibitors in the treatment of various types of heart failure.[654] A small study with carvedilol for six months in four DMD patients with reduced ejection fraction, did not show an improvement compared to controls.[655] In contrast, in a study in 22 muscular dystrophy (DMD or BMD) patients with dilated cardiomyopathy, carvedilol treatment was safe and resulted after six months in a modest improvement in systolic and diastolic functions. Another open trial where 41 patients received carvedilol versus 13 untreated patients, an improved survival rate was observed when solely looked at cardiac failure as cause of death, but not when looked at all-cause mortality. Furthermore no changes in LVEF or BNP plasma levels were observed. In addition, patients with a very low ejection fraction were also treated with an ACE-inhibitor or pimobendan (a PDE3-inhibitor).[656] A randomized, double-blinded, placebo-controlled clinical trial assessing the effect of nebivolol, a $\beta_1$-blocker, on the prevention of systolic dysfunction in DMD patients is currently ongoing (NCT01648634). Furthermore two phase IV clinical trials with carvedilol, one with carvedilol (NCT00606775) only and one (NCT00819845) comparing it with the ACE-inhibitor ramipril, should have been performed; however no results are published and their status is unknown.

As described before, combinational treatment with an ACE-inhibitor and a $\beta$-blocker is often used for DMD-related cardiomyopathy. A study in 11 symptomatic patients suggested this combination might reduce left ventricular dilation, BNP and ANP levels and may reverse symptoms; however no control group existed.[14] In a study with several types of muscular dystrophy patients, among whom DMD patients, carvedilol plus an ACE-inhibitor improved

left ventricular systolic function, whereas ACE-inhibitors alone did not.[657] A larger retrospective study on DMD patients with heart failure treated with an ACE-inhibitor and $\beta$-blocker showed a beneficial effect on long term (over ten years) survival, which was most effective in asymptomatic patients with left ventricular dysfunction.[569] A recent study confirmed this beneficial effect of combinational therapy (ACE-inhibitor and $\beta$-blocker) since a significant improvement was seen in ejection fraction compared to before the start of treatment. This was also seen by ACE-inhibitor treatment alone and no additional value of the $\beta$-blocker was observed. However, this can be due to the delayed addition of $\beta$-blocker therapy.[570]

## 2.16 Summary

In this chapter various therapeutic strategies that do not aim to restore the expression of the dystrophin protein itself, have been discussed. These vary from genetic approaches to up- or downregulate the expression of other genes to pharmaceutical compounds acting on signalling pathways disturbed in DMD pathogenesis. At the moment the only widely used treatments are corticosteroids and, if signs of cardiomyopathy become apparent, ACE-inhibitors. Unfortunately, while corticosteroids have clear beneficial effects, *i.e.* prolonging ambulation, they also have numerous side effects.

Next to these many compounds have been identified showing positive results in animal models or *in vitro* model systems. Some of these have already been tested in clinical trials, but unfortunately often without very convincing results. One of the problems is that many compounds that seem promising *in vitro* in cells or *in vivo* in animal models often fail when tested in clinical trials or already when tested in (higher) animal models. Partly this is intrinsic to translational research. However, it is to underline the international efforts for more standardization of animal models and outcome measures made in the last years in the frame of activities of the international TREAT-NMD network.[658] This in order to improve predictability of data obtained at pre-clinical stage.[659] In addition, re-evaluation of failed clinical trials is important to design more appropriate pre-clinical testing methods and improve trial protocols in the future. This helps in understanding each other's results and improving model systems.

At the moment, one promising candidate is tadalafil, a PDE5A-inhibitor that is already on the market for other disorders and targets the NO-cGMP signalling pathway to enhance vasodilation. Results in a trial with BMD patients and in a pilot study with DMD patients were promising. Another promising strategy is to target the TGF-$\beta$ pathway. Hereby, inhibiting both TGF-$\beta$ and myostatin signalling, *i.e.* by AON-mediated exon skipping of their receptors seems a better strategy than targeting myostatin alone. This has only been tested preclinically, but it could be combined with *DMD* exon skipping to restore both dystrophin expression and enhance muscle formation. However, a concern with myostatin inhibition is to increase the work load on the heart, thereby increasing cardiomyopathy. This does not only account for myostatin inhibition, but for every therapy that mainly improves skeletal muscle function without targeting cardiomyopathy.

A problem will always be that most compounds only target one or a few of the secondary defects, while the underlying cause (lack of dystrophin) is not addressed. Therefore these will only temporarily alleviate the symptoms and should, once available, be combined with a therapy restoring or substituting the dystrophin protein, as is tested for myostatin/TGF-$\beta$ (receptor) inhibition.

# Dose-dependent pharmacokinetic profiles of 2'-*O*-methyl phosphorothioate antisense oligonucleotides in *mdx* mice

Ingrid E.C. Verhaart, Christa L. Tanganyika-de Winter, Tatyana G. Karnaoukh, Ingrid G.M. Kolfschoten, Sjef J. de Kimpe, Judith C.T. van Deutekom and Annemieke Aartsma-Rus

Previously published in: Nucleic Acid Ther; 2013: 23: 228-237.

Chapter 3

## Abstract

Antisense-mediated exon skipping is a promising therapeutic approach for Duchenne muscular dystrophy. It aims to restore the dystrophin open reading frame by skipping exons with antisense oligonucleotides (AONs) to allow production of partly functional proteins. The approach is currently tested in phase III clinical trials, but dosing and maintenance regimens have not yet been well studied.

This study compared pharmacokinetic and pharmacodynamic effects of different 2'-*O*-methyl phosphorothioate RNA AON dosing and maintenance regimens in the preclinical *mdx* mouse model. When comparing different dosing regimens over a period of eight weeks, higher levels of AON, exon skipping, and protein were observed in muscle after low daily doses compared with weekly large doses. Secondly, after receiving a high loading dose (1 250 mg/kg) in the first week, mice treated with maintenance injections twice weekly for eight weeks showed higher preservation of therapeutic effects than mice receiving less or no maintenance injections.

In both cases, the regimen resulting in highest AON and exon skipping levels in muscle, also resulted in high AON levels in liver and kidneys. These studies underline the importance of balancing optimal AON efficacy and tolerable levels in non-target organs, which may be fine-tuned by further optimisation of AON treatment regimens.

## Introduction

Duchenne muscular dystrophy (DMD) is a severe, progressive muscle-wasting disorder affecting around one in 5 000 newborn boys.[660] It is caused by mutations in the *DMD* gene, located on the X-chromosome, leading to a disruption of the open reading frame, thereby causing the complete absence of the encoded dystrophin protein. Dystrophin plays an important role in the stabilization of muscle fibres during contraction, by connecting the intracellular actin cytoskeleton to the extracellular matrix. In the absence of dystrophin, muscle fibres will be damaged with normal exercise, eventually leading to loss of muscle tissue and function and premature death in the third or fourth decade of life.[5]

The exon skipping approach aims to partly correct the underlying genetic defect on RNA level by restoring the dystrophin reading frame to allow production of a slightly shorter, but largely functional, dystrophin protein as is found in Becker muscular dystrophy (BMD), a much milder form of muscular dystrophy.[661] To achieve exon skipping, antisense oligonucleotides (AONs) are used. AONs are small pieces of RNA, reverse complementary to a specific sequence in the pre-messenger RNA (pre-mRNA). By binding to its target sequence in the pre-mRNA, the AON interferes with the splicing of a specific exon, thereby preventing its incorporation in the mRNA.[164] AONs can have different chemistries. For exon skipping the development with 2'-*O*-methyl phosphorothioate RNA (2OMePS) and phosphorodiamidate morpholino oligomers (PMO) or peptide-conjugated PMOs (pPMO) is most advanced.

Proof-of-principle for exon skipping-mediated restoration of dystrophin has first been shown *in vitro*, *i.e.* in DMD patient-derived myoblast cultures, and *in vivo* in dystrophic animal models (reviewed in Aartsma-Rus *et al.*[269]). The most widely used model for DMD is the *mdx* mouse (C57Bl/10ScSn-DMD$^{mdx}$/J). These mice have a premature stopcodon in exon 23, leading to the complete absence of dystrophin protein. Although these mice display a relatively mild phenotype compared to human DMD patients,[81,159] this model well facilitates preclinical pharmacokinetic (PK) and pharmacodynamic (PD) AON studies for DMD. Treatment of *mdx* mice with 2OMePS or PMO AONs targeting exon 23 showed dystrophin restoration and improved muscle function.[161,182,197,199,200] Exon skipping and dystrophin protein levels in heart were lower compared with skeletal muscles. This is probably due to differences in the nature of the dystrophin-negative muscle fibres and cardiomyocytes. In the absence of dystrophin, skeletal muscle fibres become leaky. AONs can migrate through these holes into the muscle fibers.[161,218] However, the heart is built up of individual cardiomyocytes, which do not become leaky, thereby making the targeting of the heart more difficult and requiring higher doses.[200,203]

2OMePS (PRO051/GSK2402968/drisapersen) and PMO (AVI-4658/eteplirsen) targeting human exon 51 have been tested in clinical trials. Dystrophin restoration has been observed after local treatment[225,226] as well as after systemic treatment.[229,231] Drisapersen resulted in dystrophin restoration in ten out of 12 patients up to 15.6% of levels in healthy controls after five weeks of weekly subcutaneous injections. For eteplirsen dystrophin was restored in seven out of nine patients. The three highest responders showed levels up to 18% of controls. Further dose optimisation studies are ongoing for eteplirsen. Drisapersen is currently tested in a large, randomized, double-blinded, placebo-controlled phase III clinical study.

It is known that 2OMePS AONs have a plasma half-life of ~4 weeks in patients[229] and a half-life of ~2-6 weeks in *mdx* mouse muscle.[200] Thus, due to clearance and turnover of the AONs, but also of the dystrophin transcript and protein, repeated, life-long AON treatment will be required. In the 2OMePS clinical trials currently a weekly dose of 6 mg/kg/wk is used. However, little is known about which dose and which dosing schedule is optimal. Dividing

the same dose over multiple smaller injections might give better results than applying the same total amount at once, as has been shown for PMOs in *mdx* mice.[239] Furthermore, it is known that dystrophin transcripts and proteins have a relatively long half-life.[206,660] Thus, after having initiated the restoration of dystrophin protein, injection with lower amounts might be sufficient to maintain the same effect. Studies to optimise dosing and maintenance regimens are difficult in humans, where only limited material is available (often only a biopsy from a superficial muscle and a small amount of blood plasma). Therefore, (DMD) animal models are a useful tool for PK/PD modelling studies. These can be used to assess the ratio between AON levels and the amount of exon skipping in muscles which can be used to extrapolate the results to the human situation.

The aim of this study was to compare different dosing and maintenance regimens through assessment of PK and PD profiles for different AON treatment regimens in *mdx* mice.

## Materials and methods

All experiments were approved by the local ethical committee for animal experiments of the LUMC (project code 07151). Mice were housed in individually ventilated cages in the animal facility of the LUMC and received food and drink *ad libitum*. *Mdx* mice (C57Bl/10ScSn-DMD$^{mdx}$/J) were obtained from our own breeding facility.

*Treatment of* mdx *mice with different 23AON dosing regimens*

Four-weeks old *mdx* mice (*n*=7 or 8 per group) were injected subcutaneously with 200 mg/kg body weight/week 23AON (3'-uccauucggcuccaaaccgg-5'; a 2'-*O*-methyl phosphorothioate AON described previously as M23D(+2-18)[197] (Prosensa Therapeutics), divided evenly over 1, 2, or 7 injections per week for 8 weeks (*i.e.* 1 weekly dose of 200 mg/kg versus 2 times 100 mg/kg versus 7 times 28.6 mg/kg). The RNA 23AON was produced in 3 gram-scale batches on an ÄKTA OP-100 oligonucleotide synthesizer (GE Healthcare) using standard phosphoramidite chemistry protocols. After synthesis, a two-step cleavage and deprotection procedure was applied; on-resin 23AON was first treated with diethylamine to remove phosphorothioate protecting groups and subsequently subjected to concentrated ammonia treatment for 16 hours at 55°C. Crude 23AON (DMT-off, *i.e.* without 4,4'-dimethoxytrityl group) was purified by IEX and transformed into its sodium salt by addition of NaCl and subsequent desalting by ultrafiltration. Negative mode electrospray ionization-mass spectrometers analysis confirmed the identity of 23AON (MW 6888) and purity (ultra-high performance liquid chromatography) of all batches was found to be acceptable (>84%). Mice were sacrificed by cervical dislocation 1 week after the last injection for their group and muscles (gastrocnemius, quadriceps, tibialis anterior, triceps, diaphragm, and heart) and organs (liver, kidney, and spleen) were isolated. Muscles were snap frozen in liquid nitrogen-cooled 2-methylbutane, and all tissues were stored at -80°C.

*Treatment of* mdx *mice with different maintenance doses of 23AON*

In the first week 4-weeks old *mdx* mice were treated subcutaneously with a loading dose of 5 times (*i.e.* daily for 5 days) 250 mg/kg body weight 23AON. This was followed by treatment with different maintenance regimens for 8 weeks: weekly 2 times 100 mg/kg (*n*=6), weekly 100 mg/kg (*n*=6), 100 mg/kg biweekly (*n*=5), 100 mg/kg monthly (*n*=3), or no further injections (*n*=4). Mice were sacrificed in week 8 by cervical dislocation, and muscles

(gastrocnemius, quadriceps, tibialis anterior, triceps, diaphragm, and heart) and organs (liver, kidney, and spleen) were isolated. Muscles were snap frozen in liquid nitrogen-cooled 2-methylbutane and all tissues were stored at -80°C.

*Plasma sampling*

Blood samples were taken weekly during treatment to assess creatine kinase (CK) levels. Blood samples were taken via the tail vein and were centrifuged at 18 000 *g* for 5 minutes at 4°C to generate plasma. This was diluted 10 times in Dulbecco's phosphate-buffered saline (Invitrogen) and CK levels were measured with a Reflotron system (Roche Diagnostics) with CK-strips (Roche). For the dosing experiments additional blood samples were taken at several time points after the first injection in the first (week 0) and the last (week 7) week of treatment (0, 15 minutes, 1 hour, 5 hours, and 24 hours from 4 animals per group). Finally, for all mice, a larger blood sample was taken prior to sacrifice to determine AON levels by enzyme-linked immunosorbent assay.

*Functional testing*

Mice were functionally tested weekly by forelimb grip strength and/or rotarod analysis. A grip strength meter (Columbus Instruments) was used to assess the forelimb grip strength according to the standardized operating procedure published on the translational research in Europe to accelerate treatments for neuromuscular disorders website.[663] Mice were tested 15 times (5 rounds of 3 consecutive measurements with 2 minutes in between). The forelimb grip strength corrected for body weight was calculated by dividing the average of the 3 highest values (the absolute strength) by the body weight in grams. For rotarod analysis mice were placed on a rotarod (Ugo Basile) accelerating from 5 to 45 rotations per minute in the first 15 seconds. The longest running time until the mouse fell off in 3 trials was notated, with a maximum of 500 seconds.

*RNA extraction and analysis of exon skipping by reverse transcriptase polymerase chain reaction*

Muscles were minced in TriPure isolation reagent (Roche) using MagNA Lyser green beads (Roche Diagnostics) and a MagNA Lyser (Roche) according to the manufacturer's instructions. Total RNA was extracted and 400 ng was used for reverse transcriptase polymerase chain reaction (RT-PCR) analysis, using Transcriptor reverse transcriptase polymerase (Roche) in 20 µL at 42°C for 45 minutes with random hexamer primers (20 ng/µL). Subsequently, 1.5 µL was amplified in a 50 µL PCR-reaction with 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, as previously described.[663] PCR products were visualized on 1.5% agarose gels and quantified using a DNA 1000 LabChip on the Agilent 2100 bioanalyzer (Agilent Technologies).

*Dystrophin protein analysis*

Muscles were homogenized in treatment buffer containing 75 mM Tris-HCl pH 6.8-15% (w/v) sodium dodecyl sulphate (SDS) using zirconium beads (1.4 mm; OPS Diagnostics) by grinding in a MagNA Lyser (Roche). Protein concentrations were determined using a Pierce bicinchoninic acid protein assay kit (Thermo Fisher Scientific) according to manufacturer's instructions. Samples containing 30 µg of protein were made in treatment buffer with 20% (v/v) glycerol, 5% (w/v) β-mercaptoethanol, and 0.001% (w/v) bromophenol blue, and

heated for 5 minutes at 95°C. Samples were loaded on 1.0 mm thick native polyacrylamide gel electrophoresis Tris-acetate (polyacrylamide) gels, with a linear resolving gel gradient of 3-8% (BioRad) and run on the Trans-Blot Turbo system for 1 hour at 75 V (0.07 A) and 2 hours at 150 V (0.12 A) in running buffer (XT Tricine; Biorad) in an ice container (Hulsker *et al.*, in preparation). Proteins were blotted on a nitrocellulose membrane using the Ready to use Trans-Blot Turbo transfer packs and the Trans-Blot Turbo transfer system from BioRad at 2.5 A and ~25 V for 10 minutes. Membranes were blocked in 10 mM Tris-HCl (pH 8) and 0.15 M NaCl (Tris-buffered saline (TBS))-5% nonfat dried milk (Elk) for 1 hour, washed in TBS-0.05% (v/v) Tween20 (TBST) and incubated overnight with first antibodies 1:125 NCL-Dys1 (Dy4; NovoCastra) and, as a loading control, alpha actinin (1:5 000; AB72592) in TBS. Membranes were washed in TBST, incubated 1 h with the fluorescent secondary antibodies 1:5 000 IRDye 800CW goat α-mouse IgG (Li-Cor, Lincoln, NE, USA) and 1:10 000 IRDye 680LT donkey -α-rabbit immunoglobuline G (IgG; Li-Cor) in TBS, washed in TBST and TBS, and analysed with the Odyssey system and software (Li-Cor). Dystrophin expression in wild type control samples containing 10%, 3.3%, 1.1%, and 0.4% of protein were used as reference to determine the dystrophin percentages in the tested samples.

*Hybridization-ligation assay for measuring 23AON concentration*

For measuring the concentration of 23AON, an assay based on a previously published hybridization-ligation assay was used.[664] Tissue were homogenized in 100 mM Tris-HCl pH 8.5, 200 mM NaCl, 0.2% SDS, 5 mM ethylene-diaminetetraacetic acid, and 2 mg/mL protK using zirconium beads (1.4 mm; OPS Diagnostics) in a MagNA Lyser (Roche). Samples were diluted 10 times in PBS (plasma), 500 and 1 000 times (muscle), or 1 000 and 5 000 (liver and kidney) in pooled control *mdx* tissue in PBS. A signal probe (containing the peptide for antibody recognition) and a template (complementary to 23AON and the probe) were added to homogenized tissue samples. The subsequent ligation step only takes place when both 23AON and probe are bound to the template. Thereafter, unbound probe was washed away and enzyme-linked antibodies were used to detect the amount of probe-23AON. Calibration curves of the analysed 23AON prepared in 60 times pooled control mouse *mdx* tissue in PBS were included. All analyses were performed in duplicate.

*Statistical analysis*

Data are represented as mean ± standard deviation. 23AON levels, exon skipping levels and protein levels were compared between all different groups using one-way analysis of variance (ANOVA), followed by a Bonferroni correction for multiple testing in case of significance ($p<0.05$) in SPSS 17.0.2 (SPSS). To assess a possible treatment effect on the weight of mice over time a longitudinal analysis was perform in R,[665] using the lme4 package.[666] A baseline corrected model was used, including fixed linear and quadratic time effects; the mouse effect was considered to be random. PK plasma levels in week 0 and 7 have been compared by non-compartmental analysis on sparse sampling data, using Phoenix® WinNonlin® 6.2 (Pharsight). Data are expressed as mean ± standard error of the mean.

## Results

### *PK/PD analysis of different dosing regimens*

In this study the relation between dosing regimens, exposure, and outcome (molecular and functional) has been investigated. Therefore *mdx* mice were injected for eight weeks with the same total dose of 23AON divided over a different number of injections (1, 2, or 7 per week). None of the mice, including the mice injected daily for eight weeks, showed any sign of changes in vital parameters, including changes in weight or behaviour (data not shown). Serum CK levels were elevated compared to wild type mice, but did not differ significantly from reference values in saline-treated *mdx* mice or between different dosage groups (fig. 3.1a). In addition, both rotarod (fig. 3.1b) and forelimb grip strength (fig. 3.1c) analyses did not show differences with saline-treated *mdx* mice, wild type mice or between the various dosage groups.

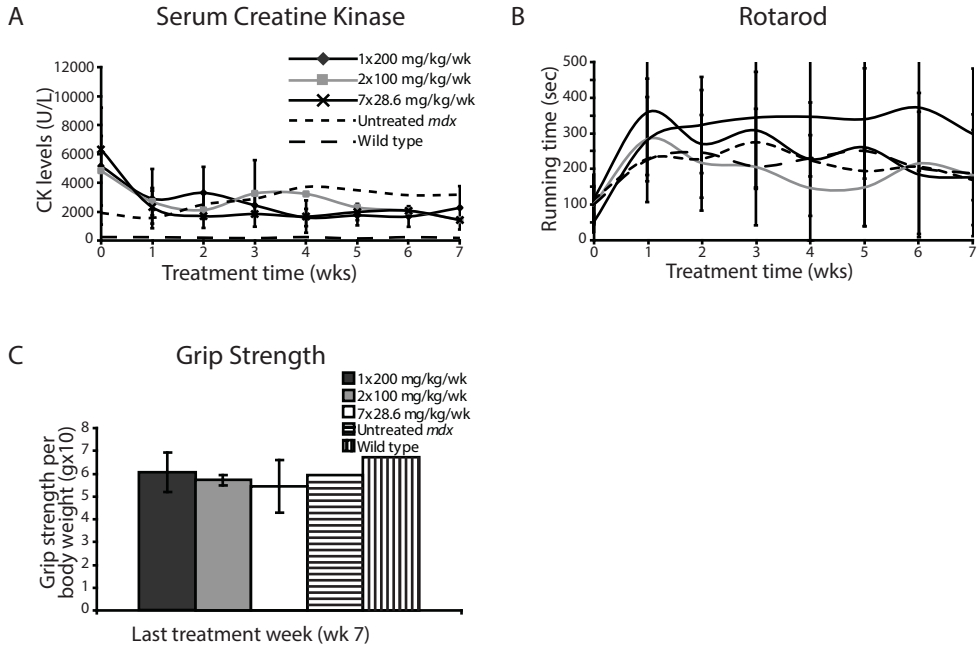


**Fig. 3.1:** Plasma creatine kinase (CK) and functional assessment during treatment
a) Serum CK levels are elevated in all *mdx* mice compared to wild type mice. No differences between mice of the different dosing regimens were observed. No differences between functional performance by rotarod (b) or forelimb grip strength (c) were seen between mice of various dosing regimens. Note that for rotarod analysis and forelimb grip strength the wild type mice and/or untreated *mdx* mice perform equally well.
Error bars represent the standard deviation.

The plasma profile of the AONs was assessed after the first AON injection in week 0 and week 7 (fig. 3.2a/b; table 3.1) by non-compartmental PK plasma analysis. Afterward, sacrifice AON levels in plasma, muscle, liver, kidney, and spleen were assessed as well (fig. 3.2c-e; supplementary table S3.1). Actual tissue exposures are the most relevant exposure parameters to take into account, since the process of uptake, distribution to tissue, or excretion into urine is relatively fast, while the stability of AONs in tissue is long (earlier publications suggested a tissue half-life in the range of two weeks or more in mice[200]). Plasma levels have

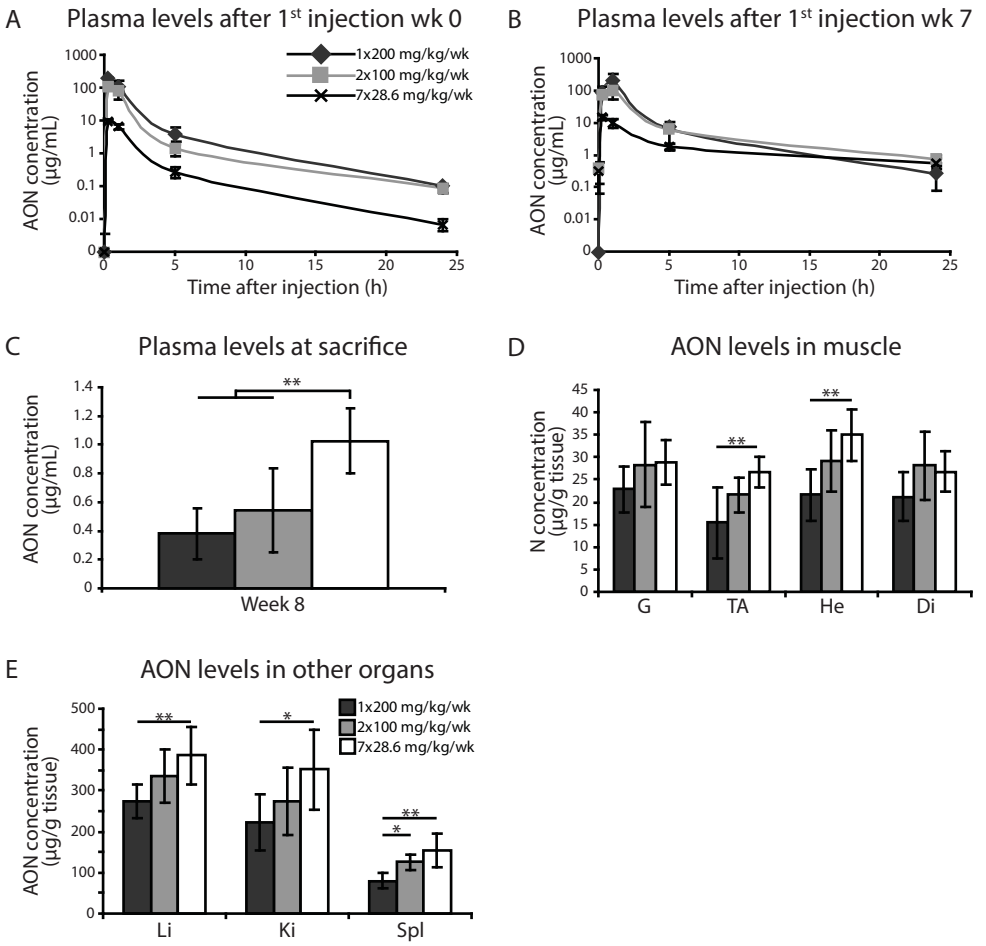


**Fig. 3.2:** Pharmacokinetic/pharmacodynamic profiles of antisense oligonucleotides (AONs) for the different dosing groups

a) In the first week high peak levels after 100 or 200 mg/kg were seen rapidly (± 15 minutes) after injection, which declined within hours. For injection with a lower amount of AON (28.6 mg/kg), a lower peak concentration was seen. b) The same pattern was observed after seven weeks of injection. Furthermore drug accumulation after repeated dosing was observed, indicated by an extension of the plasma profile and increase in area under the curve (AUC). c) Plasma levels at sacrifice were significantly higher for the group which had received daily injections compared to both other groups. d/e) In most muscles (the target tissue) and all other organs analysed (the non-target tissues) higher AON levels were observed in mice receiving seven injections per week compared to mice receiving the same total dose all at once.

Error bars represent the standard deviation. G=gastrocnemius; TA=tibialis anterior; He=heart; Di=diaphragm; Li=liver. Ki=kidney; Spl=spleen. *$p$<0.05 **$p$<0.01

been measured as a control to confirm dosing and detect potential non-linearities. Because blood sampling in mice is limited, only five time points have been used for the plasma PK profile using a sparse sampling approach. An overview of non-compartmental PK exposure parameters is given in table 3.1. After subcutaneous injection, AONs are taken up rapidly reaching maximum plasma levels after 15 minutes, followed by a decline to levels below 5%

| | *Week 0 plasma exposure* | | *Week 7 plasma exposure* | | *Ratio week 7/week 0* | |
|---|---|---|---|---|---|---|
| *Dosing and schedule* | *Cmax (µg/µL)* | *AUC0-t (µg.h/µL)* | *Cmax (µg/µL)* | *AUC0-t (µg.h/µL)* | *Cmax* | *AUC0-t* |
| 1 x 200 mg/kg/week | 174 ± 14 | 381 ± 78 | 193 ± 70 | 577 ± 162 | 1.1 | 1.5 |
| 2 x 100 mg/kg/week | 109 ± 11 | 244 ± 10 | 116 ± 3 | 407 ± 20 | 1.1 | 1.7 |
| 7 x 28.6 mg/kg/week | 10 ± 1 | 24 ± 2 | 14 ± 1 | 56 ± 5 | 1.5 | 2.4 |

**Table 3.1:** PK analysis of plasma levels in the first and last week of treatment
Non-compartmental analysis on sparse sampling data has been performed to calculate the Cmax and AUC for the different dosing regimens, showing a more than dose-proportional increase in plasma exposure levels for both parameters at higher dosing levels.
Data are expressed as mean ± standard error of the mean.

of the maximum at 5 hours after injection (fig. 3.2a/b). Upon repeated dosing, the plasma profile gets extended and the area under the curve (AUC) increases, which is indicative of the drug accumulation in tissues (fig. 3.2b/table 3.1). The plasma exposures increase at higher dosing levels, both for the maximum plasma concentration (Cmax) and the AUC. The increase is more than dose-proportional between 28.6 and 100 mg/kg (~10 times for Cmax and ~7 to 10 times for the AUC). This indicates that mechanisms responsible for distribution to tissue can be saturated, which can explain the decreasing tissue levels at the higher dosing level of injection, while the total weekly dose remains the same (fig. 3.2c-e). The increase in exposure between 100 and 200 mg/kg appears somewhat less than dose-proportional in week 7 (fig. 3.2b). This may be due to infrequent sampling after one hour, but combined with

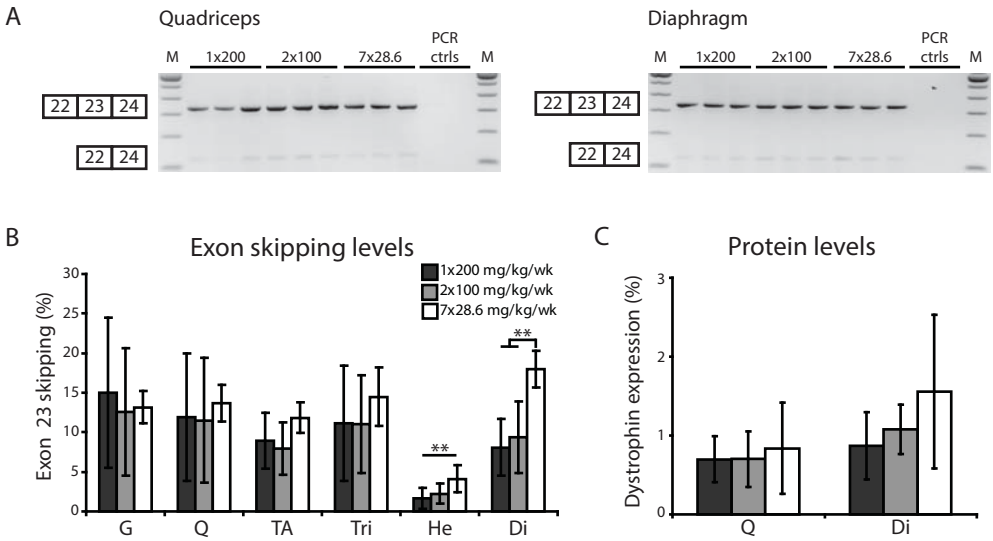


**Fig. 3.3:** Exon skipping levels and dystrophin protein expression
a) Examples of RT-PCR analysis of the quadriceps and diaphragm muscles showing the unskipped wild type (334 bp) and exon 23 skipped (121 bp) product. b) Quantification of exon skipping levels in different muscles. In skeletal muscle no differences between the different dosing regimens were observed. Significantly higher levels were seen in the heart and diaphragm of daily injected mice versus mice injected once and/or twice weekly. c) No differences in the expression levels of the dystrophin protein levels were observed between all dosing groups in the quadriceps. In the diaphragm a slight, but not significant, increase was found in the daily injected mice.
Error bars represent the standard deviation. M=size marker; PCR ctrls=negative PCR-controls; G=gastrocnemius; Q=quadriceps; TA=tibialis anterior; Tri=triceps; He=heart; Di=diaphragm. *$p$<0.05 **$p$<0.01

the further decrease in tissue levels, it suggests an enhanced renal excretion of free AONs in plasma when binding to plasma proteins gets saturated at higher plasma concentrations.

When comparing exon skipping levels between the different groups of mice, no differences were seen between the different groups in muscle (fig. 3.3a/b; supplementary table S3.1). Only in the heart and diaphragm an increase in exon skipping levels was found in the daily injected mice compared to the mice injected once and/or twice weekly (fig. 3.3b). A direct correlation between AON and exon skipping levels was not found, except for the heart (fig. 3.2d versus 3.3b). Furthermore the higher AON and/or exon skipping levels in daily injected mice did not result in higher protein levels (fig. 3.3c; supplementary table S3.1). These were low and comparable for all groups in the quadriceps. Slightly higher dystrophin protein levels were observed for daily injected animals in both quadriceps and diaphragm, but this was not significant due to the low levels in general and high interindividual differences (fig. 3.3c). No exon skipping or dystrophin restoration was observed in non-treated animals (data not shown).

## *PK/PD analysis of different maintenance regimens*

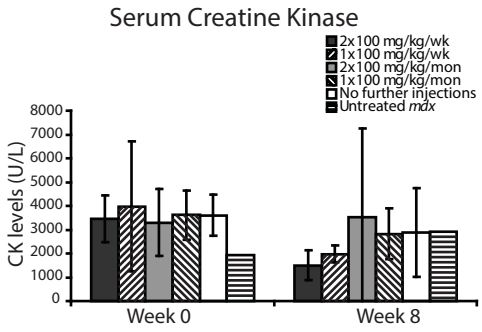


**Fig. 3.4:** Plasma CK before initial treatment and after eight weeks of maintenance treatment

Plasma CK is highly variable in mice, and no differences were observed between different maintenance regimens after treatment.

Error bars represent the standard deviation.

To study the effect of different maintenance regimens, *mdx* mice were subjected to different maintenance schemes for eight weeks. After an initial 'loading' treatment with five times 250 mg/kg 23AON in the first week (proven to be effective in previous experiments[200,259]), mice were injected twice weekly, once weekly, twice monthly, or once monthly with 100 mg/kg or did not receive any further injections for the subsequent eight weeks. During this period, no differences in weight or functional performance by rotarod analysis were seen between all groups (data not shown). Furthermore, CK levels did not show differences at the end of the eight weeks treatment period (fig. 3.4).

The amount of 23AON in several muscle groups and organs was assessed for the different regimens (fig. 3.5a/b; supplementary table S3.2). The 23AON was detectable in all samples analysed, even in those that did not receive any further injections for eight weeks after the initial treatment in the first week. For all muscles (fig. 3.5a) and organs (fig. 3.5b) levels were highest ($p<0.05$) in the mice injected twice weekly compared with other groups. Also, the mice injected once weekly, had significantly higher levels for most muscles and organs compared with the mice, which had received no injections and/or only once a month during the maintenance period. Only in the tibialis anterior and heart differences were observed between the group that received injections twice per month and the group that received no further injections. A clear trend with the number of injections and tissue levels was observed for all tissues.

In contrast to the dose optimisation phase, in this maintenance phase the exon skipping levels followed the pattern observed for AON levels (fig. 3.5a versus 3.5c; supplementary

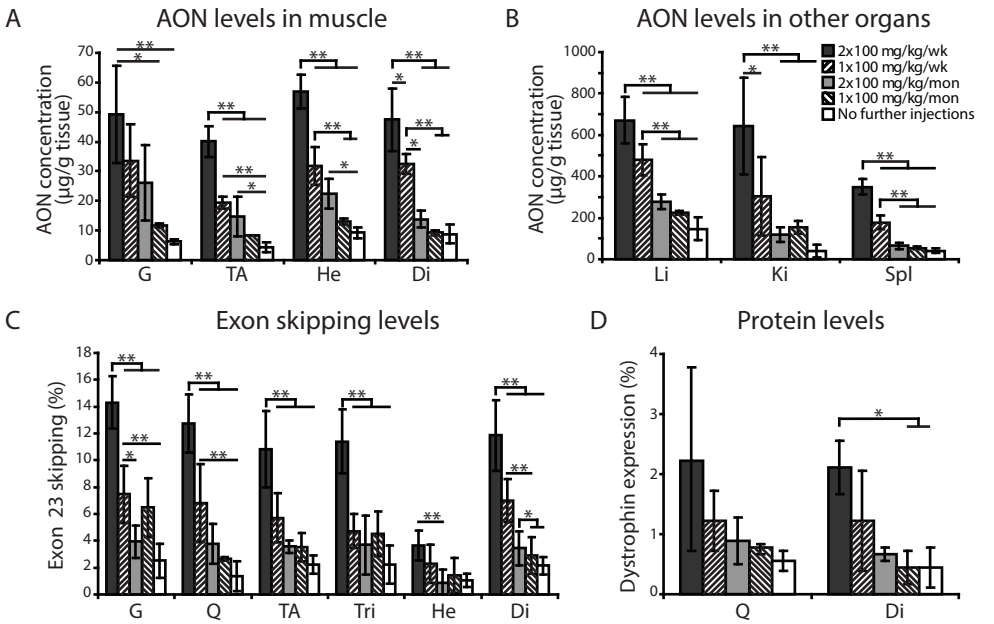


**Fig. 3.5:** Biodistribution, exon skipping levels and protein expression

a/b) A dose-dependent pattern was observed for the number of maintenance injections and the AON levels in all analysed muscles and other organs. For all muscle groups and other organs, levels were significantly higher in the mice which received two times 100 mg/kg/wk during the maintenance period compared to almost all other groups. Mice which had received one injection per week also showed higher levels compared to mice which had received less injection in all tissues, except for the gastrocnemius and kidney. c) The exon skipping levels also showed a dose-dependent pattern with the number of injections. However, levels were only significantly higher for mice injected twice and/or once per week, and less differences were observed between mice injected with less maintenance injections. d) Protein levels were only increased in the diaphragm for mice that were injected twice per week compared to mice injected once per month or that did not receive maintenance injections.

Error bars represent the standard deviation. G=gastrocnemius; TA=tibialis anterior; He=heart; Di=diaphragm; Li=liver; Ki=kidney; Spl=spleen; Q=quadriceps; Tri=triceps. *$p< 0.05$ **$p< 0.01$

table S3.2), that is, a decrease in exon skipping percentages with decreasing numbers of injections (and thus total dose). Only in heart, the differences were less clear, partly due to higher variations between individual mice and partly due to the fact that the exon skipping levels in heart were lower in mice injected twice a week compared with the skeletal muscles. For the mice that received AONs twice weekly, exon skipping levels were significantly increased compared with less frequently injected mice in all muscles, except for heart. Mice injected once weekly showed higher levels only in the gastrocnemius, quadriceps, and diaphragm and differences between the other groups were seen only for the diaphragm (fig. 3.5c). For dystrophin protein expression (fig. 3.5d; supplementary table S3.2) only in the diaphragm significantly higher protein levels were found in the twice-weekly injected mice versus the mice injected once monthly or not at all after the loading phase. The reason that no clear differences were observed in protein levels is the overall low expression levels and high variation between individual mice.

## *Detailed PK/PD analysis of dosing and maintenance regimens*

The ratios between exon skipping and AON levels observed in the different muscle groups

were assessed (fig. 3.6a/b). The ratios were much higher, *i.e.* more exon skipping for a certain amount of AON, in skeletal muscles than in heart, suggesting that in heart the majority of AON is probably trapped in the interstitium and therefore ineffective. No clear pattern was observed for the different dosing regimens (fig. 3.6a). In limb muscles the ratios were higher, albeit with large interindividual variations, for the mice injected once a week, suggesting that lower AON levels were able to induce the same amount of exon skipping levels or that more AON was taken up by the muscle fibres. By contrast, in the diaphragm a higher ratio was observed for the mice injected seven times a week, suggesting that for this organ daily injections may lead to improved uptake of AONs.

For the maintenance study mice, receiving less frequent injections (and thereby also a lower total dose) showed a higher exon skipping/AON ratio for the gastrocnemius and tibialis anterior (fig. 3.6b). This may be a reflection of the fact the exon skipping is a stepwise process (AON uptake, exon skipping, dystrophin restoration) and suggests that dystrophin transcripts have a relatively long half-life.

The ratios of the average AON levels in skeletal muscles (gastrocnemius, tibialis anterior, and diaphragm) and those in non-target organs (kidney, liver, and spleen) were also calculated (fig. 3.6c/d). For the different dosing regimens, no differences were observed (fig. 3.6c). For the comparison of the different maintenance regimens the ratios, were generally similar (fig. 3.6d).

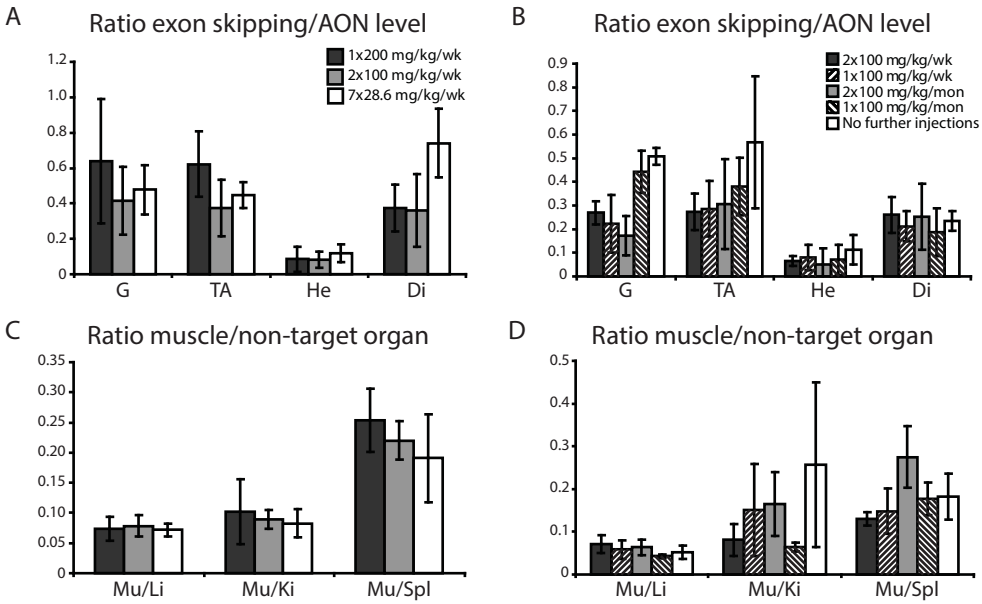


**Fig. 3.6:** Ratio of exon skipping versus AON levels in different muscle groups and AON levels in the muscle (target tissue) versus non-target organs

a) The exon skipping/AON ratios did not reveal large differences between the different dosage regimens. Only in the diaphragm relatively more exon skipping for a certain amount of AON was observed for the daily injected mice. The ratios were markedly lower in the heart compared to other muscles. b) In gastrocnemius and tibialis anterior exon skipping/AON ratios were a bit higher in mice receiving less frequent maintenance injections. Here, heart ratios were also markedly lower. c) Ratios between the AON levels in the target organ (muscle) versus the non-target organs were similar for the different dosage regimens. d) More variation was observed between muscle/non-target organ AON level ratios for the different maintenance regimens for the kidneys and spleen.

Error bars represent the standard deviation. G=gastrocnemius; TA=tibialis anterior; He=heart; Mu=muscle; Di=diaphragm; Li=liver; Ki=kidney; Spl=spleen.

## Discussion

Antisense-mediated exon skipping is currently one of the most promising therapeutic approaches for Duchenne muscular dystrophy. This approach has shown encouraging results in preclinical experiments *in vitro* and *in vivo* in animal models and in early phase clinical trials.[667] However, to improve the therapeutic effect, optimisation of the treatment regimen is necessary. In *mdx* mice, the optimal dose is around 200 mg/kg body weight for 2OMePS.[200] This is much higher than the dosages used in clinical trials (6 mg/kg body weight). However, a correction factor must be applied when translating from mice to humans (see Guidance for Industry[605]). When applying this correction factor, 200 mg/kg in mice would correspond to 16 mg/kg in humans. This is slightly higher than the dose used in humans, but this can be explained by differences in clearance and regeneration capacity between mice and humans, interexon differences, and differences in PK/PD properties of 23AONs versus 51AONs. Although some preclinical studies into the PK/PD profiles of AONs have been done in animal models,[200,239] it is uncertain whether the currently used dosing schedule in clinical trials is the best. During optimisation there will always be a trade-off between the efficacy (exon skipping and dystrophin restoration) due to AONs in the targeted muscles and the amount that ends up in other organs, like the liver and kidneys, where it potentially can have adverse effects. Therefore, detailed PK/PD analyses were done for different 2OMePS 23AON dosing and maintenance regimens in order to model the AON and exon skipping effects.

Interestingly, dividing the same total dose of 2OMePS AONs over multiple, daily injections was more effective than giving the same dose in one or two weekly injections, especially for heart and diaphragm. This is in concordance with a study on PMO AONs in *mdx* mice, which also reported increased effectiveness with multiple, low dose injections compared to a single, high dose injection.[239] In the present study, a single injection with a high amount of AON resulted almost immediately in high plasma levels, which rapidly declined. This is partly due to uptake by, and potentially saturation of the tissues, but also largely due to clearance by the kidneys. For 2OMePS AONs, in contrast to PMOs, this is partly prevented by the serum binding properties of the PS-backbone.[668] However the binding capacity of serum proteins is limited, and at high concentrations saturation is reached, leading to urinary excretion,[669] explaining the higher concentrations of AONs in the muscles after multiple injections with low dose of AONs, which were significant in tibialis anterior and heart. Due to lower peak levels, which do not exceed the binding capacity of the serum proteins, all AON can bind serum proteins, resulting in higher availability of AON, indicated by the accumulation over time seen in this group, as reflected by the plasma levels at sacrifice. However, this effect is not only seen in the muscles, but also in the other organs (liver, kidney, and spleen). When comparing the amount of AON in the muscles and non-target organs the ratio was roughly similar between the different dosage regimens, indicating that the uptake of AONs in the different tissues increases with the same proportion with increasing numbers of smaller injections.

The exon skipping levels measured here were lower than those reported in our previous studies.[161,200] This is most likely due to the fact that previously a nested PCR was used to detect exon skipping. It has now been shown that this gives an overestimation of exon skipping.[663] Therefore, to more accurately determine skipping percentages, a single PCR was used in our current study. Indeed the exon skipping levels reported here are more comparable to another report using a single PCR.[259]

For the different dosing regimens, no clear correlation was observed between the biodistribution pattern to individual muscles and the exon skipping levels observed (fig. 3.6a). A

possible explanation could be that a treatment period of eight weeks is not sufficient to reach a steady state. This is confirmed by our earlier findings that upon longer treatment exon skipping levels increase up to 12 weeks,[219] while they do not increase further after 24 weeks.[201] In the gastrocnemius and tibialis anterior, where no increase in skipping was observed, this ratio was higher for mice who received the AONs all at once. Notably, in heart multiple, small injections did result in higher AON levels, which did correlate to higher exon skipping levels. This suggests that optimising the dosing regimen is possibly a way to improve heart targeting. This is important, since previous studies have shown that targeting heart so far has been challenging.[200,203]

No clear increase in protein levels was seen for the different dosing groups, except for a small increase in the diaphragm. This is mainly due to the high standard deviation and because levels are low in general, which is expected after such a short treatment period in mice. Notably, in clinical studies higher dystrophin levels were observed already after five weeks of treatment.[229] This could be due to differences in muscle fibre permeability between DMD patients and *mdx* mice and suggests there may be dissimilarities in biodistribution between DMD patients and *mdx* mice.

Furthermore, different regimens to maintain the effect achieved by initial treatment were compared in this study. In contrast to the dosing regimen study, here a clear dose-dependent pattern was observed, where more maintenance injections resulted in higher AON, exon skipping and dystrophin levels. This difference can be due to the initial loading with a very high dose of AON, the fact that this study was longer and/or because the total dose received by the mice was similar in the dosing study, whereas different groups received different total doses in the maintenance study. Notably, the exon skipping/AON ratio was higher in the groups receiving the lowest number of injections. This could reflect differences in turnover of AON and RNA transcripts. Animals were sacrificed one week after the last injection of the group receiving maintenance injections twice weekly. This means that the animals that did not receive maintenance injections had their last AON treatment nine weeks before sacrifice. For these animals it is anticipated that part of the AON is already turned over (as reflected by the lower AON levels in the muscles, fig. 3.5a). However, apparently the turnover of exon skipped transcript is slower, resulting in a higher exon skipping/AON ratio. Our results underline the long half-life of the 2OMePS AONs in muscle, as low levels of AON and exon skipping were still detected eight weeks after the last injection. More extensive studies are required to study the turnover of the different components (AON, transcript, and proteins) in more detail.

For the selection of an optimal dosage and maintenance regimen in humans, clinical studies are indispensable. However, preclinical animal studies provide important indications of the effects of different treatment regimens and can be used in data models to enable further optimisation. Achieving high levels of exon skipping and dystrophin restoration are of course aimed for. However, as with most drugs, one also has to balance this with how much AON accumulates in non-target tissues, mainly liver, kidney, and spleen.

Additional studies are needed for more extensive PK/PD modelling and to further elucidate the long term effects of AONs on different levels (*i.e.* RNA and protein).

## Acknowledgments

The authors are grateful to Maarten van Iterson (LUMC) for assistance with the statistical analysis. This work is supported by grants from the Duchenne Parent Project, the Prinses Beatrix Spierfonds [W.OR09-12], Spieren voor Spieren, and ZonMW (the Netherlands), and utilized the infrastructure of the Center for Medical System Biology (CMSB) and the Center for Biomedical Genetics (CBG).

## Author disclosure statement

AAR discloses being employed by LUMC, which has patent applications on exon skipping that are licensed to Prosensa Therapeutics. As a co-inventor on some of these patents, AAR is entitled to a share of royalties. IV and CTdW declare no conflict of interest. TK, IK, SdK, and JvD report being employed by Prosensa Therapeutics. JvD discloses being co-inventor on exon skipping patents and being entitle to a share of royalties.

# Supplementary data

| | *AON levels* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Muscle* | | | | | *Other organs* | | |
| *Dosing and schedule* | *Plasma* | *Gastroc-nemius* | *Tibialis anterior* | *Heart* | *Dia-phragm* | *Liver* | *Kidney* | *Spleen* |
| 1 x 200 mg/kg/week | 0.38 ± 0.18 | 22.9 ± 5.1 | 15.4 ± 8.0 | 21.6 ± 5.8 | 21.2 ± 5.5 | 273 ± 41 | 223 ± 70 | 80 ± 19 |
| 2 x 100 mg/kg/week | 0.54 ± 0.29 | 28.4 ± 9.5 | 21.6 ± 3.9 | 29.3 ± 6.8 | 28.1 ± 7.6 | 336 ± 66 | 273 ± 82 | 126 ± 20 |
| 7 x 28.6 mg/kg/week | 1.03 ± 0.23 | 28.8 ± 4.9 | 26.7 ± 3.3 | 35.0 ± 5.8 | 26.7 ± 4.5 | 386 ± 70 | 352 ± 97 | 155 ± 42 |

| | *Exon skipping levels* | | | | | | *Protein levels* | |
|---|---|---|---|---|---|---|---|---|
| | *Gastrocne-mius* | *Quadri-ceps* | *Tibialis anterior* | *Triceps* | *Heart* | *Dia-phragm* | *Quadri-ceps* | *Dia-phragm* |
| 1 x 200 mg/kg/week | 15.0 ± 9.5 | 11.9 ± 8.0 | 8.9 ± 3.5 | 11.1 ± 7.3 | 1.7 ± 1.3 | 8.1 ± 3.6 | 0.7 ± 0.3 | 0.9 ± 0.4 |
| 2 x 100 mg/kg/week | 12.6 ± 8.1 | 11.5 ± 7.9 | 8.0 ± 3.3 | 11.0 ± 6.1 | 2.2 ± 1.3 | 9.4 ± 4.5 | 0.7 ± 0.3 | 1.1 ± 0.3 |
| 7 x 28.6 mg/kg/week | 13.2 ± 2.0 | 13.7 ± 2.3 | 11.8 ± 2.0 | 14.5 ± 3.7 | 4.1 ± 1.7 | 18.0 ± 2.3 | 0.8 ± 0.6 | 1.6 ± 1.0 |

**Supplementary Table S3.1:** Overview of PK/PD data of different dosing regimens
Summary of the AON, exon skipping and protein levels at sacrifice in week 8 of the different dosing regimens. 23AON levels are expressed as µg/mL (for plasma) or µg/g tissue (for organs). Exon23 skipping levels are expressed as skipped transcript as percentage of total transcript. Dystrophin protein levels are expressed as percentage of wild type levels in the same muscle.
Data are expressed as mean ± standard deviation.

| | *AON levels* | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Muscle* | | | | *Other organs* | | |
| *Dosing and schedule* | *Gastrocne-mius* | *Tibialis anterior* | *Heart* | *Diaphragm* | *Liver* | *Kidney* | *Spleen* |
| 2 x 100 mg/kg/week | 49.3 ± 16.5 | 40.1 ± 5.1 | 56.9 ± 5.8 | 47.4 ± 10.6 | 671 ± 113 | 645 ± 234 | 348 ± 38 |
| 1 x 100 mg/kg/week | 33.6 ± 12.2 | 19.4 ± 2.0 | 31.9 ± 6.4 | 32.6 ± 3.3 | 481 ± 77 | 305 ± 190 | 178 ± 35 |
| 2 x 100 mg/kg/month | 26.0 ± 12.7 | 14.8 ± 6.6 | 22.5 ± 5.0 | 13.9 ± 2.8 | 277 ± 35 | 119 ± 34 | 65 ± 16 |
| 1 x 100 mg/kg/month | 11.7 ± 0.6 | 8.5 ± 0.0 | 13.1 ± 1.1 | 9.2 ± 0.7 | 224 ± 9 | 154 ± 32 | 55 ± 5 |
| No further injections | 6.2 ± 0.8 | 4.4 ± 1.7 | 9.2 ± 1.9 | 8.9 ± 3.1 | 146 ± 55 | 41 ± 30 | 41 ± 12 |

| | *Exon skipping levels* | | | | | | *Protein levels* | |
|---|---|---|---|---|---|---|---|---|
| | *Gastroc-nemius* | *Quadri-ceps* | *Tibialis anterior* | *Triceps* | *Heart* | *Dia-phragm* | *Quadri-ceps* | *Dia-phragm* |
| 2 x 100 mg/kg/week | 14.3 ± 1.9 | 12.7 ± 2.2 | 10.8 ± 2.8 | 11.4 ± 2.4 | 3.7 ± 1.1 | 11.9 ± 2.6 | 2.2 ± 1.5 | 2.1 ± 0.4 |
| 1 x 100 mg/kg/week | 7.5 ± 2.1 | 6.8 ± 2.9 | 5.7 ± 1.8 | 4.7 ± 1.3 | 2.3 ± 1.4 | 7.0 ± 1.6 | 1.2 ± 0.5 | 1.2 ± 0.8 |
| 2 x 100 mg/kg/month | 3.9 ± 1.2 | 3.8 ± 1.5 | 3.6 ± 0.5 | 3.7 ± 2.2 | 0.9 ± 1.0 | 3.4 ± 1.3 | 0.9 ± 0.4 | 0.7 ± 0.1 |
| 1 x 100 mg/kg/month | 6.5 ± 2.2 | 2.6 ± 0.1 | 3.5 ± 1.0 | 4.5 ± 1.7 | 1.4 ± 1.3 | 2.9 ± 1.4 | 0.8 ± 0.1 | 0.4 ± 0.3 |
| No further injections | 2.5 ± 1.3 | 1.4 ± 1.1 | 2.2 ± 0.7 | 2.2 ± 1.4 | 1.0 ± 0.5 | 2.1 ± 0.7 | 0.5 ± 0.2 | 0.4 ± 0.3 |

**Supplementary Table S3.2:** Overview of PK/PD data of different maintenance regimens
Summary of the AON, exon skipping and protein levels at sacrifice in week 8 of the different dosing regimens. 23AON levels are expressed as µg/g tissue. Exon23 skipping levels are expressed as skipped transcript as percentage of total transcript. Protein levels are expressed as percentage of wild type levels in the same muscle.
Data are expressed as mean ± standard deviation.

# The dynamics of compound, transcript and protein effects after treatment with 2OMePS antisense oligonucleotides in *mdx* mice

Ingrid E.C. Verhaart, Laura van Vliet-van den Dool, Jessica A. Sipkens, Sjef J. de Kimpe, Ingrid G.M. Kolfschoten, Judith C.T. van Deutekom, Lia Liefaard, Jim E. Ridings, Steve R. Hood and Annemieke Aartsma-Rus

Previously published in: Mol Ther NA; 2014: 3.

Chapter 4

## Abstract

Antisense-mediated exon skipping is currently in clinical development for Duchenne muscular dystrophy (DMD) to amend the consequences of the underlying genetic defect and restore dystrophin expression. Due to turnover of compound, transcript and protein, chronic treatment with effector molecules (antisense oligonucleotides) will be required. To investigate the dynamics and persistence of antisense 2'-*O*-methyl phosphorothioate oligonucleotides, exon skipping, and dystrophin expression after dosing was concluded, *mdx* mice were treated subcutaneously for eight weeks with 100 mg/kg oligonucleotides twice weekly. Thereafter, mice were sacrificed at different time points after the final injection (36 hours-24 weeks). Oligonucleotide half-life was longer in heart (~65 days) compared with that in skeletal muscle, liver and kidney (~35 days). Exon skipping half-lives varied between 33 and 53 days, whereas dystrophin protein showed a long half-life (>100 days). Oligonucleotide and exon skipping levels peaked in the first week and declined thereafter. By contrast, dystrophin expression peaked after three to eight weeks and then slowly declined, remaining detectable after 24 weeks. Concordance between levels of oligonucleotides, exon skipping and proteins was observed, except in heart, wherein high oligonucleotide levels, but low exon skipping and dystrophin expression were seen. Overall these results enhance our understanding of the pharmacokinetics and pharmacodynamics of 2'-*O*-methyl phosphorothioate oligos used for the treatment of DMD.

## Introduction

Duchenne muscular dystrophy (DMD) is the most prevalent form of inherited muscular dystrophies affecting around one in 5 000 newborn boys.[1,2] Patients display severe progressive muscular weakness due to the absence of the dystrophin protein, which functions as mechanical stabiliser during muscle contraction. In the absence of dystrophin the muscle fibres are easily damaged and are gradually replaced by fibrotic and adipose tissues. First symptoms generally become apparent at two or three years of age, after which, pathology develops rapidly. Patients often die before the age of 30 years due to respiratory and/or cardiac failure.[5]

In humans, lack of dystrophin is generally caused by out-of-frame deletions or small mutations that introduce premature stop codons in the *DMD* gene.[670] Several animal models are available for DMD, among which, the *mdx* mouse (C57Bl/10ScSn-DMD$^{mdx}$/J) -which does not express dystrophin due to a premature stop codon in exon 23- is the most widely used.[79] However, the lack of dystrophin is less disastrous in *mdx* mice than in humans. These mice have a nearly normal life expectancy, only slightly impaired muscle function and a better muscle quality compared with DMD patients.[671] Nevertheless, this mouse model displays several features of the DMD pathology, *i.e.* muscle degeneration and leaky fibres due to the absence of dystrophin, and is very useful for preclinical studies.[672]

During the past several years a lot of progress has been made toward the development of antisense oligonucleotide (AON)-mediated exon skipping as a potential therapy targeting the underlying genetic defect of DMD. This aims to restore the reading frame or bypass a small mutation by skipping one or more exons. Thereby translation can continue and a largely functional protein can be formed, as is found in the related, but much milder Becker muscular dystrophy. After obtaining proof-of-principle *in vitro* in cultured cells and *in vivo* in animal models, clinical trials for AONs with two different backbone chemistries, 2'-*O*-methyl phosphorothioate RNA (2OMePS) and phosphorodiamidate morpholino oligomers (PMO), are currently ongoing.[673] Systemic (subcutaneous) treatment with drisapersen (2OMePS AON, targeting exon 51) for five weeks induced dystrophin expression in ten of 12 patients up to 15.6% of levels found in healthy persons.[229] Treatment with eteplirsen (PMO AON, targeting exon 51) by intravenous infusion resulted in dystrophin restoration in seven of 19 patients at highly variable percentages up to 18% of control levels.[231] Placebo-controlled phase 2 and 3 trials have been conducted for drisapersen. Encouraging results were reported for the phase 2 trials, suggesting improved walking distance in six minutes for treated patients compared with placebo administered after 24 and 48 weeks of treatment. However, it was recently reported that the primary outcome measure (distance walked in six minutes) in the phase 3 trial,[233] although slightly improved in the treated patients, did not differ significantly from the placebo treated patients. More detailed analysis is pending. In addition to drisapersen (targeting exon 51), AONs targeting other exons, *i.e.* exon 44, 45 and 53, are under clinical evaluation.

Because RNA-mediated therapies are subject to clearance and turnover of AONs and the dystrophin transcripts and proteins, repeated, life-long injections will be required to maintain therapeutic effects. Therefore, insight in the pharmacokinetic (PK) and pharmacodynamic (PD) properties of these compounds is essential to determine how long the effects persist and to assess dosing frequencies and regimens. Previous studies have revealed a plasma half-life for 2OMePS AONs of around four weeks in patients[229] and a tissue half-life of two to six weeks in *mdx* mouse muscle.[200] It has previously been observed that uptake of AONs is better in dystrophic muscle than in healthy muscle, probably due to the dystrophic "leaky" nature of the muscle fibres. Both subcutaneous and intravenous routes of administration resulted

in muscle uptake and exon skipping, but intravenous injections led to much higher AON levels in liver and kidneys, although AON levels in muscle were comparable with those adter subcutaneous treatment.[200] Furthermore, for 2OMePS AONs, exon skipping and dystrophin protein levels vary among different muscle tissues. Generally exon skipping levels in heart are lower, whereas the levels are comparable in limb muscle and diaphragm.[161,200,201,219,240,259] Increase in exon skipping and accumulation of dystrophin protein were seen up to 12 weeks of treatment and long term treatment for six months has been shown to be feasible.[201,219] Comparison of various maintenance regimens revealed that eight weeks after the last dose, the decline in AON levels was faster than the decline in exon skipping and protein levels, indicating differences in turnover.[240] However, the relation between clearance and turnover of the compound, the induced skipped transcript, and restored dystrophin protein and long term analysis of the persistence of different effects after subcutaneous treatment have not been studied. In order to expand our understanding of the PK/PD relationship of 2OMePS AONs, *mdx* mice, in which the mutation can be bypassed by skipping of exon 23, were dosed with 100 mg/kg twice weekly for eight weeks via the subcutaneous route, after which, tissues were harvested at different time points for the analysis of 23AON, exon skipping and protein levels.

## Materials and methods

All experiments were approved by the local ethical committee for animal experiments of the Leiden University Medical Center. Mice were housed in individually ventilated cages in the animal facility of the Leiden University Medical Center and received food and drink *ad libitum*. *Mdx* mice (C57Bl/10ScSn-DMD$^{mdx/}$J) were obtained from our own breeding facility.

### *Treatment of* mdx *mice with 23AON and sample preparation*

Starting at an age of 3-4 weeks, 36 *mdx* mice were treated subcutaneously with 100 mg of 23AON/kg body weight in 100 µL saline twice weekly for 8 weeks. The 23AON molecule, previously described as M23D(+2-18), is a 2'-*O*-methyl phosphorothioate RNA oligonucleotide with a full-length phosphorothioate backbone, specifically targeting exon 23[197] (Prosensa Therapeutics, Leiden, the Netherlands). Mice were sacrificed by cervical dislocation at different time points after the last injection: t=36 hours and 1, 3, 8, 12, and 24 weeks (6 mice (3 males, 3 females) per time point). Before sacrifice, blood samples were taken for plasma PK analysis (see below). Plasma was generated by centrifuging at 18 000 *g* for 5 min and it was stored at -80 ºC until analysis. After sacrifice, muscles (triceps, tibialis anterior, quadriceps, heart and diaphragm) and liver, kidney and spleen were isolated, snap frozen in liquid nitrogen cooled 2-methylbutane and stored at -80 °C.

### *Assessment of 23AON levels with a hybridization-ligation assay*

An assay based on a previously published hybridization-ligation assay was used for the determination of the 23AON level in different tissues at Prosensa Therapeutics.[664] Tissues were homogenized in 100 mM Tris-HCl (pH 8.5), 200 mM NaCl, 0.2% sodium dodecyl sulfate, 5 mM ethylenediaminetetraacetic, and 2 mg/mL protK using zirconium beads (1.4 mm; OPS Diagnostics, Lebanon, NJ) in a MagNA Lyser (Roche Diagnostics, Almere, the Netherlands)

according to manufacturer's protocol. Plasma samples were diluted tenfold, muscle samples 500 and 1 000 times and organs 1 000 and 5 000 in pooled control *mdx* tissue in phosphate-buffered saline. A signal probe (containing the peptide for antibody recognition) and a template (complementary to 23AON and the probe) were added to homogenized samples. Only when both 23AON and probe are bound to the template, a ligation step will take place. After this step and washing away of the unbound probe, enzyme-linked antibodies were used to detect the amount of probe-23AON. Calibration curves of the analysed 23AON prepared in 10% pooled plasma or in pooled control mouse *mdx* tissue in phosphate-buffered saline were used to quantify 23AON. All analyses were performed in duplicate.

### *RNA extraction and analysis of exon skipping by RT-PCR*

Muscles were homogenized in TriPure isolation reagent (Roche Diagnostics) using zirconium beads (1.4 mm; OPS Diagnostics) by grinding in a MagNA Lyser (Roche Diagnostics). Total RNA was extracted and 600 ng was used for reverse transcription–polymerase chain reaction analysis. Complementary DNA was generated by incubating at 42 °C for 45 min with random hexamer primers (20 ng/µL) and Transcriptor reverse transcriptase polymerase (Roche Diagnostics) in 20 µL. Subsequently, 2 µL of cDNA was amplified in a 50 µL polymerase chain reaction reaction with 30 cycles of 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec, as previously described.[663] PCR products were visualized on 1.5% agarose gels and quantified using a DNA high sensitivity chip on the LabChip GX, in combination with the LabChip GX software (Caliper Life Sciences, Teralfene, Belgium).

### *Analysis of dystrophin protein expression by Western blot*

Muscles were minced in treatment buffer containing 75 mM Tris-HCl pH 6.8-15% (w/v) sodium dodecyl sulphate using zirconium beads (1.4 mm; OPS Diagnostics) or MagNA Lyser green beads (Roche Diagnostics) in a BBY24M Bullet Blender Storm (Next Advance, Averill Park, NY). Protein concentrations were determined using a Pierce bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions. Samples containing 30 µg of protein were made in treatment buffer with 20% (v/v) glycerol, 5% (v/v) β-Mercaptoethanol and 0.001% (w/v) Bromophenol blue and heated for 5 min at 95 °C. Wild type control samples containing 10%, 3.3%, 1.1% and 0.4% of protein were used as reference. Samples were loaded on 1.0 mm thick native PAGE Tris-acetate (polyacrylamide) gels, with a linear resolving gel gradient of 3-8% (BioRad, Veenendaal, the Netherlands) and run on the Trans-Blot Turbo system for 1 h at 75 V (0.07 A) and 2½ hrs at 150 V (0.12 A) in an ice container. Proteins were blotted on a nitrocellulose membrane using the Ready to use Trans-Blot Turbo transfer packs in combination with the Trans-Blot Turbo transfer system (BioRad) at 2.5 A and ~25 V for 10 min. Membranes were blocked in 10 mM Tris-HCl (pH 8) and 0.15 M NaCl Tris-buffered saline (TBS)-5% non-fat dried milk (Elk, Campina Melkunie, Zaltbommel, the Netherlands), washed in TBS-0.05% (v/v) Tween20 (TBST). Membranes were incubated overnight with 1:125 NCL-Dys1 (Dy4; NovoCastra, Newcastle Upon Tyne, UK) and α-actinin as loading control (1:7 500; AB72592; Abcam, Cambridge, UK) in TBS. The next day, membranes were washed in TBST, incubated 1 h with the fluorescent secondary antibodies IRDye 800CW goat-α-mouse IgG (1:5 000; Li-Cor; NE) and IRDye 680LT donkey-α-rabbit IgG (1:10 000; Li-Cor) in TBS, washed in TBST and TBS and analysed with the Odyssey system and software (Li-Cor) (M. Hulsker *et al.*, manuscript in preparation).

### *Statistical analysis*

For 23AON levels, individual data are represented for each measurement and exponential curves were fitted for each tissue. For exon skipping and protein levels data are represented as mean ± SD for each tissue. PK plasma parameters (terminal half-lives of the molecule in plasma and tissue) were derived by non-compartmental analysis using Phoenix WinNonLin software (version 6.2; Pharsight Corporation, Mountain View, CA). For samples in which 23AON was not detectable, values were set at 50% of limit of quantification of mouse 23AON in neat plasma (*i.e.* 50% of 0.011 µg/ml). For statistical testing, SPSS version 20.0 (IBM Corporation, Armonk, NY) was used. A $p$ value ≤0.05 was considered significant.

## Results

### *Dynamics of 23AON levels in plasma, muscle and organs*

To investigate the dynamics of the effects induced by treatment with 2OMePS AONs, mice were treated subcutaneously with 100 mg 23AON/kg body weight twice weekly for eight weeks. Six mice per group were sacrificed at different time points after the last injection, ranging from 36 hours up to 24 weeks. To assess the uptake, turnover and clearance of the AON in plasma and different tissues, 23AON levels were determined with an AON specific hybridization-ligation assay, and average results were calculated (Fig. 4.1; Table 4.1). For all tissues, this revealed a decline in AON levels after the final injection, and 23AON was still detectable in muscle, liver, and kidney after 24 weeks (on average around five to ten percent of the 23AON levels at 36 hours). Terminal half-lives ranged from 28 days in triceps

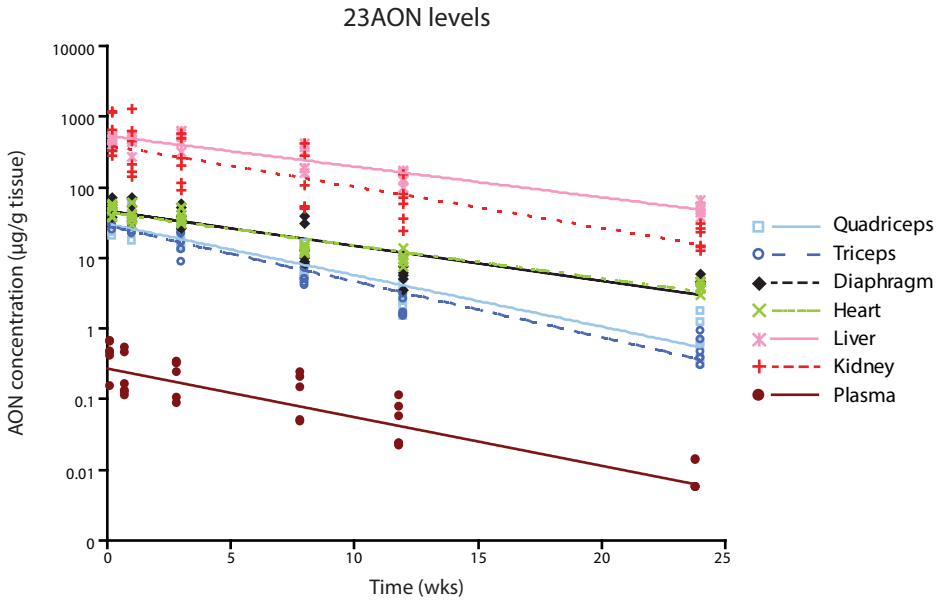


**Fig. 4.1:** 23AON levels in plasma, muscle and other organs over time

A hybridization-ligation assay was used to determine 23AON levels in plasma, muscle, and organs at different time points ranging from 36 hours up to 24 weeks after the final injection. Data are represented on a logarithmic scale. Each dot represents an individual measurement and lines are fitted through the averages for each tissue. AON, antisense oligonucleotide.

| | *AON levels*[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Muscle* | | | | | *Other organs* | |
| *Time (wks)* | *Plasma* | *Quadriceps* | *Tibialis anterior* | *Diaphragm* | *Heart* | *Liver* | *Kidney* |
| 0.2 | 0.462 ± 0.205 | 32.3 ± 9.1 | 33.0 ± 5.6 | 54.3 ± 11.4 | 49.1 ± 9.0 | 477.0 ± 49.7 | 655.7 ± 419.9 |
| 1 | 0.245 ± 0.183 | 33.9 ± 16.3 | 28.2 ± 4.6 | 51.1 ± 16.3 | 40.7 ± 10.5 | 456.1 ± 101.3 | 479.7 ± 437.9 |
| 3 | 0.236 ± 0.119 | 18.8 ± 6.1 | 18.3 ± 6.6 | 40.5 ± 12.5 | 40.6 ± 9.2 | 510.9 ± 119.6 | 289.6 ± 200.3 |
| 8 | 0.137 ± 0.092 | 8.9 ± 5.1 | 5.7 ± 1.7 | 18.1 ± 12.9 | 13.3 ± 1.6 | 270.1 ± 109.3 | 160.1 ± 155.0 |
| 12 | 0.052 ± 0.041 | 2.9 ± 1.2 | 2.0 ± 0.7 | 6.4 ± 2.5 | 10.2 ± 2.0 | 142.7 ± 29.1 | 70.3 ± 44.5 |
| 24 | 0.008 ± 0.004 | 0.8 ± 0.6 | 0.5 ± 0.2 | 4.7 ± 0.7 | 4.0 ± 0.7 | 50.4 ± 10.2 | 20.1 ± 7.2 |
| *Terminal half-life (days)* | 30 | 32 | 28 | 45 | 65 | 48 | 38 |

**Table 4.1:** Average AON levels at each time point per tissue and terminal half-life for each tissue

[a]23AON levels are expressed as µg/mL for plasma and µg/g tissue for muscle and other organs (mean ± SD).

to 65 days in heart (Table 4.1). The half-life in heart was much longer than that in the other tissues. Although the terminal half-life in diaphragm was comparable to liver and kidney, it was slightly shorter in limb muscles (quadriceps and triceps) and comparable with that found in plasma. As observed previously,[200] AON levels were higher in liver and kidney than in muscle. When comparing the different muscles with each other, 23AON levels were higher in diaphragm and heart than in limb muscles ($p<0.05$). No differences were observed in 23AON levels when comparing liver and kidney. Oligonucleotide levels in plasma decreased rapidly after the final dose and by 24 weeks, 23AON was barely detectable when levels in only two out of six mice reached the limit of detection.

## *Dynamics of exon skipping levels in muscle*

The exon 23 skipping levels were determined in several limb muscles, diaphragm and heart (Fig. 4.2; Table 4.2). Exon skipping levels were highest at the early time points (36 hours

| | *Exon skipping levels*[a] | | | | *Protein levels*[b] | |
|---|---|---|---|---|---|---|
| Time (wks) | *Quadriceps* | *Tibialis anterior* | *Diaphragm* | *Heart* | *Quadriceps* | *Diaphragm* |
| 0.2 | 11.3 ± 1.3 | 11.2 ± 2.6 | 11.3 ± 2.4 | 4.1 ± 0.5 | 0.61 ± 0.23 | 2.36 ± 0.49 |
| 1 | 9.7 ± 2.1 | 8.7 ± 1.9 | 11.7 ± 2.7 | 3.2 ± 0.7 | 0.95 ± 0.13 | 1.93 ± 0.52 |
| 3 | 6.9 ± 2.2 | 9.4 ± 1.6 | 10.6 ± 3.4 | 2.7 ± 0.5 | 1.31 ± 0.63 | 2.43 ± 0.85 |
| 8 | 5.7 ± 2.4 | 2.8 ± 1.0 | 8.6 ± 0.5 | 0.9 ± 0.7 | 0.91 ± 0.44 | 2.75 ± 1.21 |
| 12 | 3.1 ± 1.3 | 2.2 ± 1.7 | 3.7 ± 1.6 | 1.0 ± 0.3 | 0.56 ± 0.38 | 2.42 ± 0.99 |
| 24 | ND | ND | ND | ND | 0.39 ± 0.19 | 1.57 ± 0.63 |
| *Terminal half-life (days)* | 53 | 33 | 49 | 39 | 103 | 137 |

**Table 4.2:** Average exon skipping and protein levels at each time point per muscle and terminal half-life for each muscle

ND, not detectable.

[a]Exon 23 skipping levels are expressed as skipped transcript as percentage of total transcript (mean ± SD). [b]Dystrophin protein levels are expressed as percentage of wild type levels in the same muscle (mean ± SD).

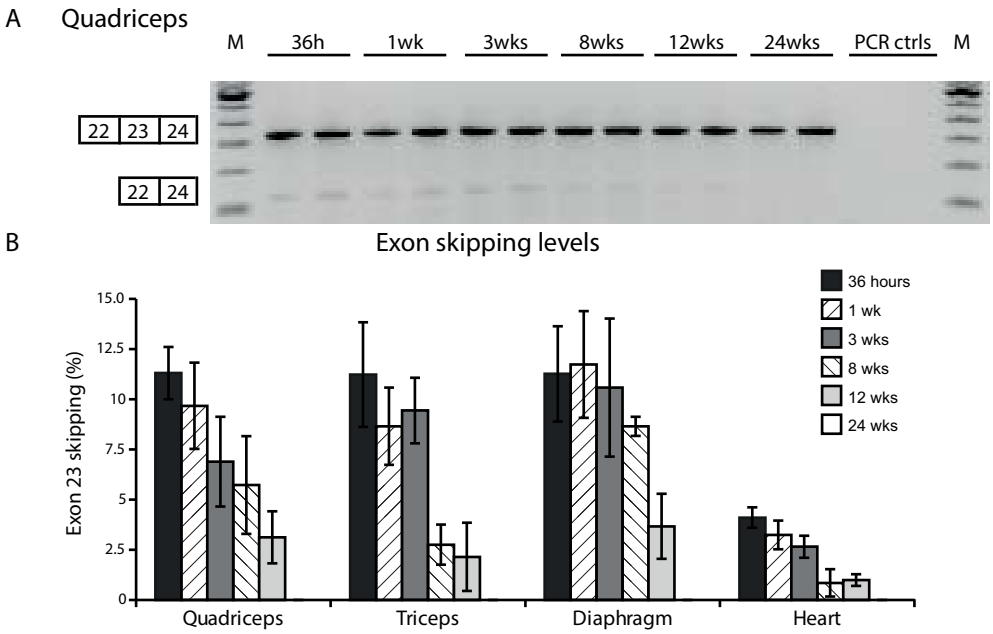


**Fig. 4.2:** Exon skipping levels in different muscles over time

a) Representative example of an RT-PCR analysis for the quadriceps. Unskipped product consists of 334 base pairs and exon 23 skipping results in a 121 base pair product. PCR ctrls indicates PCR controls, M is marker. b) Quantification of exon 23 skipping levels by RT-PCR analysis at several time points after the final 23AON injection. Data are represented as mean ± SD. AON, antisense oligonucleotide.

and one week after final AON injection) and declined thereafter. Exon skipping levels were significantly lower in heart compared to skeletal muscle ($p<0.01$). After 24 weeks skipping was undetectable in all samples. No large differences in rate of decline between individual muscles were observed, considering the interindividual variations within a tissue and the half-life varied between 33 and 53 days.

## *Dynamics of dystrophin protein expression*

Restoration of dystrophin protein expression after AON treatment was detectable at each time point in quadriceps and diaphragm (Fig. 4.3; Table 4.2). No dystrophin was observed for untreated *mdx* mice (data not shown). In treated muscles dystrophin levels were initially low, peaked somewhere between three and eight weeks after the final injection for both diaphragm and quadriceps, and then slowly declined. Levels in diaphragm were and remained significantly ($p<0.01$) higher than those in quadriceps and also were slightly more stable, with half-lives of ~100 and 130 days, respectively. In heart no dystrophin protein levels >0.5% could be detected (data not shown).

## *Correlation among 23AON levels, exon skipping, and protein expression*

For the majority of muscles analysed, a correlation among 23AON levels, exon skipping and protein expression was observed. The highest 23AON levels were observed in the diaphragm, resulting in highest exon skipping and dystrophin protein expression. Lower 23AON levels were observed in quadriceps and triceps, resulting in exon skipping levels that were at most

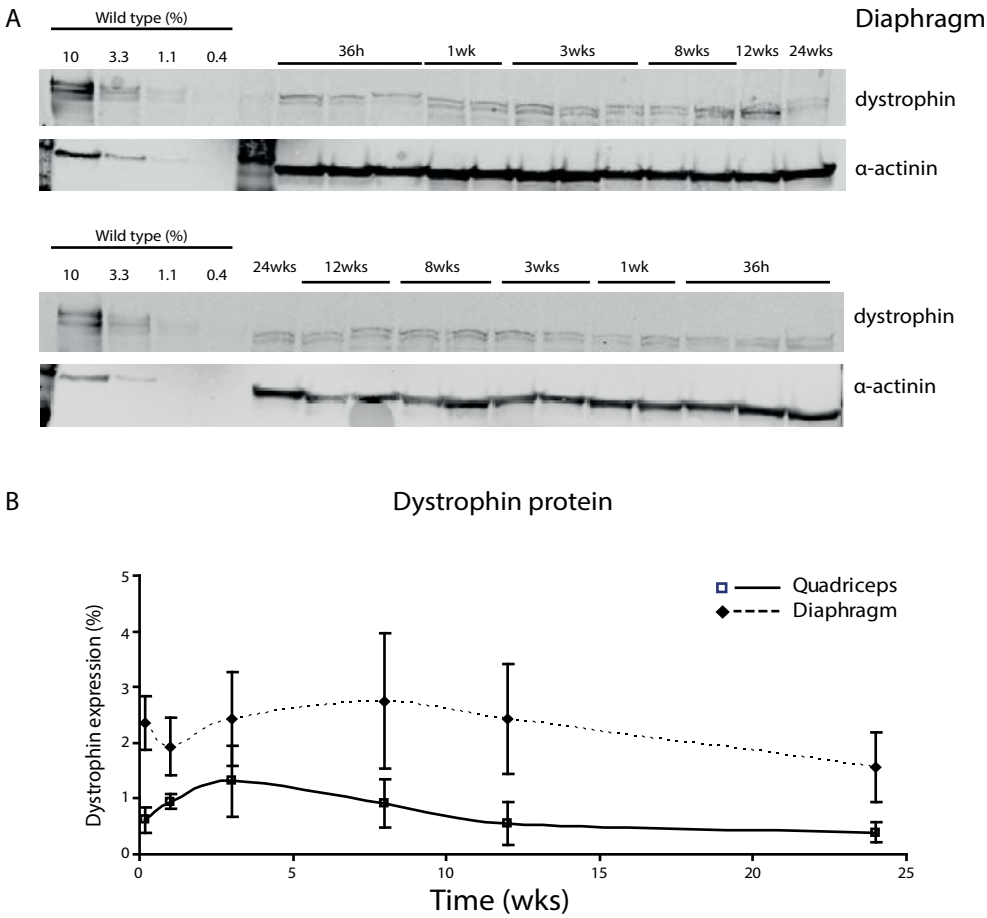


**Fig. 4.3:** Dystrophin protein levels in muscle over time

a) Representative examples of Western blot analyses for the diaphragm showing low levels of dystrophin protein (*top*) at all analysed time points. As loading control, α-actinin (*bottom*) was used. b) Quantification of dystrophin protein levels, detected by Western blot analysis. Data are represented as mean ± SD for each tissue.

time points analysed, slightly lower than or comparable with exon skipping levels in diaphragm. Furthermore, the level of dystrophin protein expression in quadriceps was lower than the corresponding values observed in diaphragm. By contrast, this correlation was not observed in heart, wherein 23AON levels were relatively high (comparable with diaphragm), while exon skipping levels were significantly lower than those in skeletal muscle; moreover dystrophin protein expression was very low.

When comparing patterns of the different parameters, both 23AON and exon skipping levels peaked shortly after treatment, within the first week, and thereafter showed a similar decline pattern, whereas the profile of dystrophin protein expression was shifted, peaking a few weeks after treatment and displaying a more prolonged effect (Fig. 4.4).

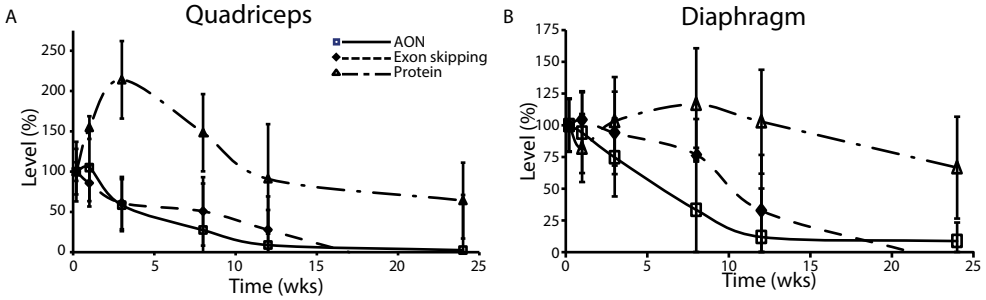


**Fig. 4.4:** Comparison of time effects at different levels
Comparison of time course of 23AON levels, exon skipping, and protein expression after the final injection for (a) quadriceps and (b) diaphragm. Data are represented as percentages of levels observed at time point t=36 hours (set at 100%) for 23AON, exon skipping ,and protein levels separately. Error bars represent the standard deviation. AON, antisense oligonucleotide

## Discussion

AON-mediated exon skipping is currently in advanced stages of clinical development as a potential therapy for DMD aiming to partly correct consequences of the genetic defect. However, due to clearance of AONs, skipped transcripts, and restored dystrophin protein, chronic treatment will be required. One way to try and prolong the effect is to deliver antisense sequences using (viral) vectors in order to achieve a stabler expression of the AONs. Adeno-associated viruses (AAVs) expressing modified small nuclear ribonucleoprotein (snRNP) particles, *i.e.* U1 and U7 small nuclear ribonucleoprotein particles, in which the small nuclear RNA is replaced by the desired AON sequence, have shown to induce long term dystrophin rescue in the *mdx* mouse.[246] This approach has also been proved to result in long term cardiac expression of dystrophin in the GRMD dog,[327] harbouring a deletion of exon 7, requiring exon 6 and 8 skipping to restore the reading frame.[122] Drawbacks of using AAVs as vectors are toxicity concerns and the possibility of an immune response against the viral vector, resulting in the loss of transduced fibres.[300] A recent five year follow-up study in GRMD dogs did not show immune rejection; however after five years, disappearance of dystrophin protein expression was observed, probably due to instability of the newly formed protein.[251] The same was observed in a study in *mdx* mice, wherein it was also shown that AAV vectors and transgene expression were lost quicker from dystrophic muscle than from healthy muscle.[252] Therefore, repetition of treatment would still be required, which is not possible due to immunization against the AAV after the first injection. This makes it unlikely this approach will be clinically applicable in the near future. Thus, the AON approach is more viable in the short term, which involves repeated injections, and knowledge about the PK and PD effects is, therefore, valuable. The detailed longitudinal analysis of different muscles, kidney and liver is ethically and practically challenging in humans but more straightforward in mice. In this study, the clearance/turnover of AON, transcript, and protein over time in *mdx* mice after treatment with 2OMePS AON, one of the two background chemistries mainly used in clinical trials, was studied.

First of all the decline in 23AON levels over time was studied in plasma, muscle, and other organs following subcutaneous injections. After injection, AON levels in plasma decline rapidly due to uptake by organs and clearance, which occurs primarily through the kidney. For 2OMePS AONs this is partly modulated by the plasma protein binding properties of the phosphorothioate backbone of the AONs, but when high doses are injected exceeding the

binding capacity, unbound AONs will be cleared rapidly.[668,669] The first time point analysed was 36 hours after the final injection, the time point when previous studies indicated that the majority of the plasma clearance has already occurred.[240] The mean half-life determined for 23AONs in mice (30 days) was comparable with that observed for 51AON in patients (29 days).[229] When comparing different muscle types, higher 23AON levels in diaphragm and heart were observed, whrereas the longest terminal half-life was found in heart. This was probably due to reduced AON turnover because the turnover rate of cardiac muscle cells is much slower than that of skeletal muscle fibres (especially dystrophic skeletal muscle fibres). This is in line with previous observations of a longer half-life of AONs in heart compared with that in skeletal muscle. In the previous study, a markedly shorter half-life in triceps (~10 days) was observed compared with that in quadriceps (~33 days), whereas in the current studies half-lives are comparable (~30 days). Furthermore, the currently calculated half-life in heart is longer (65 days versus 46 days).[200] These discrepancies are probably due to the fact that in the previous study only four time points up to 14 days after the final injection were measured, consisting of two mice per time point, making the estimations less accurate. Furthermore, in the previous study intravenous injections were used, which show higher levels of AON uptake by the kidney and liver,[200] which can also have contributed to the observed differences. As expected, AON levels in liver and kidney were much higher than those in muscle. It has been noticed before that for 2OMePS AONs levels are similar in liver and kidney[161] and here is shown that the decline pattern is comparable too. Unfortunately, when comparing the terminal half-lives in the non-target organs (liver and kidney) to the targeted organ (muscle), half-lives were slightly shorter in limb muscles compared with those in liver and kidney. A longer muscle half-life would have been advantageous for planning off-treatment periods, *e.g.* to prevent accumulation of compounds in liver and kidney. Nevertheless, the absolute reduction of AON levels in liver and kidney is greater than that in muscles.

Second, exon skipping levels were determined in several skeletal muscles and the heart. Here, highest levels were also observed shortly after the final injection (within the first week), whereupon they declined in a similar pattern as the 23AON levels. For diaphragm, the higher 23AON levels were also accompanied by high exon skipping levels whereas by contrast, this correlation was not observed in heart, wherein, although 23AON levels were relatively high (comparable to diaphragm), exon skipping levels were significantly lower than those in skeletal muscle.

Third, dystrophin protein levels were determined. These showed a delayed pattern, peaking a few weeks later, compared with AON and exon skipping levels. Importantly, although levels were low in general, dystrophin protein expression was observed up to 24 weeks. Wu *et al.* determined the half-life of the dystrophin protein in *mdx* mice after a single intravenous injection with peptide-conjugated PMOs (pPMOs) targeting exon 23. This resulted in a protein half-life of ~2 months for skeletal muscles and levels had dropped to around 10% after five months.[206] The half-lives measured in our experiments were a bit longer (three to four months), resulting in a smaller decrease in dystrophin levels; 30-50% of initial levels were still observed after ~5 months (levels at 24 weeks compared with peak levels) in both muscles. A possible explanation is the difference in experimental set-up and backbone chemistry of the AONs between both studies. In the present study, 2OMePS AONs were used, whereas the study of Wu *et al.* used pPMOs. These two chemistries probably have a different biodistribution pattern, as was also seen between 2OMePS AONs and naked PMOs.[161] Because AON PKs were not measured in the study of Wu *et al.*, it is not known how much AON remained in the muscle to induce further exon skipping and dystrophin production,

resulting in differences over time. Furthermore Wu *et al*. only used a single AON injection, whereas mice were treated for a longer period in our study. A single injection is more likely to give variation in AON levels between mice, which influences the preciseness of the half-life estimations. Therefore, both results are not directly comparable. Unfortunately, dystrophin protein was barely detectable in heart. This was also seen in a study using PMO AONs wherein dystrophin expression could be detected in all analysed muscles, except for the heart, after seven weeks of treatment.[202] Probably, overall higher skipping levels and/or longer treatment times are needed for protein restoration. This is underlined by the fact that after six months of weekly treatment with 200 mg/kg, dystrophin was readily detectable in heart.[201]

Overall there was a good correlation between AON effects at different levels. In diaphragm, the higher 23AON levels were accompanied by higher exon skipping levels and protein expression. Only in heart, the higher 23AON levels did not result in higher exon skipping and protein levels. This can be explained by inherent differences between cardiac and skeletal muscle. As mentioned before, skeletal muscles become permeable in the absence of dystrophin, facilitating the uptake of AONs. The heart is built up of individual cardiomyocytes, which do not display this leakiness, thereby making it harder for AONs to get inside.[200,203] The assay used for determination of 23AON levels does not discriminate between AON inside the heart as whole organ and AON that is actually inside the cardiomyocytes. Therefore, the presence of higher 23AON levels, but lower exon skipping levels, suggest that in heart the majority of AON is probably located in the interstitium and therefore ineffective. This might also be the reason why, although low 23AON levels were still detectable after 24 weeks, no exon skipping could be observed at this time point. Other possibilities are that this is due to differences in sensitivity of the assay used for determination of AON levels versus those for determining exon skipping levels or that the low AON levels are insufficient to induce exon skipping. Expression percentages for dystrophin protein are much lower than those for exon skipping. This is usually seen for both 2OMePS[201,240] and PMO/pPMO AONs,[161,206,211,212] and this discrepancy can be explained by the fact that exon skipping levels are compared to the total transcript levels in the *mdx* mouse, whereas dystrophin protein levels are compared with wild type dystrophin levels. It has been reported that compared with wild type dystrophin transcripts, a 5' to 3' imbalance exists for *mdx* dystrophin transcripts. This means that not all *mdx* dystrophin transcripts are complete and cannot be translated into dystrophin protein.[674]

In conclusion, 23AON and exon skipping levels show a similar decline pattern over time after the final injection. Exon skipping is not detectable anymore after six months, but its effects on dystrophin protein restoration remain quite stable. After treatment AONs are taken up by muscles and other organs, partly leading to immediate excretion without inducing further effects and partly leading to exon skipping inside the muscle fibres. Thereafter, new dystrophin protein can be formed, which displays a long half-life. The differences in rates of turnover of the compound itself, RNA and protein influence these effects, in addition to differences in composition and amount of degeneration and regeneration between different muscle groups. Our results proved further insight in how these processes interact and are useful for studying the long term effects of AON treatment.

## Acknowledgments

The authors are grateful to Rick Vermue (Prosensa Therapeutics) for assistance with the hybridization–ligation assay. This work was supported by grants from the Prinses Beatrix Spierfonds and Spieren voor Spieren (the Netherlands) and the Dutch Duchenne Parent Project. This work was carried out in Leiden, the Netherlands.

A.A.-R. discloses being employed by Leiden University Medical Center, which has patent applications on exon skipping that are licensed to Prosensa Therapeutics. As a coinventor on some of these patents, A.A.-R. is entitled to a share of royalties. J.A.S., S.d.K., I.G.M.K., and J.C.T.v.D. report being employed by Prosensa Therapeutics. J.C.T.v.D. discloses being coinventor on exon-skipping patents and being entitled to a share of royalties. L.L., J.E.R., and S.R.H. report being employed by GlaxoSmithKline. The other authors declare no conflict of interest.

# The effect of 6-thioguanine on alternative splicing and antisense-mediated exon skipping treatment for Duchenne muscular dystrophy

Ingrid E.C. Verhaart and Annemieke Aartsma-Rus

Previously published in: PLoS Curr; 2012: 4.

Chapter 5

## Abstract

The severe muscle wasting disorder Duchenne muscular dystrophy (DMD) is caused by genetic defects in the *DMD* gene, leading to a complete absence of dystrophin protein. Of the therapeutic approaches addressing the underlying genetic defect, exon skipping through antisense oligonucleotides (AONs) is the closest to clinical application. Several strategies to improve the efficiency of this approach are currently being investigated, such as the use of small chemical compounds that improve AON-mediated exon skipping levels. Recently, enhanced exon skipping in combination with a guanine analogue, 6-thioguanine (6TG) was reported for phosphorodiamidate morpholino oligomers (PMO). Here the effect of 6TG on the exon skipping efficacy of 2'-*O*-methyl phosphorothioate RNA (2OMePS) and PMO AONs *in vitro* and *in vivo* was further evaluated, as well as the effect of 6TG by itself. Results confirm an increase of exon skipping levels *in vitro*, however, in contrast to the previous report, no effect was observed *in vivo*. Importantly, 6TG treatment *in vitro* resulted in numerous additional *DMD* exon skipping events. This, in combination with the known cytotoxic effects of 6TG after incorporation in DNA, warrants reconsidering of the use of 6TG as enhancer of AON efficiency in DMD, were chronic treatment will be required.

## Introduction

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are both muscle wasting disorders caused by mutations in the *DMD* gene. However, DMD is characterized by a much more severe and progressive phenotype than BMD. In DMD, children generally become wheelchair-dependent around the age of ten, need assisted ventilation before their twenties, develop cardiac problems shortly thereafter and die before their thirties. By contrast, the BMD phenotype is much more variable with an average age of onset around 12, wheelchair-dependency in their twenties and death in their forties for the most severely affected patients.[5] However, less severely affected patients can remain asymptomatic into their fifties and have nearly normal life expectancies.[675] Furthermore, only around 50% of BMD patients develops dilated cardiomyopathy.[5] The differences between both phenotypes are due to the different nature of the underlying genetic defects. The *DMD* gene, located on Xp21, encodes dystrophin, an important protein for muscle fibres. Dystrophin forms a bridge between the actin cytoskeleton and the extracellular matrix, thereby giving mechanical stability to the fibres during contraction.[670] In DMD the reading frame is disrupted, leading to a premature stop of translation and thereby formation of a truncated, non-functional protein. Consequently, fibres are easily damaged and die, which leads to a gradual loss of muscle tissue and replacement by fibrotic and adipose tissue. By contrast, in BMD mutations do not affect the reading frame, allowing protein translation to continue, resulting in internally deleted dystrophin proteins, which are partly functional. Therefore in BMD patients the disease progression is slower.[49]

This difference underlies the rationale of the exon skipping therapy. Hereto antisense oligonucleotides (AONs) are used to hide a specific exon from the splicing machinery, inducing the skipping of this exon in order to restore the reading frame and allow the production of a shortened, Becker-like dystrophin protein. After demonstration of proof-of-principle and obtaining encouraging results *in vitro* in cultured patient-derived cells and *in vivo* in animal models, clinical trials are currently ongoing.[673] For these trials two different backbone chemistries are used: 2'-*O*-methyl phosphorothioate RNA (2OMePS) and phosphorodiamidate morpholino oligomers (PMO).

There are several ways to further optimize the efficiency of AON-mediated exon skipping. First, AON efficiency can be improved following previously identified guidelines that take into account target RNA accessibility, thermodynamic properties and the presence of known splicing motifs.[676] Furthermore the efficiency depends on the amount that reaches its target organ (muscle and heart) and organelle (cell nucleus), but also on the turnover of the compound, skipped transcripts and internally deleted dystrophin proteins, and on the muscle cells. A second approach has been to improve AON efficacy by conjugating PMOs to cell-penetrating peptides (pPMOs) or dendrimeric octaguanidine polymers (vivo-morpholinos).[163,677] These indeed lead to enhanced exon skipping and protein restoration, but also raise safety concerns.[162] Determination of a relatively low $LD_{50}$ of pPMOs indicates a high level of acute toxicity.[206] A third strategy is to optimize administration or dosing regimens [Verhaart *et al.*, manuscript submitted]. Another possibility is to enhance exon skipping levels using small chemical compounds. Chemical compounds that influence splicing have been reported, *i.e.* the cytokine kinetin to specifically correct the splice defect in familial dysautonomia and several compounds have been identified to enhance exon 7 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.[255] However this compound is specific for this mutation, so its application is rather limited. In order to identify more

general exon skipping enhancing compounds, large drug screening systems have been used. Ideally these compounds should enhance AON-induced exon skipping rather than inducing skipping by themselves, as the latter involves the risk of aspecific exon skipping events. For DMD, Hu *et al.* identified a guanine analogue, 6-thioguanine (6TG) that enhances PMO-induced exon 23 skipping *in vitro* in cultured *mdx* cells, which carry a nonsense mutation in exon 23, thereby causing the absence of dystrophin protein.[79] Increased exon skipping levels were also observed after local intramuscular injection of AONs and 6TG in *mdx* mice.[256]

6TG is widely known as an antileukemic agent due to its cytotoxic effects after incorporation in the DNA during replication.[678] The mechanism by which 6TG enhances PMO-induced exon skipping on pre-mRNA level is not known. Hu *et al.* suggested that alternative splicing could be enhanced after alteration of the structure of the *DMD* gene by incorporation of 6TG. Alternatively, 6TG, a small nucleobase, might interact with the bases of the PMO, thereby improving delivery efficiency to the nucleus and binding to the targeted sequence.[256]

To investigate whether the effect of 6TG on exon skipping also applies to other exons and AON chemistries, here the effects of 6TG treatment alone or in combination with AONs targeting several human exons *in vitro* and mouse exon 23 *in vitro* and *in vivo* were examined. *In vitro* 6TG indeed enhanced exon skipping levels for all targeted exons, but only at low AON concentrations or with suboptimal AON sequences. However, we were not able to detect the previously reported, enhanced exon skipping levels for 2OMePS or PMO AONs. Furthermore, *in vitro* 6TG treatment resulted in numerous additional, unintended *DMD* exon skipping events.

## Materials and methods

### *Cell culture*

An immortalized control human myoblast cell line 7304.1,[679] a mouse myoblast cell line C2C12 and primary DMD patient-derived myoblasts cultures with a deletion in exon 51-55 (DL589.2)[177] were used in this study. Cells were cultured on a collagen layer (1:30; Pure Col; Nutacon BV; The Netherlands). DL589.2 cells were cultured in Nutrient Mix F-10 (HAM) supplemented with GlutaMAX, 20% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (P/S) (Gibco-BRL; the Netherlands). For 7304.1 cells this medium was mixed 1:1 with Skeletal Muscle Cell Basal Medium supplemented with 10% FBS, 1.4% GlutaMAX, 1% P/S, 5 µg hEGF, 0.5 µg hFGF, 25 mg Fetuin, 5 mg Insulin and 200 µg Dexamethasone (PromoCell GMbH; Germany). C2C12 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, without phenol red) supplemented with 10% FBS, 1% P/S, 2% GlutaMAX and 1% D-glucose (Gibco-BRL). Human cells were grown at 37°C, 5% $CO_2$ and mouse cells at 37°C, 10% $CO_2$. To induce differentiation into myotubes, medium was changed into DMEM supplemented with 2% FBS, 1% P/S, 2% GlutaMAX and 1% D-glucose (Gibco-BRL) for C2C12 and patient-derived cells and into DMEM supplemented with 3% horse serum (Gibco-BRL), 1% P/S and 2% GlutaMAX for 7304.1 cells when cells were 80-90% confluent. Cells were differentiated in 3 mL differentiation medium per well of a 6-wells plate for 5 to 10 days depending on the cell type.

## *6TG treatment and AON transfection*

6-Thioguanine (6TG; Cas number 154-42-7; Sigma-Aldrich; the Netherlands) was added 48 hours before AON transfection and maintained during and after transfection at indicated concentrations (30-90 µM). For 2OMePS AONs (Eurogentec; Belgium and Prosensa Therapeutics; the Netherlands) cells were transfected in 1 mL differentiation medium for 4 hours with indicated concentrations using polyethylenimine (PEI, Exgen 500; MBI Fermentas; Germany), according to the manufacturer's instructions, using 2 µL of PEI per µg of AON for human cells and 3.5 µL of PEI per µg of AON for mouse cells. Cells were harvested >24 hours after transfection. For PMO AON (Gene Tools; OR, USA) experiments, PMOE23(+07-18) was added 72 hours before RNA analysis with or without 6TG at indicated concentrations according to manufacturer's instructions. An overview of used AONs, their backbone chemistries and sequences is given in table 5.1.

| *AON name* | *Target species* | *Chemistry*[a] | *Sequence (5'-3')* |
|---|---|---|---|
| h45AON5 | human | 2OMePS | gcccaaugccauccugg |
| h50AON1 | human | 2OMePS | cucagagcucagaucuu |
| h50AON2 | human | 2OMePS | ggcugcuuugcccuc |
| h53AON1 | human | 2OMePS | cuguugccuccgguucug |
| h55AON1 | human | 2OMePS | cuguugcaguaaucuaugag |
| M23D | mouse | 2OMePS | uccauucggcuccaaaccgg |
| PMOE23 | mouse | PMO | ggccaaaccttcggcttacctgaaat |

**Table 5.1:** Overview of AONs and their sequence used in this study
[a]2OMePS=2'-*O*-methyl phosphorothioate RNA; PMO=phosphorodiamidate morpholino oligomers

## In vivo *23AON and 6TG treatment*

All experiments were approved by and performed following the guidelines of the Dier Experimenten Commissie (Animal Experimental Commission) of the Leiden University Medical Centert (permit number: 08224). Effort was put in minimizing the amount of distress caused to the animals as much as possible. Mice were housed in individually ventilated cages in the animal facility of the LUMC and received food and drink *ad libitum*. *Mdx* mice (C57Bl/10ScSn-DMD$^{mdx}$/J) were obtained from our own breeding facility.

For the first experiment 5 weeks old *mdx* mice were anesthetized with isoflurane. Mice were intramuscularly injected in both gastrocnemius muscles on 2 consecutive days with 0, 1, 10 or 50 µg 6TG with or without 20 µg (≡2.9 nmol) M23D(+2–18) 2OMePS AONs specifically targeting exon 23[197] (Prosensa Therapeutics) in 40 µL saline ($n$=2 per condition; 6TG dosing was kept constant within one mouse). Mice were sacrificed 10 days after the last injection by cervical dislocation and muscles were isolated.

For the second experiment 7 or 8 weeks old *mdx* mice were anesthetized with isoflurane and intramuscularly injected in both gastrocnemius muscles on 2 consecutive days with 20 µg (≡2.9 nmol) M23D(+2–18) 2OMePS AON, or 5 µg (≡0.60 nmol) M23D(+07-18) PMO AON with or without 25 µg 6TG or 25 µg 6TG only ($n$=4 per condition; 6TG dosing was kept constant within one mouse). Mice were sacrificed 10 days after the last injection by cervical dislocation and muscles were isolated.

## *RNA extraction and analysis of exon skipping by RT-PCR*

Harvested cells were lysed with TriPure isolation reagent (Roche Diagnostics; Switzerland). Total RNA was extracted and 400 ng of RNA was used for RT-PCR analysis, using Transcriptor reverse transcriptase polymerase (Roche) in 20 µL at 55°C for 30 min with an appropriate primer (primer sequences on request). cDNA was amplified by nested PCR. Three microliters of cDNA were amplified in a 25 µL reaction for 20 cycles of 94°C (40 sec), 60°C (40 sec) and 72°C (80 sec), followed by 32 cycles of 94°C (40 sec), 60°C (40 sec) and 72°C (60 sec), with 1.5 µL of PCR product in a 50 µL reaction.

Muscles were homogenized in TriPure isolation reagent, using zirconium beads (1.4 mm; OPS Diagnostics; NJ, USA) by grinding in a MagNA Lyser (Roche) according to manufacturer's instructions. Total RNA was extracted and 1 µg of RNA was used for RT-PCR analysis, using Transcriptor reverse transcriptase polymerase in 20 µL at 42°C for 45 min with random hexamer primers (20 ng/µL). Then, 1.5 µL of cDNA was amplified in a 50 µL reaction for 30 cycles of 94°C (30 sec), 60°C (30 sec) and 72°C (30 sec), as previously described.[663] All PCR products were visualized on 1.5 or 2% agarose gels and exon skipping levels were quantified using a DNA 1000 LabChip on the Agilent 2100 bioanalyzer (Agilent Technologies; CA, USA) according to the manufacturer's protocol.

## *Sequence analysis*

RT-PCR products were isolated from agarose gels using the QIAquick Gel Extraction kit (Qiagen; the Netherlands) according to manufacturer's instructions. Direct DNA sequencing was performed by the Leiden Genome Technology Center (Leiden, the Netherlands) using the BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) and analysed on a 3730*xl* DNA Analyzer (PE Applied Biosystems).

## *Protein extraction and Western blot analysis*

Muscles were minced in treatment buffer containing 75 mM Tris-HCl pH 6.8-15% (w/v) sodium dodecyl sulphate (SDS) using zirconium beads (1.4 mm; OPS Diagnostics) by grinding in a MagNA Lyser. Protein concentrations were determined using a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific; MA, USA) according to manufacturer's instructions. Samples containing 30 µg of protein were made in treatment buffer with 20% (v/v) glycerol, 5% (w/v) β-Mercaptoethanol and 0.001% (w/v) Bromophenol blue and heated for 5 min at 95°C. As reference, wild type (C57Bl/10ScSnJ) control samples containing 33%, 11%, 3.7%, 1.2% and 0.4% of protein were used. Samples were loaded on 1.0 mm thick native PAGE Tris-acetate (polyacrylamide) gels, with a linear resolving gel gradient of 3-8% (Bio-Rad; the Netherlands) and run on the Trans-Blot Turbo system for 1 h at 75 V (~0.07 A) and 2 hrs at 150 V (~0.12 A) in XT Tricine running buffer (Bio-Rad) in an ice container [Hulsker *et al.*, manuscript in preparation]. Ready to use Trans-Blot Turbo transfer packs in combination with the Trans-Blot Turbo transfer system (BioRad) at 2.5 A and ~25 V for 10 minutes were used to blot the proteins on a nitrocellulose membrane, which were blocked in 10 mM Tris-HCl (pH 8) and 0.15 M NaCl (TBS)-5% non-fat dried milk (Elk, Campina Melkunie; the Netherlands) and washed in TBS-0.05% (v/v) Tween20 (TBST). Membranes were incubated overnight with primary antibodies 1:125 NCL-Dys1 (1:125; Dy4; NovoCastra,; UK) and α-actinin (loading control; 1:5 000; AB72592; Abcam; UK) in TBS. Membranes were washed in TBST, incubated 1 h with the fluorescent secondary

antibodies IRDye 800CW goatαmouse IgG (1:5 000; Li-Cor; NE, USA) and IRDye 680LT donkeyαrabbit IgG (1:10 000; Li-Cor) in TBS, washed in TBST and TBS and analysed with the Odyssey system and software (Li-Cor).

## Results

### *6TG induces exon skipping by itself and enhances exon skipping by 2OMePS AONs in human muscle cells* in vitro

The effect of 6TG on exon skipping by 2OMePS AONs was first tested *in vitro* in cultured human muscle cells derived from a healthy control. Without 6TG exon 50 skipping was observed in decreasing levels till 50 nM of h50AON1. When 6TG was added, exon skipping was also observed for lower levels (25 and 5 nM) of h50AON1 and without AON. 6TG enhanced exon skipping levels for 50 nM of AON, but not for higher AON concentrations (100 and 200 nM) (fig. 5.1a). For a less efficient AON (h50AON2) again low exon skipping levels in combination with 6TG were observed for 0-25 nM of AON. However, for higher AON concentrations (50-200 nM) a clear enhancement was observed in combination with 6TG compared to h50AON2 only (fig. 5.1b). The same effect was seen for the AONs h45AON5, h53AON1 and h55AON, where low levels of exon skipping were detected without or with doses ≤25nM of AONs and an improvement in skipping levels was seen for 50-200 nM (data not shown).

Thereafter, the effect on DMD patient cells with a deletion of exon 51-55 was investigated. For this deletion, the reading frame can be restored by exon 50 skipping. As has been reported previously, there is some very low level of exon 50 skipping detectable in untreated cells.[177] Treatment with increasing concentrations of h50AON1 (fig. 5.1c) or h50AON2 (data not shown) resulted in a dose-dependent increase of exon 50 skipping. Exon skipping levels were further increased by co-treatment with 6TG. However, 6TG treatment by itself also resulted in similar levels of exon skipping (fig. 5.1c, most right black bar).

Because exon skipping was observed after 6TG addition in the absence of AONs, other regions of the dystrophin transcript were investigated to assess exon skipping events. The exon 70-75 region is known to be subjected to alternative splicing events in a tissue and differentiation-specific manner. In untreated human control cells exon 71 and 71 to 74 skipping was observed (fig. 5.1d/e, left lane/bars). However, after 6TG treatment the alternative exon skipping events were greatly enhanced and exon 71, 74, a combination of 71 and 74, and 71-74 skipping were observed (confirmed by sequence analysis (fig. 5.1d/e)). Furthermore, exon 45, 46, 53 and 55 skipping was observed in the exon 44-55 region (data not shown), suggesting that splicing is disrupted throughout the transcript rather than that 6TG specifically enhances AON-mediated exon skipping.

### *6TG enhances mouse exon 23 skipping in cultured mouse cells* in vitro*, but not in* mdx *mice* in vivo

The effect of 6TG was then tested *in vivo* by intramuscular injections of 2OMePS mouse exon 23AONs in the gastrocnemius muscles of *mdx* mice on two consecutive days. Mice were sacrificed ten days after the last injection and the injected muscles were isolated and analysed. Exon 23 skipping was only observed after treatment with 23AON and no enhance-

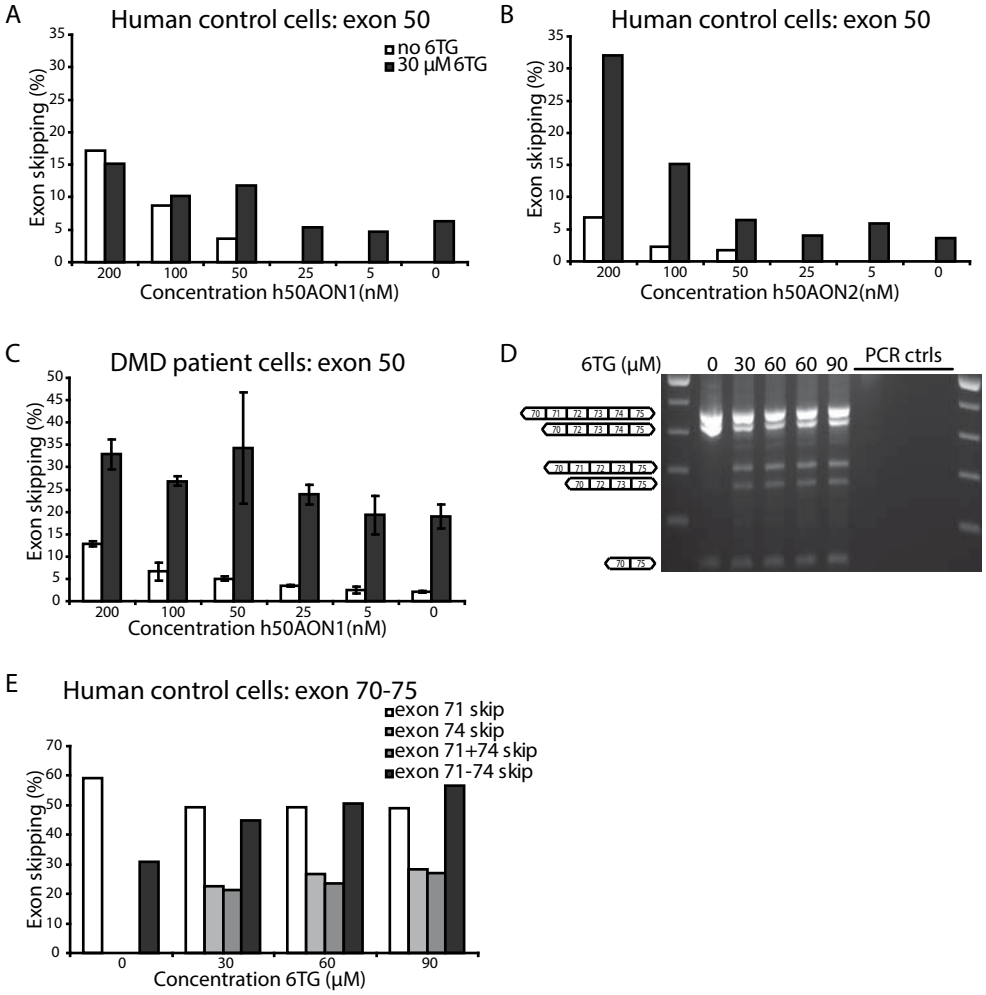


**Fig. 5.1:** Effect of 6TG on muscle cells *in vitro*.
a) In healthy control muscle cells, 6TG only enhanced exon skipping with 2OMePS AON at lower concentrations of AON (a) or with a less efficient AON (b). c) In DMD patient cells harbouring a deletion 51-55, skipping of exon 50 was observed after treatment with AONs against this exon. This was greatly enhanced by 6TG treatment. 6TG alone also induced high skipping of this exon (right bar). d/e) Example of greatly enhanced alternative exon skipping in control cells by 6TG around exon 70-75. In non-treated cells next to the wild type product (468 bp) only skipping of exon 71 (429 bp) and 71-74 (138 bp) was seen, whereas in 6TG-treated cells also skipping of 74 (309 bp) and combined skipping of exon 71+74 (270 bp) was observed.

ment was seen after co treatment with 6TG in any tested concentration (fig. 5.2a).

Since Hu *et al.* reported increased exon 23 skipping levels after combinational treatment with a PMO against exon 23 and 6TG compared to PMO alone,[256] the experiment was repeated with murine exon 23AONs of the 2OMePS and the PMO backbone. First the effect of 6TG was explored *in vitro* in mouse C2C12 cells for both chemistries. Here, only a moderate increase of exon 23 skipping was observed after treatment with low concentrations of either AON. The increase was less obvious at higher AON concentrations (fig. 5.2b). Note that exon skipping levels induced by 2OMePS and PMO AON cannot directly be compared, since for 2OMePS AONs PEI is needed as a transfection reagent (leading to very efficient

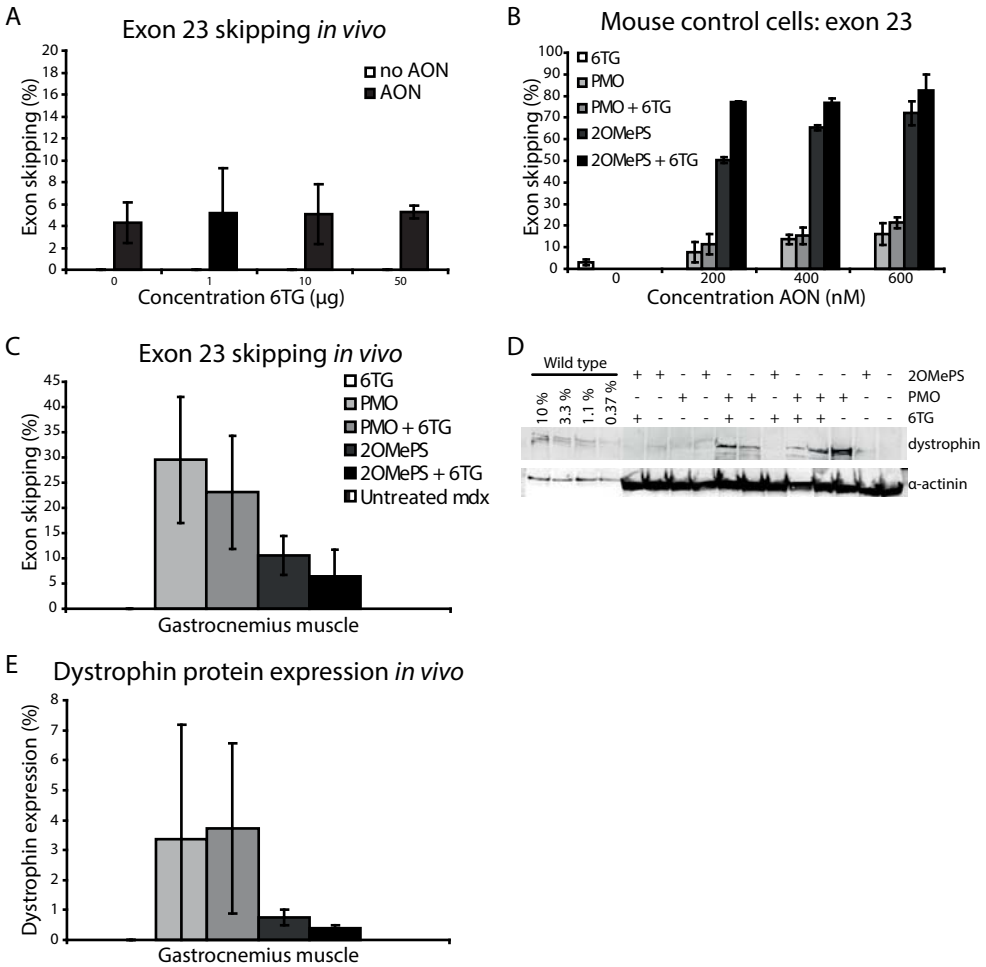


**Fig. 5.2:** Effect of 6TG on exon 23 skipping and dystrophin protein expression.
a) *In vivo* in *mdx* mice no enhancement by 6TG of exon skipping by 2OMePS AON targeting exon 23 was observed. No skipping of exon 23 was observed after injection with 6TG alone. b) *In vitro* in a mouse control cell line, 6TG enhanced exon skipping by both 2OMePS and PMO AONs only moderately at low AON concentrations. Without AON treatment no exon 23 skipping was observed. However 6TG alone induced a small percentage of exon 23 skipping by itself (left white bar). c) After local injection *in vivo*, no enhancement of exon skipping by 2OMePS or PMO AON against exon 23 was observed. No skipping of exon 23 was observed after injection with 6TG alone. d/e) No effect of 6TG on dystrophin protein expression was observed by itself or after combinational treatment with AON.

transfection), while PMOs were added to the medium. Hu *et al.* did not observe exon skipping after treatment with 60 or 90 µM 6TG by itself. However, our results showed some exon skipping with 60 µM 6TG in the absence of AON treatment (fig. 5.2b, left white bar).

Thereafter the *in vivo* experiment with local treatment was repeated now using both PMO and 2OMePS AONs. As has been observed previously, PMOs induced more efficient exon 23 skipping than 2OMePS AONs.[161] Again, no increase of exon skipping levels by 6TG was seen for 2OMePS AONs (fig. 5.2c). Furthermore, in contrast to the results obtained by Hu *et al.*, exon skipping levels by PMO AONs were also not increased (fig. 5.2c). No induction of exon 23 skipping was observed after treatment with 6TG alone. Finally, the effect of 6TG on dystrophin protein expression was investigated (fig. 5.2d/e). Untreated *mdx* mice or

mice treated with 6TG alone did not have any dystrophin expression. Treatment with PMO and 2OMePS AONs induced dystrophin expression, but combinational treatment with 6TG did not increase the dystrophin levels. In accordance with the higher exon skipping levels, dystrophin levels were higher after PMO than 2OMePS treatment. However, the differences between individual mice were larger as well after PMO treatment.

In summary, 6TG by itself induced numerous exon skipping events *in vitro* at low concentrations and only showed enhancement of exon skipping levels at low AON concentrations and/or of suboptimal AONs. *In vivo* no effect of 6TG on exon skipping levels was observed.

## Discussion

At present only palliative therapies are available for DMD. Exon skipping induced by AONs is the most promising therapeutic strategy that targets the underlying genetic effect and is currently tested in phase III clinical trials.[673] Since higher exon skipping levels will probably lead to enhanced therapeutic effects, several ways to improve efficiency are under investigation. One of the strategies is to combine AON treatment with chemical compounds. 6TG was identified as a compound that could enhance PMO mouse exon 23 skipping *in vitro* and locally *in vivo*.[256] To further explore whether the effects of 6TG on exon skipping also apply to other exons and AON chemistries, here additional experiments were performed.

*In vitro* results showed that 6TG indeed had the potential to enhance exon skipping for 2OMePS AONs as well, but only when combined with suboptimal AONs or at suboptimal concentrations. Furthermore, various additional exon skipping events were observed after treatment with 6TG alone in different regions of the dystrophin transcript. By contrast, *in vivo* after local, intramuscular injections no enhancement of AON-induced exon skipping levels or exon skipping events by 6TG alone were observed. This in contrast to previous published results where increased exon skipping was observed after intramuscular PMO and 6TG injections and no exon skipping events were detected after 6TG treatment alone.[256] Our first explanation for this discrepancy was that the enhancing effect of 6TG might be dependent on the backbone chemistry of the AON, which influences the biophysical, biochemical and biological properties (for more information see Chan *et al.* Clin Exp Pharmacol Physiol 2006[150] and Amantana *et al.* Curr Opin Pharmacol 2005[680]), but this was ruled out by repeating the experiment with AONs of both chemistries. We could still not reproduce the reported effects for PMOs, despite the fact that the conditions for the experiments were very similar: identical PMOs were used, the same route of administration was used, *mdx* mice were of comparable age (6-8 wks old) and time of analysis after injection (2 weeks/10 days) was almost identical. The only difference was the specific muscle that was injected (tibialis anterior versus gastrocnemius), but we feel it is unlikely this causes the observed discrepancy, since a correction factor (2.5 times) was used to account for the difference in size of the muscle. Absolute exon skipping percentages cannot be compared between both studies, since no values are reported in the published study and skipping percentages are known to vary with different methods of quantification.[663]

The exon skipping events we observed after 6TG treatment throughout the dystrophin transcript, suggest splicing may have been disrupted for other genes as well. As such, unintended effects of 6TG treatment cannot be ruled out. Furthermore, 6TG is known for its antileukemic effects due to cytotoxicity via several mechanisms after being incorporated in the DNA during replication. This causes miscoding, leading to recognition by

the post-replicative mismatch repair system, which cuts at the miscoded base pair.[681,682] Furthermore, the 6TG modified-DNA duplex modifies the structure very locally, which alters specific DNA-processing enzyme activities and recognition by proteins.[683,684] In addition, the presence of 6TG blocks the formation of quadruplex DNA by telomeres and other DNAs, which causes blockage of replication and thereby cytotoxicity after one round of replication.[685] All these effects explain the delayed onset of cytotoxicity by 6TG. These mutagenic effects mainly affects rapidly proliferating cells like leukemic cells,[686] but will also affect other dividing cells. Since 6TG in leukaemia is always used in treatment regimens combining several chemotherapeutic agents, it is difficult to interpret the contribution of 6TG itself to the toxicity observed after chemotherapy in most studies.[687,688] Long term treatment with 6TG alone in humans or animals has not been reported. However, there are reports about 6-mercaptopurine (6MP, a guanine analogue related to 6TG). Although there is no evidence for secondary malignancies after chronic maintenance chemotherapy with 6MP,[689] repeated treatment of mice with a low dose of 6MP resulted in lethal mutations.[690] Therefore, it is not unlikely 6TG by itself could have similar side effects. This combined with our own observations of disturbed splicing, makes us doubt whether 6TG would be a good candidate to improve the therapeutic efficiency of AON therapy.

However there are other candidates to improve exon skipping levels with more specific effects, like the aforementioned TG003 for exon 31 skipping.[255] Hopefully further research will identify a compound that can improve AON-mediated exon skipping more specifically.

## Funding Statement

IV is paid by a grant from the Prinses Beatrix Spierfonds (the Netherlands) and AAR receives funding from ZonMw, the Prinses Beatrix Spierfonds and the Duchenne Parent Project (the Netherlands). The infrastructure of the Center for Medical Systems Biology and the Center for Biomedical Genetics was utilized to conduct the reported work. AAR reports being employed by LUMC, which has patents on exon skipping. As a co-author on some of these patents AAR is entitled to a share of royalties. IV declares no conflict of interest.

# Prednisolone treatment does not interfere with 2'-*O*-methyl phosphorothioate antisense-mediated exon skipping in DMD

Ingrid E.C. Verhaart, Hans Heemskerk, Tatyana G. Karnaoukh, Ingrid G.M. Kolfschoten, Anne Vroon, Gertjan B. van Ommen, Judith C.T. van Deutekom and Annemieke Aartsma-Rus

Previously published in: Hum Gene Ther; 2012: 23: 262-273.

Chapter 6

## Abstract

In Duchenne muscular dystrophy (DMD), dystrophin deficiency leading to progressive muscular degeneration is caused by frame-shifting mutations in the *DMD* gene. Antisense oligonucleotides (AONs) aim to restore the reading frame by skipping of a specific exon(s), thereby allowing the production of a shorter, but semifunctional protein, as is found in the mostly more mildly affected Becker muscular dystrophy (BMD) patients. AONs are currently investigated in phase III placebo-controlled clinical trials. Most of the participating patients are treated symptomatically with corticosteroids (mainly predniso(lo)ne) to stabilize the muscle fibres, which might affect the uptake and/or efficiency of AONs. Therefore the effect of prednisolone on 2'-*O*-methyl phosphorothioate AON efficacy in patient-derived cultured muscle cells and the *mdx* mouse model (after local and systemic AON treatment) was assessed in this study. Both *in vitro* and *in vivo* skip efficiency and biomarker expression were comparable between saline- and prednisolone-cotreated cells and mice. After systemic 23AON treatment for eight weeks, dystrophin was detectable in all treated mice. Western blot analyses indicated slightly higher dystrophin levels in prednisolone-treated mice, which might be explained by a better muscle condition and consequently more target dystrophin pre-mRNA. In addition, fibrotic and regeneration biomarkers were normalized to some extent in prednisolone- and/or 23AON-treated mice. Overall these results show that the use of prednisone forms no barrier to participation in clinical trials with AONs.

## Introduction

Duchenne muscular dystrophy (DMD) is a severe, muscle-wasting disease, affecting around 1 in 3 500 newborn boys. First symptoms generally become visible before the age of five years, followed by wheelchair dependency around the age of ten. Most patients die at about 30 years of age, predominantly due to heart and respiratory failure.[5]

DMD is caused by mutations in the *DMD* gene, located on the short arm of the X chromosome (Xp21), leading to the complete loss of the dystrophin protein it encodes.[39,691] Dystrophin stabilizes muscle fibres by connecting intracellular actin to the extracellular matrix,[692] through a long repeat domain that intersperses the two essential binding domains. In the absence of dystrophin, muscle fibres will be continuously damaged during contraction, which leads to an influx of inflammatory cells and secretion of cytokines, in particular tumour necrosis factor (TNF)-α.[358,426,427] TNF-α activates the IκB kinase (IKK)/NF-κB signalling pathway in macrophages, which in turn increases necrosis and inflammation and reduces regeneration in muscle fibres.[428,429] Fibrotic tissue is formed by fibroblasts and even by satellite cells, which, once fibrotic tissue has been formed, start to produce collagen type I and no longer take part in regeneration.[693] Fibroblasts from dystrophin-negative *mdx* mice, a naturally occurring mouse model for DMD,[79] remain activated even when activating factors from immune cells have dissipated.[694] These processes further increase fibrosis which gradually replaces the damaged muscle fibres. Especially fibrosis in the endomysium is negatively correlated with functional performance in patients.[695] Last, muscle regeneration is reduced through induction of the transforming growth factor (TGF)-*β* pathway by the fibrotic tissue.[696]

At present there is no cure for DMD. However, there are pharmacological approaches that try to combat the symptoms caused by the underlying genetic defect. The main treatment is the use of the corticosteroids prednisone, prednisolone (the active form of prednisone), or deflazacort (an oxazoladine derivative of prednisone).[368] Their exact mechanism of action is unknown, but studies have shown that corticosteroid-treated patients have increased muscle strength and remain ambulant for about three years longer than untreated patients.[10,13] In *mdx* mice a positive effect of prednisolone on muscle strength and histology (decrease of centrally located nuclei) was seen.[371] This beneficial effect is probably through the anti-inflammatory effects and the reduction of muscle necrosis.[10,369] In a dystrophin-deficient *Caenorhabditis elegans*, the amount of degenerating cells is decreased after treatment with prednisone.[369] Because *C. elegans* only has a very simple immune system, this indicates other mechanisms are involved as well. One study suggests that deflazacort activates the calcineurin/NF-AT (nuclear factor of activated T cell) pathway and thereby increases the expression of NF-AT target genes, among which the dystrophin homologue utrophin, which can partly take over the function of dystrophin, thereby decreasing the dystrophic muscle fibre pathology.[374] An alternative possibility is that the anabolic effect of corticosteroids in patients increases muscle regeneration and growth by enhancing proliferation of myogenic precursor stem cells or myoblasts.[10] Furthermore, corticosteroids have a positive effect on calcium homeostasis, which is deregulated in patients with DMD.[368] Unfortunately, corticosteroids also have deleterious side effects[375] such as osteoporosis,[377] weight gain, growth inhibition,[13] delayed puberty, and cataracts.[376] They have a catabolic effect on muscle in unaffected individuals,[378] but in DMD this is generally abrogated by the positive effects. In *mdx* mice it has even been shown that prednisolone induced fibrosis in the heart,[381] but this is not observed in patients with DMD, in whom corticosteroid use prevents/delays ventricular dysfunction.[382,383]

Approaches aiming to restore the underlying genetic defect of DMD are currently under

investigation. At the moment, the most promising strategy is exon skipping, using antisense oligonucleotides (AONs) to restore the disrupted reading frame. These AONs target a specific region in the pre-mRNA involved in appropriate exon inclusion. In this way they prevent the exon to be recognized by the spliceosome and, consequently, it will not be incorporated in the mRNA.[164] The resulting in-frame mRNA transcript allows translation of a protein that is internally deleted, but contains the essential actin and extracellular matrix binding domains, and therefore it will be partially to largely functional.[697] These dystrophins will resemble the internally deleted proteins found in patients with Becker muscular dystrophy (BMD), who have a much milder phenotype and longer life expectancy.[42,43] Proof of principle has been obtained *in vitro* with both healthy and patient-derived cultured primary human myoblasts[176,177,188] and in *in vivo* studies in *mdx* mice.[161,205,207,209] Exon skipping can be performed using AONs with various backbone chemistries.[161] For *in vivo* studies, mainly 2'-*O*-methyl phosphorothioate (2OMePS) and morpholino phosphorodiamidate (PMO) AONs are used. These have also been used in two exploratory clinical trials with local injections which have shown positive results.[225,226] A phase I-IIa systemic clinical trial with 2OMePS AONs has been completed and a dose-dependent effect was seen after subcutaneous injections with AONs against exon 51. Treatment resulted in exon skipping and dystrophin expression up to ~15% of normal expression levels. In an open label extension study, three months of weekly injections with the highest dose resulted in an increase in functional performance, without serious adverse events.[229] The first systemic clinical trial with PMOs showed promising results as well: seven out of 19 patients, mainly in the higher dose groups, showed dystrophin restoration after treatment, albeit with a lot of variability between patients.[231]

Larger multicenter placebo-controlled trials with 2OMePS AONs have now been initiated. The majority of the patients involved in these trials will use corticosteroids, primarily predniso(lo)ne. Thus far the effect of corticosteroid treatment on AON biodistribution and skipping efficiency has not been studied. Corticosteroids are thought to stabilize the damaged muscle fibre membrane, whereas the exon skipping approach actually makes use of the fact that in patients with DMD the membrane is leaky, allowing higher uptake of AONs.[200] Thus, corticosteroid treatment could result in decreased uptake of AONs and lower levels of exon skipping and dystrophin restoration. On the other hand, the exon skipping approach requires a sufficient amount of pre-mRNA to be effective, which is expressed only by muscle tissue and not by fibrotic tissue. Therefore, more dystrophin pre-mRNA might be available when the muscle is better preserved due to corticosteroid treatment. To elucidate this, the effect of co-treatment with 2OMePS AONs and prednisolone on patient-derived muscle cell cultures and in dystrophic *mdx* mice was assessed.

The results suggest that prednisolone treatment does not interfere with 2OMePS AON uptake and exon skipping levels in patient-derived muscle cells *in vitro* and *mdx* mice *in vivo*. Prednisolone might even enhance the dystrophin expression induced by exon 23-specific AONs (23AONs) in *mdx* mice.

## Materials and methods

### *Cell cultures and AON transfection*

Two patient cell cultures, DL589.2 (deletion exon 51-55) and 53914.1 (deletion exon 52) (previously described[177,188]) were grown to 80% confluency on high-serum medium.

Differentiation was induced by switching to low-serum medium. Prednisolone in saline (Leiden University Medical Center (LUMC) pharmacy, Leiden, The Netherlands) was added in doses ranging from 0.2 to 2.25 µg/mL to determine optimal concentration. For AON transfection experiments prednisolone was added at 0.75 µg/ml to the medium. When differentiation was deemed sufficient (generally after approximately ten days), h50AON1 and PRO051[176] were transfected in the respective patient cell cultures and the control cells using polyethylenimine (PEI, Exgen 500; MBI Fermentas, Sank Leon-Rot, Germany), according to the manufacturer's instructions and using 2.5 µL PEI per µg AON ($n$=12 per condition per cell line). All AONs were 2'-*O*-methyl RNA oligonucleotides with a full-length phosphorothioate backbone (Eurogentec, Seraing, Belgium and Prosensa Therapeutics, Leiden, The Netherlands). Cells were harvested two days after transfection.

## In vivo *23AON and prednisolone treatment*

All experiments were approved by the local animal ethical experimental committees. Mice were housed in individually ventilated cages in the animal facility of the LUMC (Leiden, The Netherlands) or Laboratory of Pharmacology and Toxicology (LPT; Hamburg, Germany) and received food and drink *ad libitum*. Intramuscular experiments were performed at the LUMC and *mdx* mice (C57Bl/10ScSn-DMD$^{mdx}$/J) with one or two copies of the utrophin gene (*mdx*/*Utrn*$^{+/-}$ or *mdx*/*Utrn*$^{+/+}$) were obtained from our own breading facilities. The systemic experiments were performed at the LPT and *mdx* mice from Charles River Laboratories (Sulzfeld, Germany) were used.

For the intramuscular 23AON treatment, mice were subcutaneously[698] injected with prednisolone (1 mg/kg; LUMC pharmacy) ($n$=4) or saline ($n$=4) on weekdays from the age of four weeks until the end of the experiment. At the age of eight weeks, the mice were anaesthetized with isoflurane and intramuscularly injected via both gastrocnemius muscles on two consecutive days with 2.9 nmol (~20 µg) M23D(+2-18), 2'-*O*-methyl phosphorothioate RNA oligonucleotides with a full-length phosphorothioate backbone, specifically targeting exon 23[197] (produced by Prosensa Therapeutics) in 40 µL saline. Ten days after the second injection the mice were sacrificed by cervical dislocation and muscles were isolated.

For the systemic 23AON treatment male *mdx* mice ($n$=8-10 per group) at the age of five weeks (day 1) were anaesthetized with ether and underwent surgery to implant a prednisolone pellet (1 mg/kg/day in a 60-day slow-release subcutaneous pellet[379]) under the dorsal skin (groups 2 and 4) or underwent mock surgery (groups 1 and 3) at the start of the experiment. Mice were injected subcutaneously with 250 mg M23D(+2-18)/kg body weight once daily for five days in test week 1 and 100 mg M23D(+2-18)/kg body weight twice a week in test weeks 2 to 8 (groups 3 and 4), or saline (groups 1 and 2). Mice were sacrificed ten days after the last injections and muscles (gastrocnemius, tibialis anterior, quadriceps, heart, and diaphragm) and organs (liver and kidney) were isolated, snap frozen in liquid nitrogen-cooled 2-methylbutane, and stored at -80°C.

## *Measurement of creatine kinase levels*

Blood samples were taken weekly via the tail vein. Samples were centrifuged at 1 700 *g* for 10 min at 4°C. Serum was stored at 4°C and creatine kinase (CK) levels were measured after diluting them 10 times in Dulbecco's phosphate-buffered saline (D-PBS; Invitrogen, Carlsbad, CA) and were measured with a Reflotron system (Roche Diagnostics, Basel,

Switzerland) with CK-strips (Roche).

## *RNA extraction and analysis of exon skipping by RT-PCR*

Harvested cells were lysed with RNA-Bee (Campro Scientific, Veenendaal, The Netherlands). Total RNA was extracted and 400 ng of RNA was used for RT-PCR analysis, using Transcriptor reverse transcriptase polymerase (Roche) in 20 µL at 55°C for 30 min with an appropriate primer (primer sequences on request). cDNA was amplified with a nested PCR. Three microliter cDNA was amplified in a 25-µL reaction for 20 cycles of 94°C (40 sec), 60°C (40 sec), and 72°C (80 sec), followed by 32 cycles of 94°C (40 sec), 60°C (40 sec), and 72°C (60 sec), with 1.5 µL PCR product in a 50-µL reaction.

Muscles were minced in TriPure Isolation Reagent (Roche) using MagNA Lyser green beads (Roche) or zirconium beads (1.4 mm; OPS Diagnostics, Lebanon, NJ) according to the manufacturer's instructions. Total RNA was extracted and 1 µg RNA was used for RT-PCR analysis, using Transcriptor reverse transcriptase polymerase (Roche) in 20 µL at 42°C for 45 min using random hexamer primers (20 ng/µL). Then, 1.5 µL cDNA was amplified in a 50-µL reaction for 30 cycles of 94°C (30 sec), 60°C (30 sec), and 72°C (30 sec), as previously described.[663] All PCR products were visualized on 1.5 or 2% agarose gels and quantified with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). PCR products were quantified using DNA 1000 LabChip on the Agilent 2100 Bioanalyzer (Agilent Technologies) according to the manufacturer's protocol. Bioanalyzer analysis has been shown to be an accurate method for exon skipping quantification in *mdx* muscle.[663]

## *Hybridization-ligation assay*

The assay for measuring the concentration of the 23AON in tissue samples is based on a previously published hybridization ligation assay.[664] Briefly, a signal probe (containing a peptide for antibody recognition) and a template (complementary to 23AON and the probe) were added to homogenized tissue samples. This was followed by a ligation step that only takes place when both AON and probe are bound to the template. Unbound probe was then washed away and the amount of probe-AON was detected using enzyme-linked antibodies against the probe. Muscles/organs were homogenized in proteinase K buffer (0.1 M Tris-HCl (pH 8.5), 0.2 M NaCl, 0.2% sodium dodecyl sulphate (SDS), and 5 mM EDTA) containing proteinase K (2 mg/mL; Invitrogen), using MagNA Lyser green beads (Roche) or zirconium beads (1.4 mm; OPS Diagnostics) by grinding in a MagNA Lyser (Roche) and incubating overnight at 55°C with gentle agitation. Calibration curves of the analysed 23AON prepared in 60 times pooled control mouse *mdx* tissue in PBS were included. All tissues were diluted in pooled control *mdx* mouse tissue. The muscle samples were diluted 500 and 1000 times, and liver and kidney tissue 1000 and 5000 times. All analyses were performed in duplicate.

## *Protein extraction and Western Blot analysis*

Western blotting was performed as described.[161,177,699] Briefly, muscles were homogenized in 75 mM Tris-HCl (pH 6.8)- 15% SDS, using MagNA Lyser green beads (Roche), by grinding in a MagNA Lyser (Roche). Protein concentrations were determined with Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Samples containing 75 µg of protein were made in 75 mM

Tris-HCl (pH 6.8), 15% SDS, 5% 2-mercaptoethanol, 2% glycerol, and 0.001% bromophenol blue, boiled for 5 min, loaded on a 4-7% gradient polyacrylamide gel, and run overnight at 4°C. Control samples containing 3.75 (5%), 1.5 (2%), 0.75 (1%), and 0.075 (0.1%) µg protein was used as a reference. Gels were blotted to nitrocellulose BA83 (Whatman/Schleicher & Schuell, Dassel, Germany) for 6 hr at 600 mA at 4°C. Blots were blocked with 5% nonfat dried milk (Campina Melkunie, Zaltbommel, The Netherlands) in (Tris-buffered saline (TBS) followed by an overnight incubation at 4°C with NCL-DYS1 (dilution 1:125, NovaCastra, Newcastle-upon-tyne, UK) in TBS plus 0.05% Tween20 to detect dystrophin. As secondary antibody the fluorescent IRDye 800CW goat anti-mouse IgG (dilution 1:5 000, Li-Cor, Lincoln, NE) was used. Blots were visualized and quantified with the Odyssey system and software (Li-Cor).

## *Immuno-histochemistry and dystrophin quantification*

Sections (thickness, 8 µm) were cut with a Shandon cryotome (Thermo Fisher Scientific) on Superfrost Plus slides (Thermo Fisher Scientific) along the entire length of the gastrocnemius with a minimum interval of 240 µm between the sections. Slides were fixed for 5 min in ice-cold acetone and blocked with PBS-0.05% Tween-5% horse serum. Slides were incubated overnight with dystrophin diluted 1:50 (dystrophin (C-20) sc-7461; Santa Cruz Biotechnology, Heidelberg, Germany) and spectrin diluted 1:200 (anti-spectrin $\beta$-3 polyclonal antibody PA1-46007; Thermo Fisher Scientific) as primary antibodies. As secondary antibodies Alexa Fluor 488-conjugated donkey anti-goat IgG diluted 1:1000 (A11055; Invitrogen) for dystrophin and Alexa Fluor 594-conjugated donkey anti-rabbit IgG diluted 1:1000 (A21207; Invitrogen) for spectrin were used. Slides were mounted with VECTASHIELD HardSet mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (H-1550, Vector Laboratories, Burlingame, CA). Images were made with a fluorescence microscope (Leica DM5500; Leica Microsystems, Rijswijk, The Netherlands) at 20x magnification. Dystrophin levels were assessed with Leica MM Basic Offline software (Leica Microsystems) by calculating the average maximum intensity of ten randomly placed rounds at the membranes minus the average of the average of ten randomly placed rounds in the cytoplasm.

## *Biomarker analysis*

Total RNA was purified with a NucleoSpin RNA II kit according to the manufacturer's instructions including a DNase digestion (Macherey-Nagel, Düren, Germany). The integrity of purified RNA was checked with an RNA 6000 Nano LabChip on the Agilent 2100 Bioanalyzer (Agilent Technologies) according to the manufacturer's protocol.

One microgram of RNA was used for cDNA synthesis, using BioScript (GC biotech, Alphen aan den Rijn, The Netherlands) in 20 µL at 70°C for 10 min and 42°C for 1 hr, with random hexamer primers (20 ng/µL). Gene expression levels were determined for *Cd68*, *Lgals3* (lectin, galactoside binding, soluble, 3), *biglycan*, *Lox*, *MyoD*, *myogenin*, *Mrf4*, and *Gapdh* (Glyceraldehyde-3-phosphate dehydrogenase) by real time qPCR using SensiMix SYBR (GC biotech) and the Roche LightCycler 480 (Roche) (primer sequences on request), with a program consisting of 45 cycles of 95°C (10 sec), 60°C (30 sec), and 72°C (20 sec).

For microRNA (miRNA) analysis 1 µg of unpurified RNA was used. Input RNA was reverse transcribed using an miScript reverse transcription kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. Two microliters of ten times diluted

cDNA was used as input for the real-time qPCR, using SensiMix™ SYBR (GC biotech) on the Roche LightCycler 480 (Roche), using a program consisting of 55 cycles of 95°C (10 sec), 57°C (30 sec), and 72°C (20 sec). Specific forward primers for miR-31 and 5S were used in combination with an universal reverse primer complementary to the adapter sequence of the RT-primer (primer sequences on request).

Calculations of relative expression were done with the LinRegPCR quantitative PCR data analysis software, version 11.3.[700] *Gapdh*, stably expressed across all conditions, was used to correct for differences in cDNA input for protein-coding transcripts and 5S was used to correct for differences in starting concentrations for miR-31.

### *Statistical analysis*

Data are represented as mean ± SD. Exon skipping percentages, 23AON concentrations, and protein levels between AON and prednisolone plus AON groups were compared using an independent samples, two-tailed, Student's t test in Excel 2003 (Microsoft Office Professional Edition 2003; Microsoft, Redmond, WA). Values of $p$ less than 0.05 after correcting for multiple testing were considered significant.

Statistical significance between all four groups for dystrophin staining, plasma creatine kinase levels at the end of the experiment (day 64), and biomarker expression was assessed by one-way analysis of variance (ANOVA), followed by a Bonferroni correction for multiple testing in case of significance ($p<0.05$) in SPSS 17.0.2 (SPSS, Inc., Chicago, IL).

To asses a possible treatment effect on the weight of mice over time a longitudinal analysis was perform in R,[665] using the lme4 package.[666] A baseline corrected model was used, including fixed linear and quadratic time effects; the mouse effect was considered to be random.

## Results

### *Prednisolone does not interfere with exon skipping in cultured cells and intramuscular 23AON injection*

The effect of prednisolone on exon skipping efficiency was first determined on cell cultures. Various doses of prednisolone were added to culture medium (0.2–2.25 µg/ml) The highest dose did not induce cell death, but impaired differentiation was observed (data not shown), a known effect of corticosteroids.[378] The dose of 0.75 µg/ml was selected for further study, as it did not induce cell death, did not affect differentiation, and is comparable to the dose that is used in most patients (recommended dose, 0.75 mg/kg/day[10,368,384,701]).

Patient-derived cell cultures were pretreated for ten days with 0.75 µg/mL prednisolone, or with saline as a control. Subsequently, cells were transfected with 400 nM concentrations of the appropriate AON. Skipping of the targeted exon was seen in all cells (fig. 6.1) and no difference was seen between prednisolone- and saline-treated cells. Exon 51 skipping in cells with a deletion of exon 52 (53914.1) averaged at 40% for both saline- and prednisolone-treated cells. For exon 50 skipping in cells with a deletion of exon 51-55 (DL589.2) percentages were ~70%, with relatively small variation (fig. 6.1a). Similar exon skipping levels were also found for other AON concentrations in the presence or absence of prednisolone (data not shown).

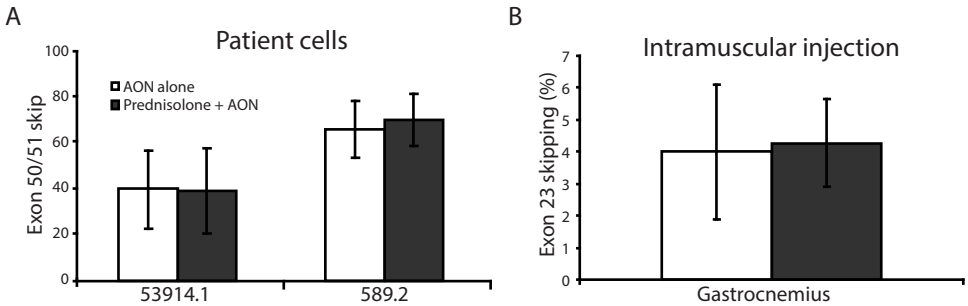


**Fig. 6.1:** Effect of prednisolone on antisense oligonucleotide mediated exon skipping *in vitro* and intramuscularly in the *mdx* mouse

Means are shown for each group. Error bars represent the standard deviation.

a) In two patient cell lines no effect on skipping percentages of addition of prednisolone was seen ($n$=12 per condition). For 53914.1 cells (deletion exon 52) AONs against exon 51 were used, and for 589.2 cells (deletion exon 51-55) AONs against exon 50. b) Prednisolone did not affect exon skipping after intramuscularly injection of 23AON in the gastrocnemius of *mdx* mice. Per group, eight muscles were analysed.

*In vivo*, the effect of systemic prednisolone treatment was first tested by local, intramuscular, injections with 23AON, a 2'-*O*-methyl phosphorothioate AON inducing exon 23 skipping (M23D(+2-18)).[197] *Mdx* mice were pretreated subcutaneously with saline or 1 mg/kg prednisolone for four weeks, starting at the age of four weeks.[698] This age was chosen because the major regeneration and degeneration cycles in *mdx* mice have been shown to take place at about this age.[702] The prednisolone dose is comparable to doses used by patients with DMD.[368] At the age of eight weeks, mice received two consecutive injections with 2.9 nmol 23AON locally in the gastrocnemius muscles. Exon skipping percentages were determined by primary PCR and bioanalyzer analysis.[661] No significant differences in exon skipping levels were observed (fig. 6.1b). Nested PCR analysis, the most commonly used method for determining exon skipping at present,[161,199,210,243,677,703] resulted in higher skipping levels of around 20% (data not shown). However, it has been shown that nested PCR gives an overestimation of the absolute exon skipping percentages.[663]

## *Prednisolone does not affect exon skipping efficiency systemically in* mdx *mice*

To investigate the effect of prednisolone on systemic treatment with 23AON, five-week-old male *mdx* mice were treated for eight weeks simultaneously with prednisolone (1 mg/kg/day via a subcutaneous slow-release pellet, shown to be an effective mode of delivering[379,704]) or saline and 23AON (250 mg/kg, five times in week 1; and 100 mg/kg, two times in week 2 to 8, subcutaneously) or saline. During the treatment the weight of the mice was monitored. In contrast to the weight increase seen in humans, prednisolone-treated mice (both prednisolone alone and prednisolone and 23AON) weighed significantly less compared with saline- or 23AON alone-treated mice (fig. 6.2a). This is in line with results described in the literature.[705] In the absence of prednisolone, no difference in weight between 23AON- and saline-treated mice was observed. Plasma creatine kinase (CK) levels (a measure of muscle damage) did not differ significantly for the various groups after treatment (data not shown). Notably, levels were generally much lower than expected (up to 2000 U/L in all mice, whereas, in general levels up to 8000 U/L are found in untreated *mdx* mice). This may have been due to the time between serum taking and measuring (1-4 weeks).

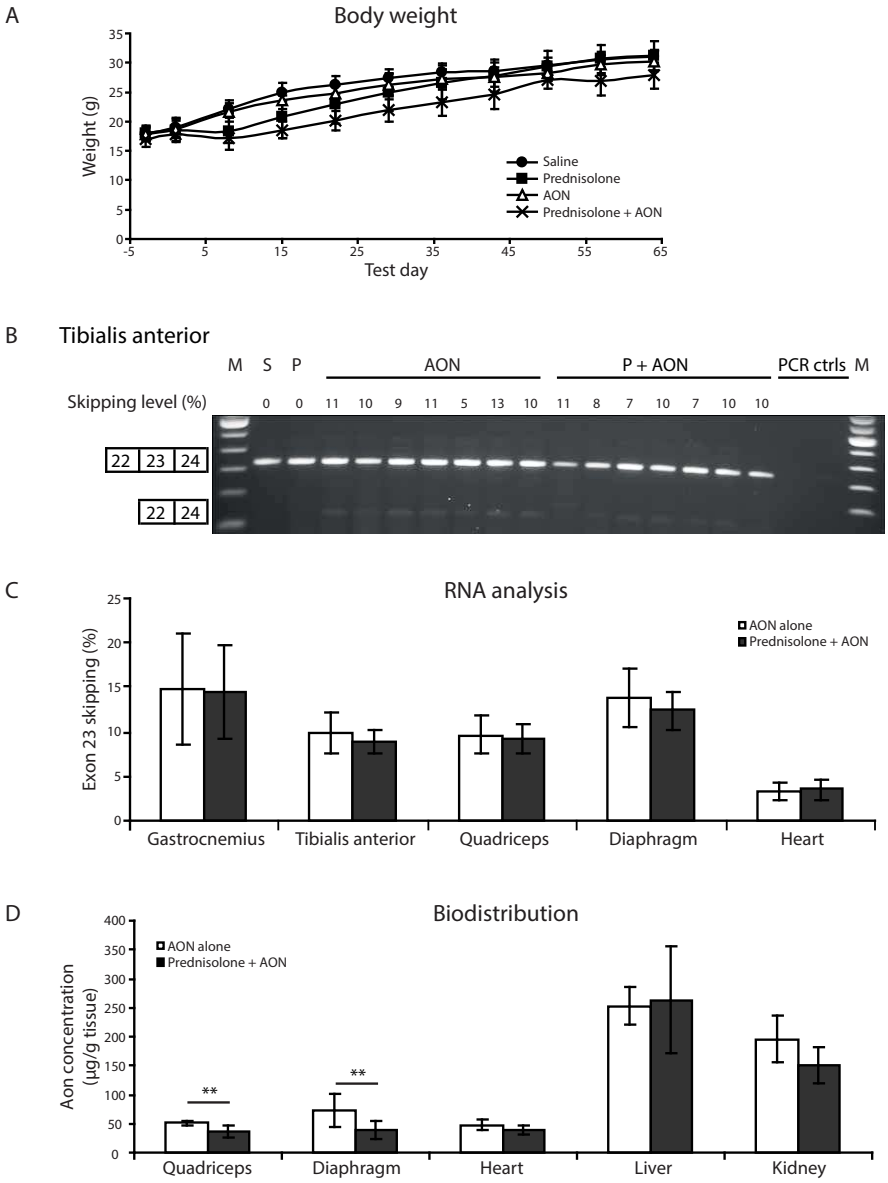


**Fig. 6.2:** Systemic treatment with prednisolone and/or 23AONs

Effect on body weight of all treatments and antisense oligonucleotide mediated exon skipping and 23AON biodistribution after co-treatment with prednisolone or saline. Per group, eight mice were analysed. Means are shown for each group. Error bars represent the standard deviation.

a) Body weight was decreased in both prednisolone treated groups compared to both saline and 23AON alone-treated mice ($p<0.01$). b) RT-PCR analysis for the tibialis anterior. Wild type product consists of 334 base pairs and exon 23 skipping results in a 122 base pair product. S=saline, P=prednisolone. c) No difference in exon skip percentages between saline and prednisolone co-treatment with 23AONs was seen. Exon skip percentages were determined by DNA 1000 lab-on-a-chip analysis on the Agilent 2100 Bioanalyzer. d) Biodistribution analysis showed a small decrease in 23AON uptake in the quadriceps and diaphragm, but not in the heart. **$p<0.01$ compared to 23AON alone-treated mice

Average skipping levels were comparable for skeletal muscles and diaphragm and skipping levels did not differ between saline- and prednisolone-treated animals (fig. 6.2b/c). In heart, exon skipping levels were much lower, as described previously,[161] but detectable in all samples. No skipping was observed without 23AON treatment for any of the muscles (data not shown).

Assessment of the concentrations of 23AON in the various tissues showed in prednisolone-treated mice a small, but significant decrease in 23AON concentration in the quadriceps and diaphragm (fig. 6.2d). In the heart the levels were almost comparable to those found in skeletal muscle, without real difference between both treatment groups. The majority of the 23AON ends up in the liver and kidneys. Levels were similar in the liver for prednisolone-treated animals versus control animals, whereas an almost significant decrease in 23AON concentration was seen in the kidneys. Overall, 23AON levels in muscle and organs were slightly lower in the prednisolone-treated mice.

## *23AON-mediated expression of dystrophin protein is slightly increased in prednisolone treated animals*

Dystrophin protein expression was determined in two ways: by Western blot analysis and by immunofluorescent staining of cross-sections. Assessment of protein levels by Western blot (fig. 6.3a/b) revealed restoration of expression in all 23AON-treated mice, albeit at low levels (<5% of wild type control). Quantification indicated slightly elevated protein levels for mice

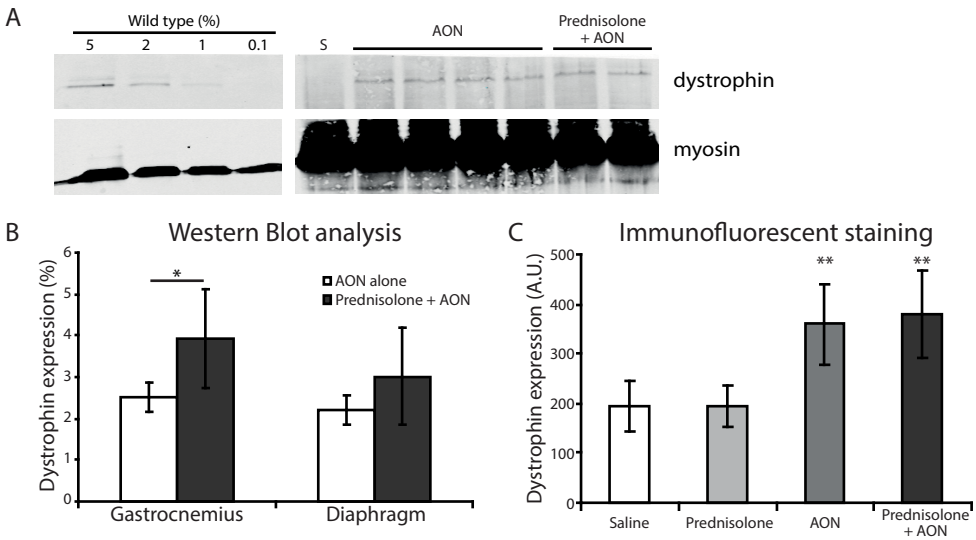


**Fig. 6.3:** Dystrophin expression detected by immunofluorescent staining and Western blot analysis

Per group, eight mice were analysed. Means are shown for each group. Error bars represent the standard deviation. a) Representative example of western blot analysis (gastrocnemius muscle), showing low levels of dystrophin protein (*top*) in all 23AON-treated mice. No band was detected in control mice. Myosin (*bottom*) was used as loading control. b) Quantification of dystrophin protein expression by Western blot showed a slight increase in protein levels in 23AON-treated mice, which was significant for the gastrocnemius muscle, but not for the diaphragm. *$p$<0.05 compared to 23AON alone treated mice. c) Immunofluorescent staining with dystrophin antibody in the gastrocnemius muscle showed dystrophin expression above background levels in both 23AON-treated groups, but no difference between prednisolone-and saline-treated mice. **$p$<0.01 compared with saline-treated mice.

treated with both prednisolone and 23AON compared with mice treated with 23AON alone; the difference was significant for the gastrocnemius. In the diaphragm the same trend was seen, but this increase was not significant (fig. 6.3a/b). However, with these low dystrophin expression levels, differences may be more difficult to observe. Immunofluorescent staining showed dystrophin expression above background levels (saline/prednisolone alone treated-mice) for both 23AON-treated groups, but no difference was observed between saline- and prednisolone-treated animals (fig. 6.3c). Untreated *mdx* mice did not express dystrophin.

### *Biomarkers*

The expression levels of multiple mRNA transcripts have been shown to be changed in the *mdx* mouse. These can be used as biomarkers to assess the effect of therapeutic interventions.[706] Expression levels for several immunological, fibrosis, and early and late regeneration markers for the tibialis anterior and diaphragm were assessed by quantitative PCR. The main changes in expression levels were seen in the tibialis anterior of 23AON- and/or prednisolone-treated mice (fig. 6.4; supplementary table S6.1). In the diaphragm the differences were relatively smaller. The immunological markers Cd68 and Lgals3 are elevated in *mdx* mice and have been shown to fall after high levels of AAV-induced exon skipping.[704] In our study, a significant decrease for both Cd68 and Lgals3 was observed in the tibialis anterior of 23AON-treated mice (fig. 6.4a/b). Prednisolone treatment did not induce a significant decrease for these immunological markers, although a trend was observed for both. Interestingly, for mice treated with both 23AONs and prednisolone, levels were higher than those in mice treated with either compound. The fibrotic markers biglycan and Lox, known to be elevated in *mdx* mice,[706,707] were lower in the tibialis anterior of prednisolone-treated mice (only for Lox), 23AON-treated mice, as well as in mice receiving both treatments (fig. 6.4c/d). Because of interindividual variations this difference was significant only for the group receiving both treatments compared with saline-treated mice for biglycan. To examine the effect of prednisolone and 23AON on regeneration, levels of the early regeneration markers MyoD and myogenin, which are elevated in *mdx* mice,[708] were measured. Both markers fell significantly after prednisolone and/or AON treatment (fig. 6.4e/f). Furthermore, the late regeneration marker Mrf4 has been shown to be inhibited by glucocorticoid treatment.[378,709] Levels were highly variable in the tibialis anterior, but in the diaphragm a significant decrease compared with saline-treated mice was seen for 23AON-treated mice with and without prednisolone (fig. 6.4g). Another regeneration marker, the microRNA miR-31, showed a small decrease after both prednisolone and 23AON treatment, and a nearly significant decrease compared with control mice was seen after combinational treatment in the diaphragm (fig. 6.4h). In conclusion, treatment with either prednisolone, 23AON, or a combination seems to normalize the levels of fibrotic and regenerative biomarkers to some extent in the tibialis anterior, while a smaller effect was observed in the more severely affected diaphragm.

## Discussion

AON-mediated exon skipping as a potential therapy for DMD is being tested in placebo-controlled clinical trials and is likely to become clinically applicable in the near future. Most patients currently use corticosteroids, mainly prednisone, to slow down disease progression. Prednisolone has been shown to have a positive effect on muscle maintenance and quality.

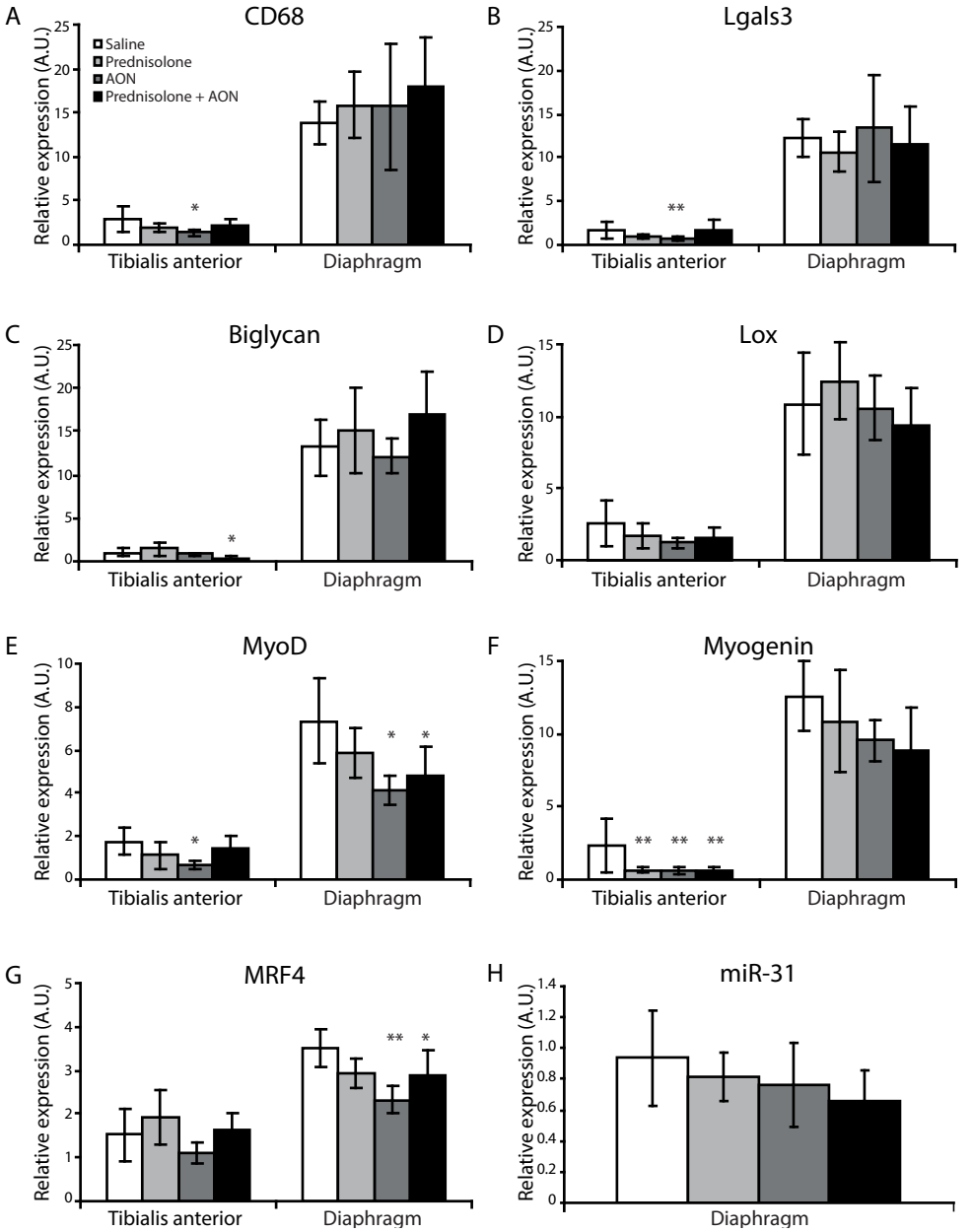


**Fig. 6.4:** Expression of biomarker levels measured by quantitative PCR

Per group, eight mice were analysed. Means are shown for each group, corrected for Gapdh or 5S expression. Values are expressed relatively to tibialis anterior levels of saline-treated mice. Error bars represent the standard deviation. Immunological markers Cd68 (a) and Lgals3 (b), fibrotic markers biglycan (c) and Lox (d), early regeneration markers MyoD (e) and myogenin (f), late regeneration marker Mrf4 (g) and miR-31 (h) were measured. miR-31 was only determined in the diaphragm (values expressed relatively to saline-treated mice)

*$p$<0.05 **$p$<0.01 compared to saline-treated mice

This could affect exon skipping efficiency either positively or negatively. It has been noticed previously that uptake of AONs and exon skipping efficiency are higher in dystrophic muscle than in wild type muscle.[200] This is thought to be caused by the leakiness of the dystrophic muscle membranes, which facilitates the entry of AONs in the muscle cells.[200,217] In fact, in mice containing only one utrophin allele in the absence of dystrophin (*mdx*/*Utrn*$^{+/-}$), which are more severely affected, exon skipping levels are higher than in the *mdx* mouse [Tanganyika-de Winter, C.L. *et al.*, unpublished observations]. Therefore stabilization of the muscle fibres by prednisolone could have a negative effect on AON uptake and thereby decrease its efficiency. Conversely, improving the quality of the muscle could lead to more muscle fibres, increasing the total amount of dystrophin pre-mRNA, the target of AONs, and thereby increase the therapeutic potential of AONs. In this study the effect of prednisolone on AON uptake and biodistribution, exon skipping levels, and dystrophin restoration was examined.

In two patient cell cultures and one healthy control cell culture no differences were seen in 2OMePS AON-induced exon skipping between prednisolone and saline cotreatment. Thus, the effect prednisolone has on muscle cells does not seem to influence skipping efficiency *in vitro*. The same appears to be true *in vivo*, where muscle cells are in their natural environment. After local injections of 23AON, skipping efficiency was the same between salin-e and prednisolone-treated mice. The same holds for systemic 23AON treatment, where exon skipping percentages were similar between both treatment groups for skeletal muscle, diaphragm, and heart.

The 2OMePS dose used in this study is much higher than that used in clinical trials using the same chemistry[229] (200 versus 6 mg/kg/week). However, for most drugs a correction factor applies when translating doses between small and larger animals based on normalization to body surface area (See Guidance for Industry[605]). When applying this correction factor, a dose of 16 mg/kg would be predicted for humans, which is in the same order of magnitude as the 6 mg/kg used in the trials. The slightly higher corrected dose for mice might be explained by a higher clearance rate for 2OMePS AONs in mice and potential differences between pharmacokinetic and pharmacodynamic properties between exon 23- and exon 51-targeting AONs.

The exon skipping levels *in vivo* after 2OMePS AON treatment (both locally and systemically) are relatively low. Vivo-morpholinos (*i.e.*, modified morpholinos, conjugated with a dendrimeric octaguanidine) have been shown to lead to much higher skip levels.[221,229] This may partly be due to differences in analysis method, primary versus nested PCR.[663] In either case, at the moment the clinical relevance of these high levels is limited, as exon skipping levels in the currently ongoing systemic trials are low.[229,231] Higher exon skip efficiency would obviously be desired, but for now only nonconjugated AONs are being tested in clinical trials. Nevertheless, low levels of dystrophin protein have already lead to histological and functional improvement in mice,[710] and the low exon skipping levels in our study did result in dystrophin restoration, which was slightly increased in prednisolone-treated mice.

As seen in previous studies[161,200] the majority of the 23AON ends up in the liver and the kidneys after systemic treatment. A small decrease in 23AON concentration was seen in the quadriceps and diaphragm in prednisolone-treated mice compared with saline-treated mice, but this did not lead to a decrease in exon skipping percentages. This might be explained by the localization of the AON within the tissue. The 23AON concentration is measured in the whole muscle and therefore reflects both AONs present in the fibres (where exon skipping can be induced) and in the extracellular matrix and interstitial spaces (where no exon skip-

ping can be induced). The lower levels observed in muscle may be due to less sequestration in the extracellular matrix caused by an improvement in muscle quality. Therefore, the higher levels in the AON-treated animals do not necessarily have to result in higher exon skipping levels, because part may be sequestered in the extracellular matrix and interstitial spaces. This is supported by the finding that more dystrophin was expressed in prednisolone-treated mice, which might be another indication of a positive effect on muscle quality (more dystrophin pre-mRNA) by prednisolone, because the same percentage of skipping in more transcripts leads to higher protein production. In this study an AON with the 2'-*O*-methyl phosphorothioate chemistry was used; however, it is anticipated that the results also hold for the other chemistry that is approaching use in large clinical trials (morpholino phosphorodiamidate AONs).

The best known effect of prednisolone is its suppressive effect on the immune system. However, no clear decrease in the immunological markers Cd68 and Lgals3 was seen in prednisolone-treated mice. By contrast, the levels of myogenic transcription factors MyoD and myogenin (regeneration markers) were decreased, although not significantly everywhere, for all treated groups, suggesting that prednisolone treatment did have an effect on muscle. This concurs with previous findings that prednisolone treatment leads to a general reduction in proliferation, and MyoD and myogenin are downregulated in methylprednisolone-treated adrenalectomized rats.[711] Because AONs interact only with their target sequence, a general effect as observed for prednisolone is unlikely. In this case regeneration might be reduced because of reduction in muscle damage, inflammation, and fibrosis as a consequence of the dystrophin restoration. The less pronounced reduction in Mrf4 levels might be explained by the timing. MyoD and myogenin are elevated early in regeneration,[712] peaking at 72 hr after induction of regeneration,[708] whereas Mrf4 is increased in maturing myofibres.[7102] The combination of prednisolone and 23AON showed mixed effects. The absence of a decrease in immunological markers, as seen in 23AON-treated mice, is probably due to conflicting results in one or two mice, which abolish the small differences observed. Fibrotic and regeneration markers followed roughly the same pattern as prednisolone and/or 23AON treatment alone. miR-31 is a microRNA that, amongst others, targets the 3' untranslated region of the dystrophin mRNA, thereby repressing its translation and expression. It has been shown to be localized in regenerating myoblasts and is almost absent in wild type muscle fibres, but is upregulated in *mdx* mice. Furthermore, repression of miR-31 led to an increase in dystrophin expression in AON-treated human DMD myoblasts.[713] In all treated mice a trend was observed toward normalisation, which seemed to be more pronounced after combinational treatment. The downregulation of miR-31 might be another explanation for the improved dystrophin levels after prednisolone treatment.

It is surprising that even the limited levels of dystrophin found in this short-term study resulted in improved muscle quality to some extent. However, long term presence of low amounts of dystrophin has been reported to result in beneficial effects before. The *mdx*$^{3cv}$ mouse model, which has approximately 5% of dystrophin since birth, performs significantly better in the grip strength test compared with *mdx* mice.[102] Our own results in mice with low levels of dystrophin (*mdx-Xist*$^{\Delta hs}$) confirm these results; here, low levels of dystrophin (<15%) were sufficient to improve histology and muscle function and to normalize biomarkers [van Putten, M. *et al.*, manuscript submitted]. Notably, in clinical trials low dystrophin levels after AON treatment appear to result in functional improvement.[229]

In conclusion, this work shows that there is no negative effect of prednisolone and 23AON on each other's therapeutic outcome in any of the tests, suggesting that patients can continue

using prednisone during exon skipping trials. Prednisolone might even have a positive effect on AON treatment.

## Acknowledgments

We are grateful to Rick Vermue and Ruurd Verheul (Prosensa Therapeutics) for assistance with the hybridisation-ligation assay, and to Maarten van Iterson (LUMC) for assistance with the statistical analysis. This work made use of the infrastructure of the Center for Medical System Biology (CMSB) and the Center for Biomedical Genetics (CBG) in which the LUMC participates. LUMC is a partner in the FP6 TREAT-NMD network of excellence (LSHM-CT-2006–036825). This work is supported by grants from the Prinses Beatrix Fonds, The Netherlands (grant W-OR09-12) and the Dutch Duchenne Parent Project.

## Author Disclosure Statement

Tatyana G. Karnaoukh, Ingrid G.M. Kolfschoten, Anne Vroon, and Judith C.T. van Deutekom report being employed by Prosensa Therapeutics. LUMC has patents on the exon skipping applications. Hans Heemskerk, Gertjan B. van Ommen, Judith C.T. van Deutekom, and Annemieke Aartsma-Rus report being coinventors on some of these patents, and as such are entitled to a share of royalties.

| | Tibialis anterior | | | | Diaphragm | | | |
|---|---|---|---|---|---|---|---|---|
| *Marker* | Saline | Prednisolone | AON | Prednisolone + AON | Saline | Prednisolone | AON | Prednisolone + AON |
| *Immunological markers* | | | | | | | | |
| *Cd68* | 0.01493 | 0.01011 | 0.00694* | 0.01064 | 0.07097 | 0.08124 | 0.08051 | 0.09158 |
| | (± 0.00773) | (± 0.00286) | (± 0.00226) | (± 0.00474) | (± 0.01294) | (± 0.01909) | (± 0.03706) | (± 0.02955) |
| *Lgals3* | 0.00691 | 0.00358 | 0.00267** | 0.00707 | 0.04953 | 0.04276 | 0.05368 | 0.04661 |
| | (± 0.00410) | (± 0.00096) | (± 0.00099) | (± 0.00423) | (± 0.00857) | (± 0.00894) | (± 0.02495) | (± 0.01723) |
| *Fibrotic markers* | | | | | | | | |
| *Biglycan* | 0.03971 | 0.05523 | 0.03346 | 0.01637* | 0.50672 | 0.58393 | 0.46490 | 0.65009 |
| | (± 0.01393) | (± 0.02722) | (± 0.00517) | (± 0.00832) | (± 0.12530) | (± 0.19527) | (± 0.08114) | (± 0.19441) |
| *Lox* | 0.00806 | 0.00545 | 0.00387 | 0.00511 | 0.03376 | 0.03861 | 0.03282 | 0.02915 |
| | (± 0.00484) | (± 0.00277) | (± 0.00106) | (± 0.00218) | (± 0.01094) | (± 0.00829) | (± 0.00692) | (± 0.00814) |
| *Early regeneration markers* | | | | | | | | |
| *MyoD* | 0.02938 | 0.01870 | 0.01142* | 0.02364 | 0.12266 | 0.09889 | 0.06963* | 0.07987* |
| | (± 0.01077) | (± 0.01000) | (± 0.00271) | (± 0.00982) | (± 0.03316) | (± 0.01919) | (± 0.01163) | (± 0.02280) |
| *Myogenin* | 0.02357 | 0.00384 | 0.00597 | 0.00627 | 0.12826 | 0.11027** | 0.09706** | 0.09033** |
| | (± 0.01851) | (± 0.00225) | (± 0.00267) | (± 0.00211) | (± 0.02458) | (± 0.03575) | (± 0.01395) | (± 0.02986) |
| *Late regeneration marker* | | | | | | | | |
| *Mrf4* | 0.04188 | 0.05293 | 0.03030 | 0.04538 | 0.09650 | 0.08069 | 0.06404** | 0.07943* |
| | (± 0.01649) | (± 0.01719) | (± 0.00667) | (± 0.00995) | (± 0.01229) | (± 0.00974) | (± 0.00830) | (± 0.01628) |
| *Micro-RNA* | | | | | | | | |
| *miR-31* | ND | ND | ND | ND | 0.00020 | 0.00017 | 0.00016 | 0.00014 |
| | | | | | (± 0.00007) | (± 0.00003) | (± 0.00006) | (± 0.00004) |

**Supplementary Table S6.1:** Average expression of mRNA severity biomarkers.
Expression of biomarker levels measured by quantitative PCR. Per group eight mice were analysed. Average expression levels (± standard deviation) relative to Gapdh/5S for each group in the tibialis anterior and the diaphragm. ND= not determined.
*$p$<0.05 **$p$<0.01 compared to saline treated mice

# The effect of losartan *in vitro* and *in vivo* on muscle signalling and function and antisense oligonucleotide-mediated exon skipping

Chapter 7

## Introduction

The renin-angiotensin system (RAS) plays a role in the maintenance of blood pressure and fluid balances and is, amongst others, involved in the development of cardiomyopathy. Upon activation, renin is secreted by the kidneys, which converts angiotensinogen, an α-glycoprotein released by the liver, into angiotensin I. Angiotensin I in turn is converted in its active form angiotensin II by the membrane-bound metalloproteinase angiotensin converting enzyme (ACE), which has numerous effects, amongst others on vascular smooth muscle cells to induce vasoconstriction. It exerts its main effects by binding to the angiotensin II type 1 (AT1) receptor, but also binds to angiotensin II receptor type 2 (AT2).[714] This system has shown to be upregulated in DMD and increases fibrosis by both transforming growth factor-$\beta$ (TGF-$\beta$)-dependent and independent mechanisms.[554,555]

TGF-$\beta$ is a member of the TGF-$\beta$ superfamily, proteins that are involved in the control of many cellular processes such as growth and differentiation. TGF-$\beta$ mainly signals via the type II receptor Tgfbr2 in combination with the type I receptor Tgfbr1 (ALK5). TGF-$\beta_1$, one of the three mammalian isoforms, is known to be induced in regenerating muscle and is a potent inducer of fibrosis. Downstream signalling occurs via the canonical Smad2/3-dependent pathway and non-canonical Smad-independent pathways.[478] TGF-$\beta_1$ is upregulated in patients and mice lacking dystrophin, a protein that is crucial to maintain muscle fibre stability during contraction and lack of which leads to severe muscle damage and replacement of muscle tissue by fibrotic and adipose tissue. The level of TGF-$\beta_1$ expression has shown to be correlated with disease severity in dystrophin negative Duchenne muscular dystrophy (DMD) patients and *mdx* mice.[715,716]

AT1 stimulates TGF-$\beta$ via the NADPH oxidase/P38/MAPK pathway, thereby enhancing downstream signalling.[558] TGF-$\beta$-independently AT1 activation works directly on the PI3K/Akt/mTOR and Raf/MEK/ERK pathways, which also induce fibrosis.[561,562]

Losartan is a selective AT1 receptor antagonist, thereby anticipated to have an antagonistic effect on TGF-$\beta$. In *mdx* mice it improved regeneration and reduced fibrosis after acute injury. Six to nine months of treatment ameliorated the disease progression evidenced by a decrease in fibrosis and improving muscle strength.[260]

Antisense oligonucleotide (AON)-mediated exon skipping, described in more detail in previous chapters, targets *DMD* transcripts, which are only expressed in muscle fibres and not in fibrotic/adipose tissue that gradually replaces the muscle fibres when the disease progresses. Indeed the first clinical trial in DMD patients using local intramuscular injection of AONs targeting exon 51 already showed that patients with better muscle quality were able to produce more dystrophin.[225] Therefore combining AON-treatment with a muscle preserving agent might increase the therapeutic effects of AON-mediated exon skipping. In light of the described effects of losartan for preserving muscle quality, in this chapter the effects of losartan itself and in combination with AONs were examined.

## Materials and methods

*Cell culture*

A mouse myoblast cell line (C2C12) and primary human myoblasts derived from a healthy control (KM109)[697] and a DMD patient with a deletion of exon 51-55 (DL589.2)[177] were used in this study.

Mouse C2C12 myoblasts were cultured on a collagen layer (1:30; Pure Col; Nutacon

BV; the Netherlands)at 37ºC 10% $CO_2$ in Dulbecco's Modified Eagle Medium (DMEM, without phenol red; Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin streptomycin (P/S), 2% GlutaMAX and 1% D-glucose (all from Gibco-BRL, Life Technologies; the Netherlands).

Human myoblast were cultured on a collagen layer (1:30; Pure Col; Nutacon BV) in Nutrient Mix F-10 (HAM) supplemented with GlutaMAX (Gibco-BRL), 20% FBS and 1% P/S at 37ºC 5% $CO_2$. To induce differentiation into myotubes medium was switched into DMEM without phenol red supplemented with 2% FBS, 1% P/S, 2% GlutaMAX and 1% D-glucose (Gibco-BRL) when cells reached 80-90% confluence.

*Smad phosphorylation assay*

For the Smad phosphorylation assay C2C12 cells were seeded in a 6-well plate at a density of $3 \cdot 10^5$ cells/well. After overnight serum starvation, cells were treated for 1 hour with 0, 0.5 or 50 µg/mL losartan potassium (Cozaar®; Merck Sharp & Dohme BV; the Netherlands) or 10 µM Transforming Growth Factor-$\beta$ type I receptor kinase inhibitor (LY-364947; Sigma-Aldrich; the Netherlands) dissolved in serum-free medium. Cells were stimulated with 1 ng/ml of TGF-$\beta_1$ (kindly provided by Ken Iwata, OSI Pharmaceuticals, Melville, NY, USA) for 45 min. Cells were lysed with sample buffer containing 100 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 0.1% (w/v) bromophenol blue, 20% (v/v) glycerol, and 8% (v/v) β-mercaptoethanol and stored at -20ºC for protein analysis.

*Losartan treatment and AON transfection*

Losartan potassium (Merck Sharp & Dohme BV) was dissolved in differentiation medium. KM109/DL589.2 cells were differentiated in 6-wells plates for 7-10 days before transfection. Losartan was added at concentrations ranging from 0 to 50 µg/mL and maintained during and after transfection. Cells were transfected with 500 pmol h49AON1 targeting exon 49 or h50AON1 targeting exon 50 (2'-*O*-methyl RNA oligonucleotides with a full-length phosphorothioate backbone (2OMePS) previously described[176]; Prosensa Therapeutics; the Netherlands) in 1 mL differentiation medium for 4 hours using 2 µL polyethylenimine (PEI, Exgen 500; MBI Fermentas; Germany) per µg AON, according to the manufacturer's instructions. Cells were harvested >24 hours after transfection.

In vivo *losartan and 23AON treatment*

All experiments were approved by and performed following the guidelines of the local animal ethics experimental committee of the Leiden University Medical Center (permit number: 08224). Mice were housed in individually ventilated cages in the animal facility of the LUMC and received food and drink *ad libitum*. *Mdx* mice (C57Bl/10ScSn-DMD$^{mdx}$/J) with one or two copies of the utrophin gene (*mdx*/*Utrn*$^{+/-}$ or *mdx*/*Utrn*$^{+/+}$) were obtained from our own breeding facilities.

Four weeks old mice were treated orally (via the drinking water) with 0.6 g/L losartan potassium (Sigma-Aldrich) till the end of the experiments. Control mice received normal drinking water.

For intramuscular 23AON experiments, *mdx* mice (M/F) were, after 6 weeks of losartan/control treatment, anesthetized with isoflurane and intramuscularly injected in both gastrocnemius muscles on two consecutive days with 2.9 nmol (≡20 µg) of M23D(+2–18), a 2OMePS RNA oligonucleotide specifically targeting exon 23,[197] in 40 µL saline (*n*=2-3 per

condition). Mice were sacrificed 10 days after the last injection by cervical dislocation and muscles were isolated.

For systemic 23AON experiments, *mdx*/*Utrn*$^{+/-}$ mice (M/F) were treated for 8 weeks with subcutaneous injections of saline, losartan in drinking water, subcutaneous injections of 23AON or losartan in drinking water and subcutaneous injections of 23AON ($n$=3-4 per group). 200 mg/kg body weight/week M23D(+2–18) divided over 4 injections was injected subcutaneously. During treatment functional performance was tested weekly by rotarod and two limb hanging wire testing and in the last week by grip strength testing.[717] Blood samples were taken weekly via the tail vein for creatine kinase (CK) level measurements. Mice were sacrificed 10 days after the last 23AON injection and muscles were isolated, snap frozen in liquid nitrogen-cooled 2-methylbutane, and stored at -80ºC.

*Creatine kinase level measurements*

Blood samples were centrifuged at 18 000 *g* for 5 min at 4ºC to generate plasma. CK levels were measured after diluting the samples 10 times in Dulbecco's phosphate-buffered saline (D-PBS; Invitrogen; CA, USA) and were measured with a Reflotron system (Roche Diagnostics; Switzerland) with CK-strips (Roche).

*RNA isolation and exon skipping analysis by RT-PCR*

For RNA isolation harvested cells were lysed with RNA-Bee (Campro Scientific; the Netherlands) and total RNA was extracted. RT-PCR analysis was performed using Transcriptor reverse transcriptase polymerase (Roche) in a 20 µL volume at 55ºC for 30 min with an appropriate primer. cDNA was amplified by nested PCR. Three microliters of cDNA was amplified in a 25 µL reaction for 20 cycles of 94ºC (40 sec), 60ºC (40 sec) and 72ºC (80 sec), followed by 32 cycles of 94ºC (40 sec), 60ºC (40 sec) and 72ºC (60 sec), with 1.5 µl of PCR product in a 50 µL reaction.

Muscles were minced in RNA-Bee (Campro Scientific) using MagNA lyser green beads (Roche) according to manufacturer's instructions. Total RNA was extracted and 1 µg of RNA was used for RT-PCR analysis, using Transcriptor reverse transcriptase polymerase (Roche) in 20 µl at 42ºC for 45 min with random hexamer primers (20 ng/µl). Then, 1.5 µl of cDNA was amplified in a 50 µL reaction for 30 cycles of 94ºC (30 sec), 60ºC (30 sec) and 72ºC (30 sec), as previously described.[663] All PCR products were visualized on 1.5% agarose gels and exon skipping levels were quantified using a DNA 1000 LabChip on the Agilent 2100 bioanalyzer (Agilent Technologies; CA, USA) according to the manufacturer's instructions.

*Protein extraction and Western blot analysis*

Cells were lysed with sample buffer containing 100 mM Tris-HCl (pH 6.8), 4% w/v sodium dodecyl sulphate (SDS), 0.1% w/v bromophenol blue, 20% v/v glycerol and 8% v/v β-mercaptoethanol. Muscles were homogenized in lysis buffer containing 50 mM Tris-HCl (pH 7.5). 50 mM NaCl, 1 mM EDTA, 1% Triton X100, 0.1% SDS, cOmplete EDTA-free protease inhibitor cocktail (Roche) and PhosSTOP phosphatase inhibitor cocktail (Roche) using MagNA lyser green beads (Roche) according to manufacturer's instructions. Protein concentrations were determined using a Pierce bicinchoninic acid protein assay kit (Thermo Fisher Scientific; IL, USA) according to manufacturer's instructions, samples containing equal amount of protein were made and mixed 1:1 with sample buffer. Protein lysates were separated on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose mem-

branes (Amersham; Belgium). Membranes were blocked in TBST-5% milk for 45 min at 4°C and incubated in primary antibody overnight at 4°C, followed by incubation with IgG-horseradish peroxidase-conjugated secondary antibody for 1 h. The detection was performed using SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific). As primary antibodies rabbit polyclonal anti-phosphorylated Smad2 (1:1000; Ludwig Institute for Cancer Research; Sweden); rabbit polyclonal anti-phosphorylated ERK1/2 (1:1 000; Cell Signaling Technology, Inc.), mouse monoclonal anti-α-actin (Sigma-Aldrich) and rabbit monoclonal anti-ERK1/2 (1:1 000; Cell Signaling Technology Inc.) were used. As secondary antibodies goat-anti-rabbit and goat-anti-mouse IgG-HRP (1:2 500; SantaCruz Biotechnology; Germany) were used.

*Biomarker analysis*

For biomarker analysis total RNA was purified with a NucleoSpin RNA II kit according to the manufacturer's instructions, including a DNase digestion (Macherey-Nagel; Germany). One microgram of RNA was used for cDNA synthesis using random hexamer primers (20 ng/µl) and BioScript (GC biotech; the Netherlands) in 20 µl at 70ºC for 10 min and 42ºC for 1 h. Gene expression levels were determined for *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase), *AT1* (angiotensin II type 1) receptor, *Ctgf* (connective tissue growth factor), *Collagen1a1* (collagen type I α1), *PAI1* (plasminogen activator inhibitor 1)/*Serpine1*, *Myogenin* and *TGF*-$\beta_1$ (transforming growth factor $\beta_1$) by real-time qPCR using 2 µL of 10x diluted cDNA, 1 µL of forward primer (0.8 pmol/µL), 1 µL of reverse primer (0.8 pmol/µL) and 4 µL SensiMix SYBR (GC biotech) per sample and the Roche LightCycler 480 (Roche) with a program consisting of 45 cycles of 95ºC (10 sec), 60ºC (30 sec) and 72ºC (20 sec). Relative expression was calculated with Lin-RegPCR quantitative PCR data analysis software, version 11.3.[700] *Gapdh*, stably expressed across all conditions, was used to correct for differences in cDNA input.

*Histology*

Sections of 8 µm were cut from the gastrocnemius muscle with a Shandon cryotome (Thermo Fisher Scientific Co.; PA, USA) on Superfrost Plus slides (Thermo Fisher Scientific, Menzel-Gläser; Germany) with a minimum interval of 240 µm between the sections. Slides were fixed for 5 min in ice-cold acetone and stained with Harris haematoxylin and eosin (H&E) (Sigma–Aldrich) according to conventional histological procedures. Pictures were taken at 5x magnifications with a Leica DC500 camera and Leica IM50 software connected to a Leica DM LB light microscope (Leica Microsystems; The Netherlands). For blending and background correction Adobe Photoshop CS3 version 10.0.1 was used. Freely available ImageJ software with the haematoxylin/eosin (H&E) colour deconvolution plugin (Rasband, W.S., ImageJ, US National Institutes of Health; MD, USA) was used to determine the fibrotic/necrotic percentage of the entire cross section.[717]

## Results and Discussion

Since losartan has been shown to act by antagonising TGF-$\beta$ signalling as indicated by attenuating its downstream targets, including phosphorylation of Smad2,[260] the effect of losartan on TGF-$\beta$-induced pSmad2 was analysed *in vitro*. Differentiated mouse myocytes (C2C12 cells), were serum starved overnight and incubated for one hour with different concentrations

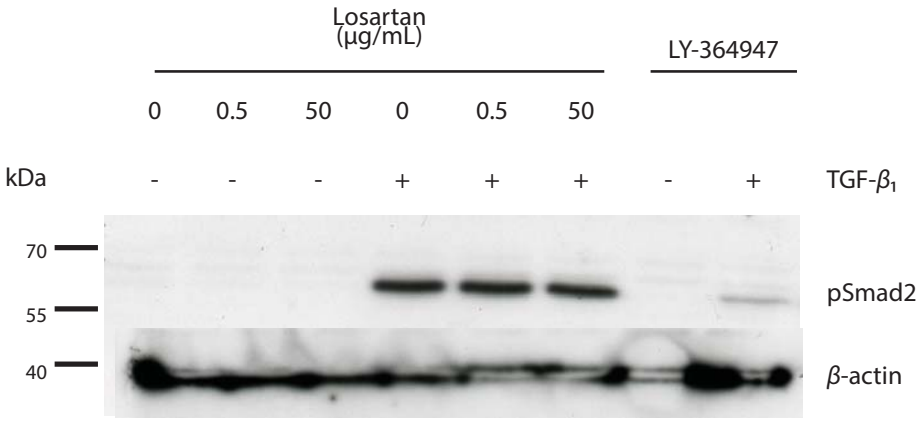


**Fig. 7.1:** Losartan does not influence Smad2 phosphorylation upon TGF-$\beta_1$ stimulation
A large increase in pSmad2 (60 kDa) is observed in stimulated muscle cells, which was not attenuated by pre-treatment with losartan. LY-364947, a TGF-$\beta_1$ receptor kinase inhibitor, was used as a positive control for TGF-$\beta_1$ inhibition. B-actin (42 kDa) was used as loading control.

of losartan before stimulation with TGF-$\beta$. pSmad2 analysis showed a potent induction of Smad2 phosphorylation upon TGF-$\beta$ stimulation, which was not observed in non-TGF-$\beta$ stimulated cells (fig. 7.1). Losartan did not attenuate this effect, which might be explained by the fact that no AT1 receptor expression could be detected in C2C12 cells (data not shown).

Healthy and DMD-derived muscle cells were treated with AONs targeting exon 49 and 50 in the absence and presence of varying concentrations of losartan. Losartan treatment did not influence exon skipping efficiencies (data not shown). Then the effect of losartan on local 23AON injection was studied. Four weeks old *mdx* mice were treated orally with losartan for six weeks after which they received two intramuscular injections with AONs against exon 23 in their gastrocnemius muscles. RNA analysis did not show differences in exon skipping levels between losartan-treated and control mice (fig. 7.2).

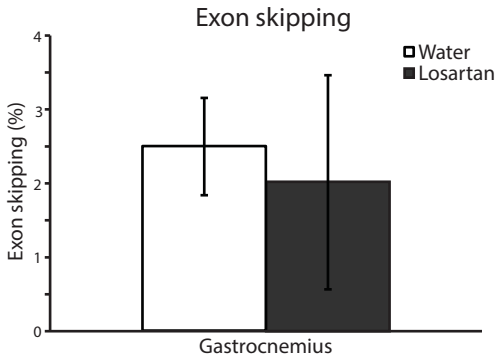


**Fig. 7.2:** Losartan has no effect on exon skipping levels after intramuscular injection of 23AONs in *mdx* mice
Quantification of exon 23 skipping levels after two intramuscular injections of 23AON in the gastrocnemius muscle. Six weeks pre-treatment with losartan did not change exon 23 skipping levels.
Data are represented as mean±SD.

To determine if there was any effect of losartan itself on signalling and muscle quality pSmad2 protein analysis and biomarker expression were performed after eight weeks of losartan treatment. pSmad2 protein might be reduced in losartan treated mice (fig. 7.3a), however group sizes were too small to draw conclusions. Expression pattern of most biomarkers did not differ between both groups in several skeletal muscles. Only a moderate reduction of some fibrotic markers (*i.e.* PAI1/Serpine1) was observed, while no change in TGF-$\beta_1$ expression itself was seen (fig. 7.3b-g).

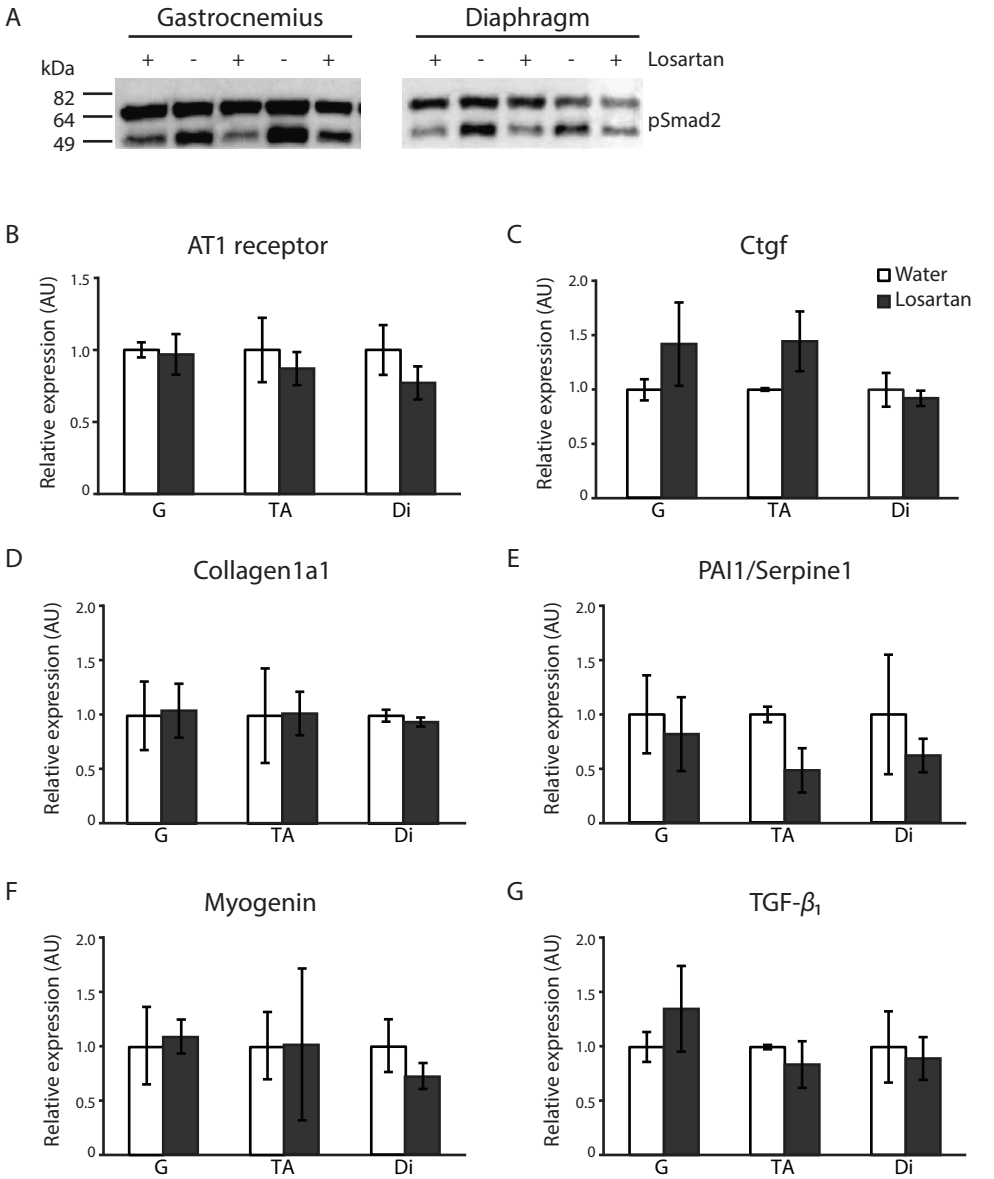


**Fig. 7.3:** pSmad2 protein levels and biomarker expression in losartan-treated *mdx* mice

a) After eight weeks of losartan treatment some reduction in pSmad2 protein expression (60 kDa; lower band) might be observed in the gastrocnemius and diaphragm of losartan-treated mice compared to control mice. However group sizes are small. b-g) Biomarker expression analysis showed no changes in several skeletal muscle in expression of the Angiotension type I receptor itself (b), several fibrotic markers (c-e), a regeneration marker (f) and TGF-$\beta_1$ (g), known to be differentially expressed in *mdx* mice. The housekeeping gene *Gapdh* was used to correct for differences in cDNA input and average expression is plotted relatively to control mice.

Error bars represent the SD. G=gastrocnemius; TA=tibialis anterior; Di=diaphragm; AT1=angiotensin II type 1 receptor; Ctgf=connective tissue growth factor; PAI1=plasminogen activator inhibitor 1; TGF=transforming growth factor

Thereafter the effect of losartan treatment on systemic 23AON treatment was analysed. Four weeks old *mdx*/*Utrn*$^{+/-}$ mice were treated for eight weeks with losartan, 23AON, a combination of both or control. *Mdx*/*Utrn*$^{+/-}$ mice were used, since pathology has shown to be aggravated compared to *mdx* mice, which display a relatively mild phenotype.[88] When we had included several animals in this experiment, additional reports on losartan in dystrophic mice were published that were much less promising than the initial publication. The first report showed only preservation of cardiac function after two years of treatment, but no effect on skeletal muscle.[261] Long term treatment did improve respiratory function, but increase of fore limb strength was only observed at two months of age and not at nine months.[263] Another group also did not observe improvement of skeletal muscle function, although a decrease in fibrosis was observed. They did see improvement of cardiac function, combined with decreased fibrosis in the heart.[264] Given these controversial results, we decided to perform an interim analysis to determine the effects of losartan itself. During treatment no changes in creatine kinase levels, a measure of muscle damage, and functional performance were observed between groups (data not shown). Histological analysis of the gastrocnemius by haematoxylin-eosin staining did not show attenuation of fibrosis by either losartan or 23AON treatment (fig. 7.4a/b).

Notably, the new literature also described that the positive effects observed in the first experiments were not mediated via blocking of TGF-$\beta$ signalling via the AT1 receptor, but

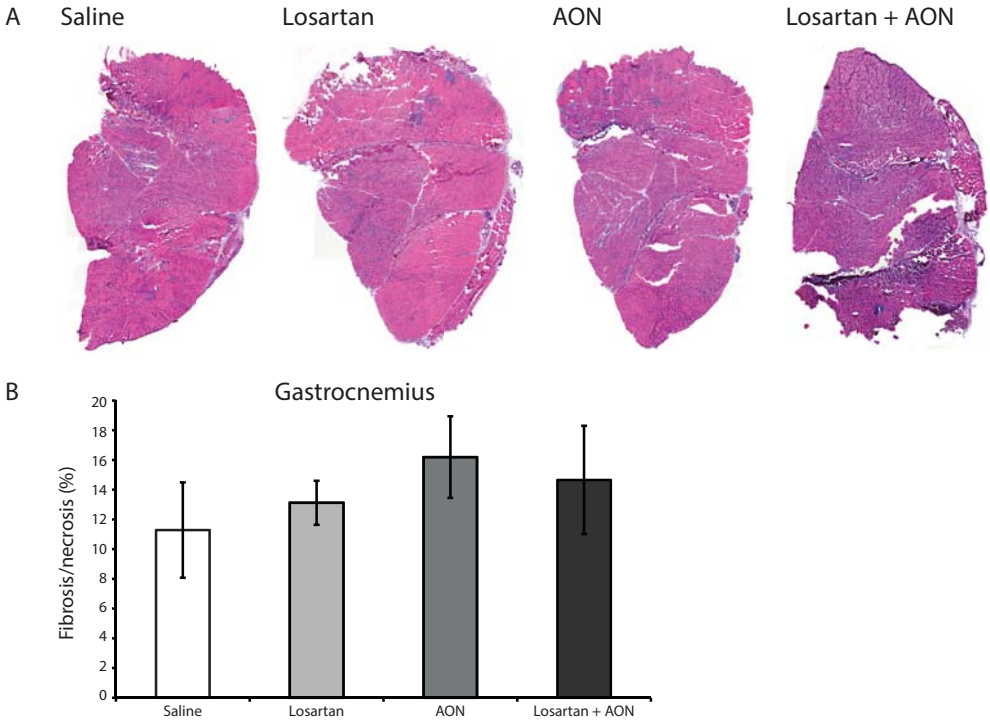


**Fig. 7.4:** Histological analysis of losartan and/or AON treated *mdx*/*Utrn*$^{+/-}$ mice.
No changes in fibrotic/necrotic area as measured by haematoxylin-eosin staining of the gastrocnemius muscle in mice treated with losartan and/or 23AON were observed.
a) Representative example for each group. Fibrotic/necrotic area is stained by haematoxylin (blue/purple), whereas eosin (pink) represents healthy tissue. b) Quantification of fibrotic/necrotic area.
Data are represented as mean±SD.

by stimulating the signalling via the AT2 receptor after AT1 receptor blockage, which has anti-fibrotic effects by blocking pERK.[262] Therefore, the effect of losartan on pERK1/2 was examined. Indeed in losartan treated cells a decrease in pERK1/2 (p44/p42 mitogen activated protein kinase or MAPK) was observed, independently of TGF-$\beta$ stimulation (fig. 7.5a), however in mice results showed large variation between different mice, independently of treatment (fig. 7.5b). Furthermore in studies in animal models for other diseases, *e.g.* disuse atrophy, no changes were observed in either pSmad2 or pERK1/2 expression.[561]

In conclusion no effect of losartan on pSmad2 phosphorylation *in vitro* was observed. *In vivo* no signs of improved muscle quality, *e.g.* by histology or biomarker expression, were seen. These results and the building evidence in literature that the main, if any, beneficial effects of losartan are at cardiac function after long term treatment, made us decide to discontinue further treatment in combination with AONs. Hopefully in the future more effective muscle quality preserving agents will be discovered that can enhance the therapeutic effect of AON treatment.

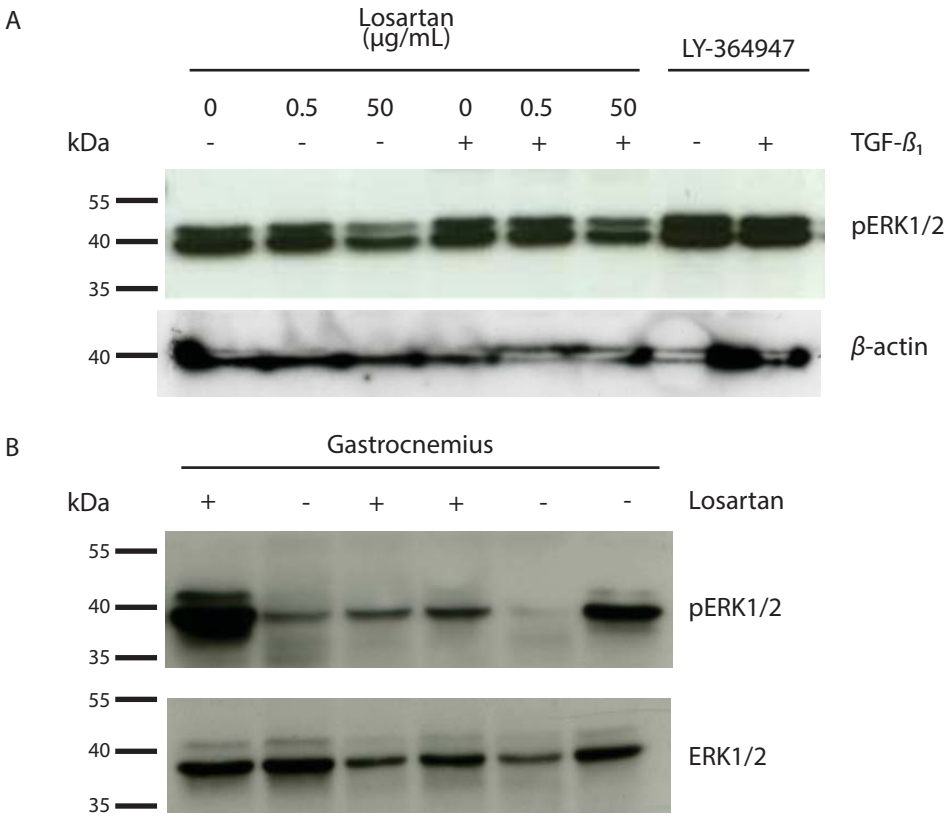


**Fig. 7.5:** Effect of losartan on ERK1/2 phosphorylation
a) In mouse myotubes a decrease in ERK1/2 phosphorylation (44/42 kDa) was observed, independently of TGF-$\beta$ stimulation. This was not observed with a specific, a TGF-$\beta_1$ receptor kinase inhibitor (LY-364947). B-actin (42 kDa) was used as loading control. b) A large variation in pERK1/2 levels was observed between individual mice, showing no clear effect of losartan treatment versus control. Total ERK1/2 was used as a loading control.

# General discussion

Chapter 8

# 8 General discussion

During the past years major steps forward have been made in developing antisense-mediated exon skipping, a potential therapy for DMD, which hopefully can turn the very severe progressive disease in a milder disease course and improve the quality of life. In addition, the knowledge about disease pathology and AONs is increasing. The antisense compounds are continuously developed further. However, there is still a lot to discover and hurdles to overcome to make the therapy successful. There are several strategies to try to improve its efficacy. One way is to improve the AONs itself or their administration. Another way is to enhance the therapeutic outcome by improving the amount/quality of their target, *i.e.* muscle.

Towards the first approach in **chapter 3 and 4** more detailed studies into the pharmacokinetic and pharmacodynamic properties of AONs in *mdx* mice are described. In **chapter 3** the effect of different dosage schemes in *mdx* mice at several levels was tested. Compared to single weekly injections, dividing the same total amount of AON over multiple injections showed to enhance its effects. Furthermore this chapter covered the influence of the frequency of repetition of injections on the maintenance of these effects after initial treatment. In both cases it revealed that increasing the desired effects, *i.e.* exon skipping and dystrophin restoration also increases the AON load on non-target organs, *i.e.* the liver, kidney and spleen, thereby increasing the risk of side effects on the long term. Therefore a balance between both effects has to be found. Towards this aim, in **chapter 4** a more detailed analysis of the pharmacokinetic and pharmacodynamic profile of AONs in *mdx* mice was performed by studying the turnover of the compound, skipped transcripts and newly formed dystrophin protein. This highlighted in particular the long half-life of the dystrophin protein. These studies may have implications for optimising AON administration schemes.

In the second part of this thesis several compounds were tested to directly or indirectly influence antisense-mediated exon skipping effects. In **chapter 5** an agent described in the literature to directly enhance AON-mediated exon skipping, 6-thioguanine (6TG), was tested in cultured cells and locally in the *mdx* mouse. *In vitro* a large number of undesired skipping events were observed, underlining that caution must be taken when using such compounds, since the chance of side effects is high. *In vitro* enhancement was only observed with suboptimally designed AONs or at suboptimal AON-concentrations, while *in vivo* no differences were observed. Here only 6TG has been tested, but other small molecules, like the recently described dantrolene, could be more effective and specific.

In **chapter 6 and 7** pharmaceutical compounds, which could potentially improve muscle quality, were tested in combination with AONs. In **chapter 6** prednisolone was used. At the moment, since a therapy targeting the underlying genetic defect is lacking, corticosteroids (predniso(lo)ne or deflazacort) are the main (pharmacological) treatment for DMD patients. Therefore most participants in clinical trials are using these compounds, which might influence the uptake and/or efficiency of AONs. Results showed that both therapies did not negatively influence each other and that prednisolone might even slightly enhance the therapeutic outcome of AON treatment. In **chapter 7** another pharmaceutical compound that showed promising results in literature on preserving muscle quality and functionality in *mdx* mice, losartan, was tested in combination with AONs to see if it could enhance efficacy. However these experiments were stopped prematurely, since interim analyses did not show any beneficial effects of losartan itself or on the working of AONs. This was supported by new literature. The results of these losartan experiments are an example of how sometimes published results can be difficult to reproduce, but at the same time underline the value of

trying to reproduce other (or your own) results and the importance of publishing negative outcomes, to avoid unnecessary repetition or clinical development of suboptimal drugs. In addition to the compounds described in these chapters, several compounds have been tested in cell models for their effect on exon skipping or *in vivo* for their effect on muscle quality in the *mdx* mouse, but unfortunately no compound has been found so far that can enhance exon skipping itself or improve the therapeutic outcome *in vivo* by improving muscle quality.

The hypothesis behind combining AONs with muscle quality improving agents was that AONs can only be effective if their target, dystrophin RNA, is present, which is only produced by muscle cells. First clinical trials had shown that the amount of dystrophin production and functional outcome depended on the amount of muscle tissue left, *i.e.* the muscle quality. However, improving muscle quality could also have a negative effect on AON efficiency. It is known from studies comparing the uptake of AONs between wild type and *mdx* mice that the uptake is much lower in wild type mice, since the AON uptake is facilitated by the leakiness of the dystrophic muscle fibres. If this permeability decreases by improving muscle quality, the uptake might be hampered. Therefore the reason no enhancement of AON effects could be found, can either be that there is no effect or that the positive and negative effects are neutralizing each other. Furthermore large interindividual variation causes that only large improvements will be picked up and longer treatment time might be needed, before differences will be found. This also raises the question how meaningful very small differences would be in the clinic at the end.

Probably larger improvements can be made by improving the effectiveness of the AON therapy itself. One strategy is to use viral vectors to get a higher and longer lasting expression of AONs. However, this approach has many drawbacks as viral vectors can evoke immune responses, as a result of which repetition of treatment is not possible. At the moment, a better strategy seems to be to modify the AONs themselves. Preclinical studies *in vitro* and *in vivo* have shown that large improvements can be made by for example conjugating muscle-targeting peptides to them. Although also here prudence is called for, since some of these peptides have shown to be toxic in higher animals, like primates.

An issue that appears in every study is the difficulty of targeting the heart, where observed exon skipping levels and restored dystrophin protein are much lower compared to skeletal muscle. This will become more and more important once the therapy becomes more effective, since higher activity levels will result in a higher workload on the heart. Also here conjugation of the AON with a specific heart-targeting peptide might be an option.

Nevertheless results from recent clinical trials have shown the importance of muscle quality. It indicates that starting treatment as early as possible would be recommended. The therapy can only put a hold on or slow down muscle degeneration, not bring back muscle that is already lost. Reaching a level of dystrophin restoration high enough to do so, is needed. Therefore more detailed studies on the effect of different levels of dystrophin restoration on muscle overall muscle functioning, but also on differences between individual muscles are very helpful.

All these matters require further investigation and optimisation of the AON-mediated exon skipping therapy. Especially the clinical trials will give more insights on the translation from animal models to humans and on the problems that have to be tackled in order to make it an effective therapy.

# References


1. Moat SJ, Bradley DM, Salmon R, Clarke A, Hartley L (2013), *Newborn bloodspot screening for Duchenne Muscular Dystrophy: 21 years experience in Wales (UK).* Eur J Hum Genet; 21: 1049-1053.
2. Mendell JR, Lloyd-Puryear M (2013), *Report of MDA muscle disease symposium on newborn screening for Duchenne muscular dystrophy.* Muscle Nerve; 48: 21-26.
3. Moser H (1984), *Duchenne muscular dystrophy: pathogenetic aspects and genetic prevention.* Hum Genet; 66: 17-40.
4. Gowers WR (1895), *A manual of the nervous system.* 2nd ed. Philadelphia.
5. Emery AE (2002), *The muscular dystrophies.* Lancet; 359: 687-695.
6. Passamano L, Taglia A, Palladino A, Viggiano E, D'Ambrosio P, Scutifero M *et al.* (2012), *Improvement of survival in Duchenne Muscular Dystrophy: retrospective analysis of 835 patients.* Acta Myol; 31: 121-125.
7. Angelini C, Peterle E (2012), *Old and new therapeutic developments in steroid treatment in Duchenne muscular dystrophy.* Acta Myol; 31: 9-15.
8. Politano L, Nigro G (2012), *Treatment of dystrophinopathic cardiomyopathy: review of the literature and personal results.* Acta Myol; 31: 24-30.
9. Alman BA, Raza SN, Biggar WD (2004), *Steroid treatment and the development of scoliosis in males with duchenne muscular dystrophy.* J Bone Joint Surg Am; 86-A: 519-524.
10. Angelini C (2007), *The role of corticosteroids in muscular dystrophy: a critical appraisal.* Muscle Nerve; 36: 424-435.
11. Barber BJ, Andrews JG, Lu Z, West NA, Meaney FJ, Price ET *et al.* (2013), *Oral corticosteroids and onset of cardiomyopathy in duchenne muscular dystrophy.* J Pediatr; 163: 1080-1084.
12. Bushby K, Finkel R, Birnkrant DJ, Case LE, Clemens PR, Cripe L *et al.* (2010), *Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and pharmacological and psychosocial management.* Lancet Neurol; 9: 77-93.
13. Merlini L, Cicognani A, Malaspina E, Gennari M, Gnudi S, Talim B *et al.* (2003), *Early prednisone treatment in Duchenne muscular dystrophy.* Muscle Nerve; 27: 222-227.
14. Ishikawa Y, Bach JR, Minami R (1999), *Cardioprotection for Duchenne's muscular dystrophy.* Am Heart J; 137: 895-902.
15. Ramaciotti C, Heistein LC, Coursey M, Lemler MS, Eapen RS, Iannaccone ST *et al.* (2006), *Left ventricular function and response to enalapril in patients with duchenne muscular dystrophy during the second decade of life.* Am J Cardiol; 98: 825-827.
16. Rall S, Grimm T (2012), *Survival in Duchenne muscular dystrophy.* Acta Myol; 31: 117-120.
17. Eagle M, Baudouin SV, Chandler C, Giddings DR, Bullock R, Bushby K (2002), *Survival in Duchenne muscular dystrophy: improvements in life expectancy since 1967 and the impact of home nocturnal ventilation.* Neuromuscul Disord; 12: 926-929.
18. Eagle M, Bourke J, Bullock R, Gibson M, Mehta J, Giddings D *et al.* (2007), *Managing Duchenne muscular dystrophy--the additive effect of spinal surgery and home nocturnal ventilation in improving survival.* Neuromuscul Disord; 17: 470-475.
19. Yasuma F, Sakai M, Matsuoka Y (1996), *Effects of noninvasive ventilation on survival in patients with Duchenne's muscular dystrophy.* Chest; 109: 590.
20. Finsterer J, Stollberger C (2008), *Cardiac involvement in Becker muscular dystrophy.* Can J Cardiol; 24: 786-792.
21. Nigro G, Comi LI, Politano L, Limongelli FM, Nigro V, De Rimini ML *et al.* (1995), *Evaluation of the cardiomyopathy in Becker muscular dystrophy.* Muscle Nerve; 18: 283-291.
22. Hoogerwaard EM, Bakker E, Ippel PF, Oosterwijk JC, Majoor-Krakauer DF, Leschot NJ *et al.* (1999), *Signs and symptoms of Duchenne muscular dystrophy and Becker muscular dystrophy among carriers in The Netherlands: a cohort study.* Lancet; 353: 2116-2119.
23. Norman A, Harper P (1989), *A survey of manifesting carriers of Duchenne and Becker muscular dystrophy in Wales.* Clin Genet; 36: 31-37.
24. Schade van Westrum SM, Hoogerwaard EM, Dekker L, Standaar TS, Bakker E, Ippel PF *et al.* (2011), *Cardiac abnormalities in a follow-up study on carriers of Duchenne and Becker muscular dystrophy.* Neurology; 77: 62-66.
25. Grain L, Cortina-Borja M, Forfar C, Hilton-Jones D, Hopkin J, Burch M (2001), *Cardiac abnormalities and skeletal muscle weakness in carriers of Duchenne and Becker muscular dystrophies and controls.* Neuromuscul Disord; 11: 186-191.
26. Mercier S, Toutain A, Toussaint A, Raynaud M, de BC, Marcorelles P *et al.* (2013), *Genetic and clinical specificity of 26 symptomatic carriers for dystrophinopathies at pediatric age.* Eur J Hum Genet; 21: 855-863.

27. Viggiano E, Picillo E, Cirillo A, Politano L (2013), *Comparison of X-chromosome inactivation in Duchenne muscle/myocardium-manifesting carriers, non-manifesting carriers and related daughters.* Clin Genet; 84: 265-270.
28. Boyd Y, Buckle V, Holt S, Munro E, Hunter D, Craig I (1986), *Muscular dystrophy in girls with X;autosome translocations.* J Med Genet; 23: 484-490.
29. Juan-Mateu J, Rodriguez MJ, Nascimento A, Jimenez-Mallebrera C, Gonzalez-Quereda L, Rivas E *et al.* (2012), *Prognostic value of X-chromosome inactivation in symptomatic female carriers of dystrophinopathy.* Orphanet J Rare Dis; 7: 82.
30. Burghes AH, Logan C, Hu X, Belfall B, Worton RG, Ray PN (1987), *A cDNA clone from the Duchenne/Becker muscular dystrophy gene.* Nature; 328: 434-437.
31. Davies KE, Pearson PL, Harper PS, Murray JM, O'Brien T, Sarfarazi M *et al.* (1983), *Linkage analysis of two cloned DNA sequences flanking the Duchenne muscular dystrophy locus on the short arm of the human X chromosome.* Nucleic Acids Res; 11: 2303-2312.
32. Jacobs PA, Hunt PA, Mayer M, Bart RD (1981), *Duchenne muscular dystrophy (DMD) in a female with an X/autosome translocation: further evidence that the DMD locus is at Xp21.* Am J Hum Genet; 33: 513-518.
33. Monaco AP, Bertelson CJ, Middlesworth W, Colletti CA, Aldridge J, Fischbeck KH *et al.* (1985), *Detection of deletions spanning the Duchenne muscular dystrophy locus using a tightly linked DNA segment.* Nature; 316: 842-845.
34. Murray JM, Davies KE, Harper PS, Meredith L, Mueller CR, Williamson R (1982), *Linkage relationship of a cloned DNA sequence on the short arm of the X chromosome to Duchenne muscular dystrophy.* Nature; 300: 69-71.
35. van Ommen GJ, Verkerk JM, Hofker MH, Monaco AP, Kunkel LM, Ray P *et al.* (1986), *A physical map of 4 million bp around the Duchenne muscular dystrophy gene on the human X-chromosome.* Cell; 47: 499-504.
36. Kingston HM, Sarfarazi M, Thomas NS, Harper PS (1984), *Localisation of the Becker muscular dystrophy gene on the short arm of the X chromosome by linkage to cloned DNA sequences.* Hum Genet; 67: 6-17.
37. Koenig M, Hoffman EP, Bertelson CJ, Monaco AP, Feener C, Kunkel LM (1987), *Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals.* Cell; 50: 509-517.
38. den Dunnen JT, Bakker E, Breteler EG, Pearson PL, van Ommen GJ (1987), *Direct detection of more than 50% of the Duchenne muscular dystrophy mutations by field inversion gels.* Nature; 329: 640-642.
39. Hoffman EP, Brown Jr. RH, Kunkel LM (1987), *Dystrophin: the protein product of the Duchenne muscular dystrophy locus.* Cell; 51: 919-928.
40. Hoffman EP, Kunkel LM, Angelini C, Clarke A, Johnson M, Harris JB (1989), *Improved diagnosis of Becker muscular dystrophy by dystrophin testing.* Neurology; 39: 1011-1017.
41. Hoffman EP, Fischbeck KH, Brown RH, Johnson M, Medori R, Loike JD *et al.* (1988), *Characterization of dystrophin in muscle-biopsy specimens from patients with Duchenne's or Becker's muscular dystrophy.* N Engl J Med; 318: 1363-1368.
42. Koenig M, Beggs AH, Moyer M, Scherpf S, Heindrich K, Bettecken T *et al.* (1989), *The molecular basis for Duchenne versus Becker muscular dystrophy: correlation of severity with type of deletion.* Am J Hum Genet; 45: 498-506.
43. Monaco AP, Bertelson CJ, Liechti-Gallati S, Moser H, Kunkel LM (1988), *An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus.* Genomics; 2: 90-95.
44. Aartsma-Rus A, van Deutekom JC, Fokkema IF, van Ommen GJ, den Dunnen JT (2006), *Entries in the Leiden Duchenne muscular dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule.* Muscle Nerve; 34: 135-144.
45. Roberts RG, Coffey AJ, Bobrow M, Bentley DR (1993), *Exon structure of the human dystrophin gene.* Genomics; 16: 536-538.
46. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J *et al.* (2001), *Initial sequencing and analysis of the human genome.* Nature; 409: 860-921.
47. Laing NG (1993), *Molecular genetics and genetic counselling for Duchenne/Becker muscular dystrophy.* Mol Cell Biol Hum Dis Ser; 3: 37-84.
48. den Dunnen JT, Bakker E, van Ommen GJ, Pearson PL (1989), *The DMD gene analysed by field inversion gel electrophoresis.* Br Med Bull; 45: 644-658.
49. Muntoni F, Torelli S, Ferlini A (2003), *Dystrophin and mutations: one gene, several proteins, multiple phenotypes.* Lancet Neurol; 2: 731-740.
50. Neri M, Torelli S, Brown S, Ugo I, Sabatelli P, Merlini L *et al.* (2007), *Dystrophin levels as low as 30% are sufficient to avoid muscular dystrophy in the human.* Neuromuscul Disord; 17: 913-918.
51. Anthony K, Cirak S, Torelli S, Tasca G, Feng L, Arechavala-Gomeza V *et al.* (2011), *Dystrophin quantifi-*

*cation and clinical correlations in Becker muscular dystrophy: implications for clinical trials.* Brain; 134: 3547-3559.

52. Bushby KM, Gardner-Medwin D, Nicholson LV, Johnson MA, Haggerty ID, Cleghorn NJ *et al.* (1993), *The clinical, genetic and dystrophin characteristics of Becker muscular dystrophy. II. Correlation of phenotype with genetic and protein abnormalities.* J Neurol; 240: 105-112.
53. Comi GP, Prelle A, Bresolin N, Moggio M, Bardoni A, Gallanti A *et al.* (1994), *Clinical variability in Becker muscular dystrophy. Genetic, biochemical and immunohistochemical correlates.* Brain; 117 ( Pt 1): 1-14.
54. Beggs AH, Hoffman EP, Snyder JR, Arahata K, Specht L, Shapiro F *et al.* (1991), *Exploring the molecular basis for variability among patients with Becker muscular dystrophy: dystrophin gene and protein studies.* Am J Hum Genet; 49: 54-67.
55. Amann KJ, Renley BA, Ervasti JM (1998), *A cluster of basic repeats in the dystrophin rod domain binds F-actin through an electrostatic interaction.* J Biol Chem; 273: 28419-28423.
56. Lai Y, Thomas GD, Yue Y, Yang HT, Li D, Long C *et al.* (2009), *Dystrophins carrying spectrin-like repeats 16 and 17 anchor nNOS to the sarcolemma and enhance exercise performance in a mouse model of muscular dystrophy.* J Clin Invest; 119: 624-635.
57. Ehmsen J, Poon E, Davies K (2002), *The dystrophin-associated protein complex.* J Cell Sci; 115: 2801-2803.
58. Gumerson JD, Michele DE (2011), *The dystrophin-glycoprotein complex in the prevention of muscle damage.* J Biomed Biotechnol; 2011: 210797.
59. Bellinger AM, Reiken S, Carlson C, Mongillo M, Liu X, Rothman L *et al.* (2009), *Hypernitrosylated ryanodine receptor calcium release channels are leaky in dystrophic muscle.* Nat Med; 15: 325-330.
60. Miller G, Wang EL, Nassar KL, Peter AK, Crosbie RH (2007), *Structural and functional analysis of the sarcoglycan-sarcospan subcomplex.* Exp Cell Res; 313: 639-651.
61. Hezel M, de Groat WC, Galbiati F (2010), *Caveolin-3 promotes nicotinic acetylcholine receptor clustering and regulates neuromuscular junction activity.* Mol Biol Cell; 21: 302-310.
62. Brenman JE, Chao DS, Xia H, Aldape K, Bredt DS (1995), *Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy.* Cell; 82: 743-752.
63. Gee SH, Madhavan R, Levinson SR, Caldwell JH, Sealock R, Froehner SC (1998), *Interaction of muscle and brain sodium channels with multiple members of the syntrophin family of dystrophin-associated proteins.* J Neurosci; 18: 128-137.
64. Newey SE, Howman EV, Ponting CP, Benson MA, Nawrotzki R, Loh NY *et al.* (2001), *Syncoilin, a novel member of the intermediate filament superfamily that interacts with alpha-dystrobrevin in skeletal muscle.* J Biol Chem; 276: 6645-6655.
65. Nicolas A, Lucchetti-Miganeh C, Yaou RB, Kaplan JC, Chelly J, Leturcq F *et al.* (2012), *Assessment of the structural and functional impact of in-frame mutations of the DMD gene, using the tools included in the eDystrophin online database.* Orphanet J Rare Dis; 7: 45.
66. Chelly J, Hamard G, Koulakoff A, Kaplan JC, Kahn A, Berwald-Netter Y (1990), *Dystrophin gene transcribed from different promoters in neuronal and glial cells.* Nature; 344: 64-65.
67. Gorecki DC, Monaco AP, Derry JM, Walker AP, Barnard EA, Barnard PJ (1992), *Expression of four alternative dystrophin transcripts in brain regions regulated by different promoters.* Hum Mol Genet; 1: 505-510.
68. D'Souza VN, Nguyen TM, Morris GE, Karges W, Pillers DA, Ray PN (1995), *A novel dystrophin isoform is required for normal retinal electrophysiology.* Hum Mol Genet; 4: 837-842.
69. Byers TJ, Lidov HG, Kunkel LM (1993), *An alternative dystrophin transcript specific to peripheral nerve.* Nat Genet; 4: 77-81.
70. Hugnot JP, Gilgenkrantz H, Vincent N, Chafey P, Morris GE, Monaco AP *et al.* (1992), *Distal transcript of the dystrophin gene initiated from an alternative first exon and encoding a 75-kDa protein widely distributed in nonmuscle tissues.* Proc Natl Acad Sci USA; 89: 7506-7510.
71. Tadayoni R, Rendon A, Soria-Jasso LE, Cisneros B (2012), *Dystrophin Dp71: the smallest but multifunctional product of the Duchenne muscular dystrophy gene.* Mol Neurobiol; 45: 43-60.
72. Snow WM, Anderson JE, Jakobson LS (2013), *Neuropsychological and neurobehavioral functioning in Duchenne muscular dystrophy: a review.* Neurosci Biobehav Rev; 37: 743-752.
73. Waite A, Brown SC, Blake DJ (2012), *The dystrophin-glycoprotein complex in brain development and disease.* Trends Neurosci; 35: 487-496.
74. Taylor PJ, Betts GA, Maroulis S, Gilissen C, Pedersen RL, Mowat DR *et al.* (2010), *Dystrophin gene mutation location and the risk of cognitive impairment in Duchenne muscular dystrophy.* PLoS One; 5: e8803.
75. Tinsley JM, Blake DJ, Davies KE (1993), *Apo-dystrophin-3: a 2.2kb transcript from the DMD locus encoding the dystrophin glycoprotein binding site.* Hum Mol Genet; 2: 521-524.

76. Tozawa T, Itoh K, Yaoi T, Tando S, Umekage M, Dai H *et al.* (2012), *The shortest isoform of dystrophin (Dp40) interacts with a group of presynaptic proteins to form a presumptive novel complex in the mouse brain.* Mol Neurobiol; 45: 287-297.
77. Moizard MP, Billard C, Toutain A, Berret F, Marmin N, Moraine C (1998), *Are Dp71 and Dp140 brain dystrophin isoforms related to cognitive impairment in Duchenne muscular dystrophy?* Am J Med Genet; 80: 32-41.
78. Pillers DA, Fitzgerald KM, Duncan NM, Rash SM, White RA, Dwinnell SJ *et al.* (1999), *Duchenne/Becker muscular dystrophy: correlation of phenotype by electroretinography with sites of dystrophin mutations.* Hum Genet; 105: 2-9.
79. Sicinski P, Geng Y, Ryder-Cook AS, Barnard EA, Darlison MG, Barnard PJ (1989), *The molecular basis of muscular dystrophy in the mdx mouse: a point mutation.* Science; 244: 1578-1580.
80. Verhaart IE, van Duijn RJ, den Adel B, Roest AA, Verschuuren JJ, Aartsma-Rus A *et al.* (2012), *Assessment of cardiac function in three mouse dystrophinopathies by magnetic resonance imaging.* Neuromuscul Disord; 22: 418-426.
81. Chamberlain JS, Metzger J, Reyes M, Townsend D, Faulkner JA (2007), *Dystrophin-deficient mdx mice display a reduced life span and are susceptible to spontaneous rhabdomyosarcoma.* FASEB J; 21: 2195-2204.
82. Khurana TS, Watkins SC, Chafey P, Chelly J, Tome FM, Fardeau M *et al.* (1991), *Immunolocalization and developmental expression of dystrophin related protein in skeletal muscle.* Neuromuscul Disord; 1: 185-194.
83. Khurana TS, Davies KE (2003), *Pharmacological strategies for muscular dystrophy.* Nat Rev Drug Discov; 2: 379-390.
84. Tinsley JM, Blake DJ, Roche A, Fairbrother U, Riss J, Byth BC *et al.* (1992), *Primary structure of dystrophin-related protein.* Nature; 360: 591-593.
85. Mizuno Y, Nonaka I, Hirai S, Ozawa E (1993), *Reciprocal expression of dystrophin and utrophin in muscles of Duchenne muscular dystrophy patients, female DMD-carriers and control subjects.* J Neurol Sci; 119: 43-52.
86. Deconinck AE, Rafael JA, Skinner JA, Brown SC, Potter AC, Metzinger L *et al.* (1997), *Utrophin-dystrophin-deficient mice as a model for Duchenne muscular dystrophy.* Cell; 90: 717-727.
87. Zhou L, Rafael-Fortney JA, Huang P, Zhao XS, Cheng G, Zhou X *et al.* (2008), *Haploinsufficiency of utrophin gene worsens skeletal muscle inflammation and fibrosis in mdx mice.* J Neurol Sci; 264: 106-111.
88. van Putten M, Kumar D, Hulsker M, Hoogaars WM, Plomp JJ, van Opstal A. *et al.* (2012), *Comparison of skeletal muscle pathology and motor function of dystrophin and utrophin deficient mouse strains.* Neuromuscul Disord; 22: 406-417.
89. Willmann R, Possekel S, Dubach-Powell J, Meier T, Ruegg MA (2009), *Mammalian animal models for Duchenne muscular dystrophy.* Neuromuscul Disord; 19: 241-249.
90. Durbeej M, Campbell KP (2002), *Muscular dystrophies involving the dystrophin-glycoprotein complex: an overview of current mouse models.* Curr Opin Genet Dev; 12: 349-361.
91. Megeney LA, Kablar B, Garrett K, Anderson JE, Rudnicki MA (1996), *MyoD is required for myogenic stem cell function in adult skeletal muscle.* Genes Dev; 10: 1173-1183.
92. Garry DJ, Meeson A, Elterman J, Zhao Y, Yang P, Bassel-Duby R *et al.* (2000), *Myogenic stem cell function is impaired in mice lacking the forkhead/winged helix protein MNF.* Proc Natl Acad Sci USA; 97: 5416-5421.
93. Grady RM, Grange RW, Lau KS, Maimone MM, Nichol MC, Stull JT *et al.* (1999), *Role for alpha-dystrobrevin in the pathogenesis of dystrophin-dependent muscular dystrophies.* Nat Cell Biol; 1: 215-220.
94. Guo C, Willem M, Werner A, Raivich G, Emerson M, Neyses L *et al.* (2006), *Absence of alpha 7 integrin in dystrophin-deficient mice causes a myopathy similar to Duchenne muscular dystrophy.* Hum Mol Genet; 15: 989-998.
95. Raymackers JM, Debaix H, Colson-Van SM, De BF, Tajeddine N, Schwaller B *et al.* (2003), *Consequence of parvalbumin deficiency in the mdx mouse: histological, biochemical and mechanical phenotype of a new double mutant.* Neuromuscul Disord; 13: 376-387.
96. Crosbie RH, Straub V, Yun HY, Lee JC, Rafael JA, Chamberlain JS *et al.* (1998), *Mdx muscle pathology is independent of nNOS perturbation.* Hum Mol Genet; 7: 823-829.
97. Fukada S, Morikawa D, Yamamoto Y, Yoshida T, Sumie N, Yamaguchi M *et al.* (2010), *Genetic background affects properties of satellite cells and mdx phenotypes.* Am J Pathol; 176: 2414-2424.
98. Chapman VM, Miller DR, Armstrong D, Caskey CT (1989), *Recovery of induced mutations for X chromosome-linked muscular dystrophy in mice.* Proc Natl Acad Sci USA; 86: 1292-1296.
99. Im WB, Phelps SF, Copen EH, Adams EG, Slightom JL, Chamberlain JS (1996), *Differential expression of dystrophin isoforms in strains of mdx mice with different mutations.* Hum Mol Genet; 5: 1149-1153.

100. Cox GA, Phelps SF, Chapman VM, Chamberlain JS (1993), *New mdx mutation disrupts expression of muscle and nonmuscle isoforms of dystrophin.* Nat Genet; 4: 87-93.
101. Danko I, Chapman V, Wolff JA (1992), *The frequency of revertants in mdx mouse genetic models for Duchenne muscular dystrophy.* Pediatr Res; 32: 128-131.
102. Li D, Yue Y, Duan D (2008), *Preservation of muscle force in Mdx3cv mice correlates with low-level expression of a near full-length dystrophin protein.* Am J Pathol; 172: 1332-1341.
103. Beastrom N, Lu H, Macke A, Canan BD, Johnson EK, Penton CM *et al.* (2011), *Mdx((5)cv) mice manifest more severe muscle dysfunction and diaphragm force deficits than do mdx mice.* Am J Pathol; 179: 2464-2474.
104. Morrison J, Lu QL, Pastoret C, Partridge T, Bou-Gharios G (2000), *T-cell-dependent fibrosis in the mdx dystrophic mouse.* Lab Invest; 80: 881-891.
105. Morrison J, Partridge T, Bou-Gharios G (2005), *Nude mutation influences limb skeletal muscle development.* Matrix Biol; 23: 535-542.
106. Bosma GC, Custer RP, Bosma MJ (1983), *A severe combined immunodeficiency mutation in the mouse.* Nature; 301: 527-530.
107. Torrente Y, Belicchi M, Sampaolesi M, Pisati F, Meregalli M, D'Antona G *et al.* (2004), *Human circulating AC133(+) stem cells restore dystrophin expression and ameliorate function in dystrophic skeletal muscle.* J Clin Invest; 114: 182-195.
108. Arpke RW, Darabi R, Mader TL, Zhang Y, Toyama A, Lonetree CL *et al.* (2013), *A new immuno-, dystrophin-deficient model, the NSG-mdx(4Cv) mouse, provides evidence for functional improvement following allogeneic satellite cell transplantation.* Stem Cells; 31: 1611-1620.
109. Araki E, Nakamura K, Nakao K, Kameya S, Kobayashi O, Nonaka I *et al.* (1997), *Targeted disruption of exon 52 in the mouse dystrophin gene induced muscle degeneration similar to that observed in Duchenne muscular dystrophy.* Biochem Biophys Res Commun; 238: 492-497.
110. Sarig R, Mezger-Lallemand V, Gitelman I, Davis C, Fuchs O, Yaffe D *et al.* (1999), *Targeted inactivation of Dp71, the major non-muscle product of the DMD gene: differential activity of the Dp71 promoter during development.* Hum Mol Genet; 8: 1-10.
111. Cox GA, Cole NM, Matsumura K, Phelps SF, Hauschka SD, Campbell KP *et al.* (1993), *Overexpression of dystrophin in transgenic mdx mice eliminates dystrophic symptoms without toxicity.* Nature; 364: 725-729.
112. Cox GA, Sunada Y, Campbell KP, Chamberlain JS (1994), *Dp71 can restore the dystrophin-associated glycoprotein complex in muscle but fails to prevent dystrophy.* Nat Genet; 8: 333-339.
113. Greenberg DS, Sunada Y, Campbell KP, Yaffe D, Nudel U (1994), *Exogenous Dp71 restores the levels of dystrophin associated proteins but does not alleviate muscle damage in mdx mice.* Nat Genet; 8: 340-344.
114. Rafael JA, Sunada Y, Cole NM, Campbell KP, Faulkner JA, Chamberlain JS (1994), *Prevention of dystrophic pathology in mdx mice by a truncated dystrophin isoform.* Hum Mol Genet; 3: 1725-1733.
115. Banks GB, Gregorevic P, Allen JM, Finn EE, Chamberlain JS (2007), *Functional capacity of dystrophins carrying deletions in the N-terminal actin-binding domain.* Hum Mol Genet; 16: 2105-2113.
116. Phelps SF, Hauser MA, Cole NM, Rafael JA, Hinkle RT, Faulkner JA *et al.* (1995), *Expression of full-length and truncated dystrophin mini-genes in transgenic mdx mice.* Hum Mol Genet; 4: 1251-1258.
117. Dunant P, Larochelle N, Thirion C, Stucka R, Ursu D, Petrof BJ *et al.* (2003), *Expression of dystrophin driven by the 1.35-kb MCK promoter ameliorates muscular dystrophy in fast, but not in slow muscles of transgenic mdx mice.* Mol Ther; 8: 80-89.
118. Stillwell E, Vitale J, Zhao Q, Beck A, Schneider J, Khadim F *et al.* (2009), *Blastocyst injection of wild type embryonic stem cells induces global corrections in mdx mice.* PLoS One; 4: e4759.
119. van Putten M, Hulsker M, Nadarajah VD, van Heiningen SH, van Huizen E, van Iterson M. *et al.* (2012), *The effects of low levels of dystrophin on mouse muscle function and pathology.* PLoS One; 7: e31937.
120. van Putten M, Hulsker M, Young C, Nadarajah VD, Heemskerk H, van der Weerd L *et al.* (2013), *Low dystrophin levels increase survival and improve muscle pathology and function in dystrophin/utrophin double-knockout mice.* FASEB J; 27: 2484-2495.
121. 't Hoen PA, de Meijer EJ, Boer JM, Vossen RH, Turk R, Maatman RG *et al.* (2008), *Generation and characterization of transgenic mice with the full-length human DMD gene.* J Biol Chem; 283: 5899-5907.
122. Sharp NJ, Kornegay JN, Van Camp SD, Herbstreith MH, Secore SL, Kettle S *et al.* (1992), *An error in dystrophin mRNA processing in golden retriever muscular dystrophy, an animal homologue of Duchenne muscular dystrophy.* Genomics; 13: 115-121.
123. Howell JM, Fletcher S, Kakulas BA, O'Hara M, Lochmuller H, Karpati G (1997), *Use of the dog model for Duchenne muscular dystrophy in gene therapy trials.* Neuromuscul Disord; 7: 325-328.
124. Ambrosio CE, Valadares MC, Zucconi E, Cabral R, Pearson PL, Gaiad TP *et al.* (2008), *Ringo, a Golden Retriever Muscular Dystrophy (GRMD) dog with absent dystrophin but normal strength.* Neuromuscul Disord; 18: 892-893.

125. Valentine BA, Winand NJ, Pradhan D, Moise NS, de LA, Kornegay JN *et al.* (1992), *Canine X-linked muscular dystrophy as an animal model of Duchenne muscular dystrophy: a review.* Am J Med Genet; 42: 352-356.
126. Shimatsu Y, Yoshimura M, Yuasa K, Urasawa N, Tomohiro M, Nakura M *et al.* (2005), *Major clinical and histopathological characteristics of canine X-linked muscular dystrophy in Japan, CXMDJ.* Acta Myol; 24: 145-154.
127. Yugeta N, Urasawa N, Fujii Y, Yoshimura M, Yuasa K, Wada MR *et al.* (2006), *Cardiac involvement in Beagle-based canine X-linked muscular dystrophy in Japan (CXMDJ): electrocardiographic, echocardiographic, and morphologic studies.* BMC Cardiovasc Disord; 6: 47.
128. Shimatsu Y, Katagiri K, Furuta T, Nakura M, Tanioka Y, Yuasa K *et al.* (2003), *Canine X-linked muscular dystrophy in Japan (CXMDJ).* Exp Anim; 52: 93-97.
129. Schatzberg SJ, Olby NJ, Breen M, Anderson LV, Langford CF, Dickens HF *et al.* (1999), *Molecular analysis of a spontaneous dystrophin 'knockout' dog.* Neuromuscul Disord; 9: 289-295.
130. Walmsley GL, Arechavala-Gomeza V, Fernandez-Fuente M, Burke MM, Nagel N, Holder A *et al.* (2010), *A duchenne muscular dystrophy gene hot spot mutation in dystrophin-deficient cavalier king charles spaniels is amenable to exon 51 skipping.* PLoS One; 5: e8647.
131. Bergman RL, Inzana KD, Monroe WE, Shell LG, Liu LA, Engvall E *et al.* (2002), *Dystrophin-deficient muscular dystrophy in a Labrador retriever.* J Am Anim Hosp Assoc; 38: 255-261.
132. Van Ham LML, Desmidt M, Tshamata M. (1993), *Canine X linked muscular dystrophy in Belgian Groendaeler Shepherds.* J Am Anim Hosp Assoc; 570-574.
133. Van Ham LML, Roels S, Hoorensm J.K. (1995), *Congenital dystrophy-like myopathy in a Brittany spaniel puppy.* Prog Vet Neurol; 135-138.
134. Wentick GH, Vanderlinde-Sipman JS, Meijer AEFH (1972), *Myopathy with a possible recessive x-linked inheritance in a litter of Irish terriers.* Vet Pathol; 328-349.
135. Paola JP, Podell M, Shelton GD (1993), *Muscular dystrophy in a miniature Schnauzer.* Prog Vet Neurol; 14-18.
136. Wetterman CA, Harkin KR, Cash WC, Nietfield JC, Shelton GD (2000), *Hypertrophic muscular dystrophy in a young dog.* J Am Vet Med Assoc; 216: 878-81, 864.
137. Jones BR, Brennan S, Mooney CT, Callanan JJ, McAllister H, Guo LT *et al.* (2004), *Muscular dystrophy with truncated dystrophin in a family of Japanese Spitz dogs*. J Neurol Sci; 217: 143-149.
138. Carpenter JL, Hoffman EP, Romanul FC, Kunkel LM, Rosales RK, Ma NS *et al.* (1989), *Feline muscular dystrophy with dystrophin deficiency.* Am J Pathol; 135: 909-919.
139. Winand NJ, Edwards M, Pradhan D, Berian CA, Cooper BJ (1994), *Deletion of the dystrophin muscle promoter in feline muscular dystrophy.* Neuromuscul Disord; 4: 433-445.
140. Gaschen FP, Hoffman EP, Gorospe JR, Uhl EW, Senior DF, Cardinet GH, III *et al.* (1992), *Dystrophin deficiency causes lethal muscle hypertrophy in cats.* J Neurol Sci; 110: 149-159.
141. Gaschen L, Lang J, Lin S, Ade-Damilano M, Busato A, Lombard CW *et al.* (1999), *Cardiomyopathy in dystrophin-deficient hypertrophic feline muscular dystrophy.* J Vet Intern Med; 13: 346-356.
142. Roberts RG, Bobrow M (1998), *Dystrophins in vertebrates and invertebrates.* Hum Mol Genet; 7: 589-595.
143. Bessou C, Giugia JB, Franks CJ, Holden-Dye L, Segalat L (1998), *Mutations in the Caenorhabditis elegans dystrophin-like gene dys-1 lead to hyperactivity and suggest a link with cholinergic transmission.* Neurogenetics; 2: 61-72.
144. Gieseler K, Grisoni K, Segalat L (2000), *Genetic suppression of phenotypes arising from mutations in dystrophin-related genes in Caenorhabditis elegans.* Curr Biol; 10: 1092-1097.
145. Bassett DI, Currie PD (2003), *The zebrafish as a model for muscular dystrophy and congenital myopathy.* Hum Mol Genet; 12 Spec No 2: R265-R270.
146. Guyon JR, Goswami J, Jun SJ, Thorne M, Howell M, Pusack T *et al.* (2009), *Genetic isolation and characterization of a splicing mutant of zebrafish dystrophin.* Hum Mol Genet; 18: 202-211.
147. Klymiuk N, Blutke A, Graf A, Krause S, Burkhardt K, Wuensch A *et al.* (2013), *Dystrophin-deficient pigs provide new insights into the hierarchy of physiological derangements of dystrophic muscle.* Hum Mol Genet; 22: 4368-4382.
148. Aigner B, Renner S, Kessler B, Klymiuk N, Kurome M, Wunsch A *et al.* (2010), *Transgenic pigs as models for translational biomedical research.* J Mol Med (Berl); 88: 653-664.
149. Baker BF, Monia BP (1999), *Novel mechanisms for antisense-mediated regulation of gene expression.* Biochim Biophys Acta; 1489: 3-18.
150. Chan JH, Lim S, Wong WS (2006), *Antisense oligonucleotides: from design to therapeutic application.* Clin Exp Pharmacol Physiol; 33: 533-540.
151. Sproat BS, Lamond AI, Beijer B, Neuner P, Ryder U (1989), *Highly efficient chemical synthesis of 2'-O-methyloligoribonucleotides and tetrabiotinylated derivatives; novel probes that are resistant to deg-*

*radation by RNA or DNA specific nucleases.* Nucleic Acids Res; 17: 3373-3386.
152. van Ommen GJ, van DJ, Aartsma-Rus A (2008), *The therapeutic potential of antisense-mediated exon skipping.* Curr Opin Mol Ther; 10: 140-149.
153. Watanabe TA, Geary RS, Levin AA (2006), *Plasma protein binding of an antisense oligonucleotide targeting human ICAM-1 (ISIS 2302).* Oligonucleotides; 16: 169-180.
154. Larsen HJ, Bentin T, Nielsen PE (1999), *Antisense properties of peptide nucleic acid.* Biochim Biophys Acta; 1489: 159-166.
155. Aartsma-Rus A, Kaman WE, Bremmer-Bout M, Janson AA, den Dunnen JT, van Ommen GJ *et al.* (2004), *Comparative analysis of antisense oligonucleotide analogs for targeted DMD exon 46 skipping in muscle cells.* Gene Ther; 11: 1391-1398.
156. Fabani MM, Gait MJ (2008), *miR-122 targeting with LNA/2'-O-methyl oligonucleotide mixmers, peptide nucleic acids (PNA), and PNA-peptide conjugates.* RNA; 14: 336-346.
157. Morita K, Hasegawa C, Kaneko M, Tsutsumi S, Sone J, Ishikawa T *et al.* (2002), *2'-O,4'-C-ethylene-bridged nucleic acids (ENA): highly nuclease-resistant and thermodynamically stable oligonucleotides for antisense drug.* Bioorg Med Chem Lett; 12: 73-76.
158. Yagi M, Takeshima Y, Surono A, Takagi M, Koizumi M, Matsuo M (2004), *Chimeric RNA and 2'-O, 4'-C-ethylene-bridged nucleic acids have stronger activity than phosphorothioate oligodeoxynucleotides in induction of exon 19 skipping in dystrophin mRNA.* Oligonucleotides; 14: 33-40.
159. Geary RS, Watanabe TA, Truong L, Freier S, Lesnik EA, Sioufi NB *et al.* (2001), *Pharmacokinetic properties of 2'-O-(2-methoxyethyl)-modified oligonucleotide analogs in rats.* J Pharmacol Exp Ther; 296: 890-897.
160. Oberbauer R, Schreiner GF, Meyer TW (1995), *Renal uptake of an 18-mer phosphorothioate oligonucleotide.* Kidney Int; 48: 1226-1232.
161. Heemskerk HA, de Winter CL, de Kimpe SJ, van Kuik-Romeijn P, Heuvelmans N, Platenburg GJ *et al.* (2009), *In vivo comparison of 2'-O-methyl phosphorothioate and morpholino antisense oligonucleotides for Duchenne muscular dystrophy exon skipping.* J Gene Med; 11: 257-266.
162. Moulton HM, Moulton JD (2010), *Morpholinos and their peptide conjugates: therapeutic promise and challenge for Duchenne muscular dystrophy.* Biochim Biophys Acta; 1798: 2296-2303.
163. Wu B, Li Y, Morcos PA, Doran TJ, Lu P, Lu QL (2009), *Octa-guanidine morpholino restores dystrophin expression in cardiac and skeletal muscles and ameliorates pathology in dystrophic mdx mice.* Mol Ther; 17: 864-871.
164. Aartsma-Rus A, van Vliet L, Hirschi M, Janson AA, Heemskerk H, de Winter CL *et al.* (2009), *Guidelines for antisense oligonucleotide design and insight into splice-modulating mechanisms.* Mol Ther; 17: 548-553.
165. Kole R, Williams T, Cohen L (2004), *RNA modulation, repair and remodeling by splice switching oligonucleotides.* Acta Biochim Pol; 51: 373-378.
166. Tanaka K, Watakabe A, Shimura Y (1994), *Polypurine sequences within a downstream exon function as a splicing enhancer.* Mol Cell Biol; 14: 1347-1354.
167. Aartsma-Rus A, de Winter CL, Janson AA, Kaman WE, van Ommen GJ, den Dunnen JT *et al.* (2005), *Functional analysis of 114 exon-internal AONs for targeted DMD exon skipping: indication for steric hindrance of SR protein binding sites.* Oligonucleotides; 15: 284-297.
168. Matsuo M, Masumura T, Nakajima T, Kitoh Y, Takumi T, Nishio H *et al.* (1990), *A very small frame-shifting deletion within exon 19 of the Duchenne muscular dystrophy gene.* Biochem Biophys Res Commun; 170: 963-967.
169. Matsuo M, Masumura T, Nishio H, Nakajima T, Kitoh Y, Takumi T *et al.* (1991), *Exon skipping during splicing of dystrophin mRNA precursor due to an intraexon deletion in the dystrophin gene of Duchenne muscular dystrophy Kobe.* J Clin Invest; 87: 2127-2131.
170. Pramono ZA, Takeshima Y, Alimsardjono H, Ishii A, Takeda S, Matsuo M (1996), *Induction of exon skipping of the dystrophin transcript in lymphoblastoid cells by transfecting an antisense oligodeoxynucleotide complementary to an exon recognition sequence.* Biochem Biophys Res Commun; 226: 445-449.
171. Takeshima Y, Nishio H, Sakamoto H, Nakamura H, Matsuo M (1995), *Modulation of in vitro splicing of the upstream intron by modifying an intra-exon sequence which is deleted from the dystrophin gene in dystrophin Kobe.* J Clin Invest; 95: 515-520.
172. Takeshima Y, Wada H, Yagi M, Ishikawa Y, Ishikawa Y, Minami R *et al.* (2001), *Oligonucleotides against a splicing enhancer sequence led to dystrophin production in muscle cells from a Duchenne muscular dystrophy patient.* Brain Dev; 23: 788-790.
173. van Deutekom JC, Bremmer-Bout M, Janson AA, Ginjaar IB, Baas F, den Dunnen JT *et al.* (2001), *Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells.* Hum Mol Genet; 10: 1547-1554.

174. Dunckley MG, Manoharan M, Villiet P, Eperon IC, Dickson G (1998), *Modification of splicing in the dystrophin gene in cultured mdx muscle cells by antisense oligoribonucleotides.* Hum Mol Genet; 7: 1083-1090.
175. Wilton SD, Lloyd F, Carville K, Fletcher S, Honeyman K, Agrawal S *et al.* (1999), *Specific removal of the nonsense mutation from the mdx dystrophin mRNA using antisense oligonucleotides.* Neuromuscul Disord; 9: 330-338.
176. Aartsma-Rus A, Bremmer-Bout M, Janson AA, den Dunnen JT, van Ommen GJ, van Deutekom JC (2002), *Targeted exon skipping as a potential gene correction therapy for Duchenne muscular dystrophy.* Neuromuscul Disord; 12 Suppl 1:S71-7.: S71-S77.
177. Aartsma-Rus A, Janson AA, Kaman WE, Bremmer-Bout M, den Dunnen JT, Baas F *et al.* (2003), *Therapeutic antisense-induced exon skipping in cultured muscle cells from six different DMD patients.* Hum Mol Genet; 12: 907-914.
178. Aartsma-Rus A, Janson AA, van Ommen GJ, van Deutekom JC (2007), *Antisense-induced exon skipping for duplications in Duchenne muscular dystrophy.* BMC Med Genet; 8: 43.
179. Gurvich OL, Tuohy TM, Howard MT, Finkel RS, Medne L, Anderson CB *et al.* (2008), *DMD pseudoexon mutations: splicing efficiency, phenotype, and potential therapy.* Ann Neurol; 63: 81-89.
180. Spitali P, Rimessi P, Fabris M, Perrone D, Falzarano S, Bovolenta M *et al.* (2009), *Exon skipping-mediated dystrophin reading frame restoration for small mutations.* Hum Mutat; 30: 1527-1534.
181. Yang L, Niu H, Gao X, Wang Q, Han G, Cao L *et al.* (2013), *Effective exon skipping and dystrophin restoration by 2'-o-methoxyethyl antisense oligonucleotide in dystrophin-deficient mice.* PLoS One; 8: e61584.
182. Gebski BL, Mann CJ, Fletcher S, Wilton SD (2003), *Morpholino antisense oligonucleotide induced dystrophin exon 23 skipping in mdx mouse muscle.* Hum Mol Genet; 12: 1801-1811.
183. Arechavala-Gomeza V, Graham IR, Popplewell LJ, Adams AM, Aartsma-Rus A, Kinali M *et al.* (2007), *Comparative analysis of antisense oligonucleotide sequences for targeted skipping of exon 51 during dystrophin pre-mRNA splicing in human muscle.* Hum Gene Ther; 18: 798-810.
184. Popplewell LJ, Adkin C, Arechavala-Gomeza V, Aartsma-Rus A, de Winter CL, Wilton SD *et al.* (2010), *Comparative analysis of antisense oligonucleotide sequences targeting exon 53 of the human DMD gene: Implications for future clinical trials.* Neuromuscul Disord; 20: 102-110.
185. Takagi M, Yagi M, Ishibashi K, Takeshima Y, Surono A, Matsuo M *et al.* (2004), *Design of 2'-O-Me RNA/ENA chimera oligonucleotides to induce exon skipping in dystrophin pre-mRNA.* Nucleic Acids Symp Ser (Oxf); 297-298.
186. Yin H, Lu Q, Wood M (2008), *Effective exon skipping and restoration of dystrophin expression by peptide nucleic acid antisense oligonucleotides in mdx mice.* Mol Ther; 16: 38-45.
187. Aartsma-Rus A, Fokkema I, Verschuuren J, Ginjaar I, van Deutekom J, van Ommen GJ *et al.* (2009), *Theoretic applicability of antisense-mediated exon skipping for Duchenne muscular dystrophy mutations.* Hum Mutat; 30: 293-299.
188. Aartsma-Rus A, Janson AA, Kaman WE, Bremmer-Bout M, van Ommen GJ, den Dunnen JT *et al.* (2004), *Antisense-induced multiexon skipping for Duchenne muscular dystrophy makes more sense.* Am J Hum Genet; 74: 83-92.
189. McClorey G, Moulton HM, Iversen PL, Fletcher S, Wilton SD (2006), *Antisense oligonucleotide-induced exon skipping restores dystrophin expression in vitro in a canine model of DMD.* Gene Ther; 13: 1373-1381.
190. Yokota T, Lu QL, Partridge T, Kobayashi M, Nakamura A, Takeda S *et al.* (2009), *Efficacy of systemic morpholino exon-skipping in Duchenne dystrophy dogs.* Ann Neurol; 65: 667-676.
191. Fall AM, Johnsen R, Honeyman K, Iversen P, Fletcher S, Wilton SD (2006), *Induction of revertant fibres in the mdx mouse using antisense oligonucleotides.* Genet Vaccines Ther; 4: 3.
192. Aartsma-Rus A, Kaman WE, Weij R, den Dunnen JT, van Ommen GJ, van Deutekom JC (2006), *Exploring the frontiers of therapeutic exon skipping for Duchenne muscular dystrophy by double targeting within one or multiple exons.* Mol Ther; 14: 401-407.
193. van Vliet L, de Winter CL, van Deutekom JC, van Ommen GJ, Aartsma-Rus A (2008), *Assessment of the feasibility of exon 45-55 multiexon skipping for Duchenne muscular dystrophy.* BMC Med Genet; 9: 105.
194. Aoki Y, Yokota T, Nagata T, Nakamura A, Tanihata J, Saito T *et al.* (2012), *Bodywide skipping of exons 45-55 in dystrophic mdx52 mice by systemic antisense delivery.* Proc Natl Acad Sci USA; 109: 13763-13768.
195. Forrest S, Meloni PL, Muntoni F, Kim J, Fletcher S, Wilton SD (2010), *Personalized exon skipping strategies to address clustered non-deletion dystrophin mutations.* Neuromuscul Disord; 20: 810-816.
196. Mann CJ, Honeyman K, Cheng AJ, Ly T, Lloyd F, Fletcher S *et al.* (2001), *Antisense-induced exon skipping and synthesis of dystrophin in the mdx mouse.* Proc Natl Acad Sci USA; 98: 42-47.
197. Mann CJ, Honeyman K, McClorey G, Fletcher S, Wilton SD (2002), *Improved antisense oligonucleotide induced exon skipping in the mdx mouse model of muscular dystrophy.* J Gene Med; 4: 644-654.

198. Lu QL, Mann CJ, Lou F, Bou-Gharios G, Morris GE, Xue SA *et al.* (2003), *Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse.* Nat Med; 9: 1009-1014.
199. Lu QL, Rabinowitz A, Chen YC, Yokota T, Yin H, Alter J *et al.* (2005), *Systemic delivery of antisense oligoribonucleotide restores dystrophin expression in body-wide skeletal muscles.* Proc Natl Acad Sci USA; 102: 198-203.
200. Heemskerk H, de Winter C, van Kuik P, Heuvelmans N, Sabatelli P, Rimessi P *et al.* (2010), *Preclinical PK and PD studies on 2'-O-methyl-phosphorothioate RNA antisense oligonucleotides in the mdx mouse model.* Mol Ther; 18: 1210-1217.
201. Tanganyika-de Winter CL, Heemskerk H, Karnaoukh TG, van Putten M, de Kimpe SJ, van Deutekom J *et al.* (2012), *Long-term exon skipping studies with 2'-O-methyl phosphorothioate antisense oligonucleotides in dystrophic mouse models.* Mol Ther Nucleic Acids; 1: e43.
202. Alter J, Lou F, Rabinowitz A, Yin H, Rosenfeld J, Wilton SD *et al.* (2006), *Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology.* Nat Med; 12: 175-177.
203. Wu B, Lu P, Benrashid E, Malik S, Ashar J, Doran TJ *et al.* (2010), *Dose-dependent restoration of dystrophin expression in cardiac muscle of dystrophic mice by systemically delivered morpholino.* Gene Ther; 17: 132-140.
204. Jearawiriyapaisarn N, Moulton HM, Buckley B, Roberts J, Sazani P, Fucharoen S *et al.* (2008), *Sustained dystrophin expression induced by peptide-conjugated morpholino oligomers in the muscles of mdx mice.* Mol Ther; 16: 1624-1629.
205. Wu B, Moulton HM, Iversen PL, Jiang J, Li J, Li J *et al.* (2008), *Effective rescue of dystrophin improves cardiac function in dystrophin-deficient mice by a modified morpholino oligomer.* Proc Natl Acad Sci USA; 105: 14814-14819.
206. Wu B, Lu P, Cloer C, Shaban M, Grewal S, Milazi S *et al.* (2012), *Long-term rescue of dystrophin expression and improvement in muscle pathology and function in dystrophic mdx mice by peptide-conjugated morpholino.* Am J Pathol; 181: 392-400.
207. Goyenvalle A, Babbs A, Powell D, Kole R, Fletcher S, Wilton SD *et al.* (2010), *Prevention of dystrophic pathology in severely affected dystrophin/utrophin-deficient mice by morpholino-oligomer-mediated exon-skipping.* Mol Ther; 18: 198-205.
208. Sazani P, Ness KP, Weller DL, Poage DW, Palyada K, Shrewsbury SB (2011), *Repeat-dose toxicology evaluation in cynomolgus monkeys of AVI-4658, a phosphorodiamidate morpholino oligomer (PMO) drug for the treatment of duchenne muscular dystrophy.* Int J Toxicol; 30: 313-321.
209. Yin H, Moulton HM, Betts C, Seow Y, Boutilier J, Iverson PL *et al.* (2009), *A fusion peptide directs enhanced systemic dystrophin exon skipping and functional restoration in dystrophin-deficient mdx mice.* Hum Mol Genet; 18: 4405-4414.
210. Ivanova GD, Arzumanov A, Abes R, Yin H, Wood MJ, Lebleu B *et al.* (2008), *Improved cell-penetrating peptide-PNA conjugates for splicing redirection in HeLa cells and exon skipping in mdx mouse muscle.* Nucleic Acids Res; 36: 6418-6428.
211. Yin H, Saleh AF, Betts C, Camelliti P, Seow Y, Ashraf S *et al.* (2011), *Pip5 transduction peptides direct high efficiency oligonucleotide-mediated dystrophin exon skipping in heart and phenotypic correction in mdx mice.* Mol Ther; 19: 1295-1303.
212. Betts C, Saleh AF, Arzumanov AA, Hammond SM, Godfrey C, Coursindel T *et al.* (2012), *Pip6-PMO, a new generation of peptide-oligonucleotide conjugates with improved cardiac exon skipping activity for DMD treatment.* Mol Ther Nucleic Acids; 1: e38.
213. Rimessi P, Sabatelli P, Fabris M, Braghetta P, Bassi E, Spitali P *et al.* (2009), *Cationic PMMA nanoparticles bind and deliver antisense oligoribonucleotides allowing restoration of dystrophin expression in the mdx mouse.* Mol Ther; 17: 820-827.
214. Ferlini A, Sabatelli P, Fabris M, Bassi E, Falzarano S, Vattemi G *et al.* (2010), *Dystrophin restoration in skeletal, heart and skin arrector pili smooth muscle of mdx mice by ZM2 NP-AON complexes.* Gene Ther; 17: 432-438.
215. Bassi E, Falzarano S, Fabris M, Gualandi F, Merlini L, Vattemi G *et al.* (2012), *Persistent dystrophin protein restoration 90 days after a course of intraperitoneally administered naked 2'OMePS AON and ZM2 NP-AON complexes in mdx mice.* J Biomed Biotechnol; 2012: 897076.
216. Mitrpant C, Fletcher S, Iversen PL, Wilton SD (2009), *By-passing the nonsense mutation in the 4 CV mouse model of muscular dystrophy by induced exon skipping.* J Gene Med; 11: 46-56.
217. Bremmer-Bout M, Aartsma-Rus A, de Meijer EJ, Kaman WE, Janson AA, Vossen RH *et al.* (2004), *Targeted exon skipping in transgenic hDMD mice: A model for direct preclinical screening of human-specific antisense oligonucleotides.* Mol Ther; 10: 232-240.
218. Hoffman EP (2007), *Skipping toward personalized molecular medicine.* N Engl J Med; 357: 2719-2722.

219. Heemskerk H, de Winter CL, van Ommen GJ, van Deutekom JC, Aartsma-Rus A (2009), *Development of antisense-mediated exon skipping as a treatment for duchenne muscular dystrophy.* Ann N Y Acad Sci; 1175: 71-79.
220. Aoki Y, Nagata T, Yokota T, Nakamura A, Wood MJ, Partridge T *et al.* (2013), *Highly efficient in vivo delivery of PMO into regenerating myotubes and rescue in laminin-alpha2 chain-null congenital muscular dystrophy mice.* Hum Mol Genet; 22: 4914-4928.
221. Wu B, Benrashid E, Lu P, Cloer C, Zillmer A, Shaban M *et al.* (2011), *Targeted skipping of human dystrophin exons in transgenic mouse model systemically for antisense drug development.* PLoS One; 6: e19906.
222. Partridge T (2010), *The potential of exon skipping for treatment for Duchenne muscular dystrophy.* J Child Neurol; 25: 1165-1170.
223. Scheuerbrandt G (2009 Oct 3), *Research approaches for a therapy of Duchenne Muscular Dystrophy. Part 1: Exon Skipping.* First update.
224. Yokota T, Nakamura A, Nagata T, Saito T, Kobayashi M, Aoki Y *et al.* (2012), *Extensive and prolonged restoration of dystrophin expression with vivo-morpholino-mediated multiple exon skipping in dystrophic dogs.* Nucleic Acid Ther; 22: 306-315.
225. van Deutekom JC, Janson AA, Ginjaar IB, Frankhuizen WS, Aartsma-Rus A, Bremmer-Bout M *et al.* (2007), *Local dystrophin restoration with antisense oligonucleotide PRO051.* N Engl J Med; 357: 2677-2686.
226. Kinali M, Arechavala-Gomeza V, Feng L, Cirak S, Hunt D, Adkin C *et al.* (2009), *Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study.* Lancet Neurol; 8: 918-928.
227. Aartsma-Rus A, van Ommen GJ (2009), *Less is more: therapeutic exon skipping for Duchenne muscular dystrophy.* Lancet Neurol; 8: 873-875.
228. Takeshima Y, Yagi M, Wada H, Ishibashi K, Nishiyama A, Kakumoto M *et al.* (2006), *Intravenous infusion of an antisense oligonucleotide results in exon skipping in muscle dystrophin mRNA of Duchenne muscular dystrophy.* Pediatr Res; 59: 690-694.
229. Goemans NM, Tulinius M, van den Akker JT, Burm BE, Ekhart PF, Heuvelmans N *et al.* (2011), *Systemic administration of PRO051 in Duchenne's muscular dystrophy.* N Engl J Med; 364: 1513-1522.
230. Flanigan KM, Voit T, Rosales XQ, Servais L, Kraus JE, Wardell C *et al.* (2013), *Pharmacokinetics and safety of single doses of drisapersen in non-ambulant subjects with Duchenne muscular dystrophy: Results of a double-blind randomized clinical trial.* Neuromuscul Disord.
231. Cirak S, Arechavala-Gomeza V, Guglieri M, Feng L, Torelli S, Anthony K *et al.* (2011), *Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study.* Lancet; 378: 595-605.
232. http://www.gsk-clinicalstudyregister.com/result_compounds.jsp
233. http://ir.prosensa.eu/releasedetail.cfm?ReleaseID=791929
234. Mendell JR, Rodino-Klapac LR, Sahenk Z, Roush K, Bird L, Lowes LP *et al.* (2013), *Eteplirsen for the treatment of Duchenne muscular dystrophy.* Ann Neurol; 74: 637-647.
235. http://investorrelations.sareptatherapeutics.com/phoenix.zhtml?c=64231&p=irol-newsArticle_print&ID=1831214&highlight=
236. van den Bergen JC, Schade van Westrum SM, Dekker L, van der Kooi AJ, de VM, Wokke BH *et al.* (2014), *Clinical characterisation of Becker muscular dystrophy patients predicts favourable outcome in exon-skipping therapy.* J Neurol Neurosurg Psychiatry; 85: 92-98.
237. Henricson EK, Abresch RT, Cnaan A, Hu F, Duong T, Arrieta A *et al.* (2013), *The cooperative international neuromuscular research group Duchenne natural history study: glucocorticoid treatment preserves clinically meaningful functional milestones and reduces rate of disease progression as measured by manual muscle testing and other commonly used clinical trial outcome measures.* Muscle Nerve; 48: 55-67.
238. McDonald CM, Henricson EK, Abresch RT, Han JJ, Escolar DM, Florence JM *et al.* (2013), *The cooperative international neuromuscular research group Duchenne natural history study: a longitudinal investigation in the era of glucocorticoid therapy: design of protocol and the methods used.* Muscle Nerve; 48: 32-54.
239. Malerba A, Thorogood FC, Dickson G, Graham IR (2009), *Dosing regimen has a significant impact on the efficiency of morpholino oligomer-induced exon skipping in mdx mice.* Hum Gene Ther; 20: 955-965.
240. Verhaart IE, Tanganyika-de Winter CL, Karnaoukh TG, Kolfschoten IG, de Kimpe SJ, van Deutekom JC *et al.* (2013), *Dose-dependent pharmacokinetic profiles of 2'-O-methyl phosphorothioate antisense oligonucleotidesin mdx mice.* Nucleic Acid Ther; 23: 228-237.
241. Moreno PM, Wenska M, Lundin KE, Wrange O, Stromberg R, Smith CI (2009), *A synthetic snRNA m3G-CAP enhances nuclear delivery of exogenous proteins and nucleic acids.* Nucleic Acids Res; 37: 1925-1935.
242. De Angelis FG, Sthandier O, Berarducci B, Toso S, Galluzzi G, Ricci E *et al.* (2002), *Chimeric snRNA*

*molecules carrying antisense sequences against the splice junctions of exon 51 of the dystrophin pre-mRNA induce exon skipping and restoration of a dystrophin synthesis in Delta 48-50 DMD cells.* Proc Natl Acad Sci USA; 99: 9456-9461.

243. Denti MA, Rosa A, D'Antona G, Sthandier O, De Angelis FG, Nicoletti C *et al.* (2006), *Body-wide gene therapy of Duchenne muscular dystrophy in the mdx mouse model.* Proc Natl Acad Sci USA; 103: 3758-3763.
244. Denti MA, Incitti T, Sthandier O, Nicoletti C, De Angelis FG, Rizzuto E *et al.* (2008), *Long-term benefit of adeno-associated virus/antisense-mediated exon skipping in dystrophic mice.* Hum Gene Ther; 19: 601-608.
245. Goyenvalle A, Vulin A, Fougerousse F, Leturcq F, Kaplan JC, Garcia L *et al.* (2004), *Rescue of dystrophic muscle through U7 snRNA-mediated exon skipping.* Science; 306: 1796-1799.
246. Brun C, Suter D, Pauli C, Dunant P, Lochmuller H, Burgunder JM *et al.* (2003), *U7 snRNAs induce correction of mutated dystrophin pre-mRNA by exon skipping.* Cell Mol Life Sci; 60: 557-566.
247. Incitti T, De Angelis FG, Cazzella V, Sthandier O, Pinnaro C, Legnini I *et al.* (2010), *Exon skipping and duchenne muscular dystrophy therapy: selection of the most active U1 snRNA antisense able to induce dystrophin exon 51 skipping.* Mol Ther; 18: 1675-1682.
248. Goyenvalle A, Babbs A, Wright J, Wilkins V, Powell D, Garcia L *et al.* (2012), *Rescue of severely affected dystrophin/utrophin-deficient mice through scAAV-U7snRNA-mediated exon skipping.* Hum Mol Genet; 21: 2559-2571.
249. Eckenfelder A, Tordo J, Babbs A, Davies KE, Goyenvalle A, Danos O (2012), *The cellular processing capacity limits the amounts of chimeric U7 snRNA available for antisense delivery.* Mol Ther Nucleic Acids; 1: e31.
250. Goyenvalle A, Wright J, Babbs A, Wilkins V, Garcia L, Davies KE (2012), *Engineering multiple U7snRNA constructs to induce single and multiexon-skipping for Duchenne muscular dystrophy.* Mol Ther; 20: 1212-1221.
251. Vulin A, Barthelemy I, Goyenvalle A, Thibaud JL, Beley C, Griffith G *et al.* (2012), *Muscle function recovery in golden retriever muscular dystrophy after AAV1-U7 exon skipping.* Mol Ther; 2120-2133.
252. le Hir M, Goyenvalle A, Peccate C, Precigout G, Davies KE, Voit T *et al.* (2013), *AAV genome loss from dystrophic mouse muscles during AAV-U7 snRNA-mediated exon-skipping therapy.* Mol Ther; 21: 1551-1558.
253. Shetty RS, Gallagher CS, Chen YT, Hims MM, Mull J, Leyne M *et al.* (2011), *Specific correction of a splice defect in brain by nutritional supplementation.* Hum Mol Genet; 20: 4093-4101.
254. Zhou J, Zheng X, Shen H (2012), *Targeting RNA-splicing for SMA treatment.* Mol Cells; 33: 223-228.
255. Nishida A, Kataoka N, Takeshima Y, Yagi M, Awano H, Ota M *et al.* (2011), *Chemical treatment enhances skipping of a mutated exon in the dystrophin gene.* Nat Commun; 2: 308.
256. Hu Y, Wu B, Zillmer A, Lu P, Benrashid E, Wang M *et al.* (2010), *Guanine analogues enhance antisense oligonucleotide-induced exon skipping in dystrophin gene in vitro and in vivo.* Mol Ther; 18: 812-818.
257. Verhaart IE, Aartsma-Rus A (2012), *The effect of 6-thioguanine on alternative splicing and antisense-mediated exon skipping treatment for duchenne muscular dystrophy.* PLoS Curr MD; 4.
258. Kendall GC, Mokhonova EI, Moran M, Sejbuk NE, Wang DW, Silva O *et al.* (2012), *Dantrolene enhances antisense-mediated exon skipping in human and mouse models of duchenne muscular dystrophy.* Sci Transl Med; 4: 164ra160.
259. Verhaart IE, Heemskerk H, Karnaoukh TG, Kolfschoten IG, Vroon A, van Ommen GJ *et al.* (2012), *Prednisolone treatment does not interfere with 2'-O-methyl phosphorothioate antisense-mediated exon skipping in Duchenne muscular dystrophy.* Hum Gene Ther; 23: 262-273.
260. Cohn RD, van Erp C, Habashi JP, Soleimani AA, Klein EC, Lisi MT *et al.* (2007), *Angiotensin II type 1 receptor blockade attenuates TGF-beta-induced failure of muscle regeneration in multiple myopathic states.* Nat Med; 13: 204-210.
261. Bish LT, Yarchoan M, Sleeper MM, Gazzara JA, Morine KJ, Acosta P *et al.* (2011), *Chronic losartan administration reduces mortality and preserves cardiac but not skeletal muscle function in dystrophic mice.* PLoS One; 6: e20856.
262. Habashi JP, Doyle JJ, Holm TM, Aziz H, Schoenhoff F, Bedja D *et al.* (2011), *Angiotensin II type 2 receptor signaling attenuates aortic aneurysm in mice through ERK antagonism.* Science; 332: 361-365.
263. Nelson CA, Hunter RB, Quigley LA, Girgenrath S, Weber WD, McCullough JA *et al.* (2011), *Inhibiting TGF-beta activity improves respiratory function in mdx mice.* Am J Pathol; 178: 2611-2621.
264. Spurney CF, Sali A, Guerron AD, Iantorno M, Yu Q, Gordish-Dressman H *et al.* (2011), *Losartan decreases cardiac muscle fibrosis and improves cardiac function in dystrophin-deficient mdx mice.* J Cardiovasc Pharmacol Ther; 16: 87-95.
265. Howard MT, Anderson CB, Fass U, Khatri S, Gesteland RF, Atkins JF *et al.* (2004), *Readthrough of dystrophin stop codon mutations induced by aminoglycosides.* Ann Neurol; 55: 422-426.

266. Barton-Davis ER, Cordier L, Shoturma DI, Leland SE, Sweeney HL (1999), *Aminoglycoside antibiotics restore dystrophin function to skeletal muscles of mdx mice.* J Clin Invest; 104: 375-381.
267. Dunant P, Walter MC, Karpati G, Lochmuller H (2003), *Gentamicin fails to increase dystrophin expression in dystrophin-deficient muscle.* Muscle Nerve; 27: 624-627.
268. Politano L, Nigro G, Nigro V, Piluso G, Papparella S, Paciello O *et al.* (2003), *Gentamicin administration in Duchenne patients with premature stop codon. Preliminary results.* Acta Myol; 22: 15-21.
269. Aartsma-Rus A, den Dunnen JT, van Ommen GJ (2010), *New insights in gene-derived therapy: the example of Duchenne muscular dystrophy.* Ann N Y Acad Sci; 1214: 199-212.
270. Malik V, Rodino-Klapac LR, Viollet L, Mendell JR (2010), *Aminoglycoside-induced mutation suppression (stop codon readthrough) as a therapeutic strategy for Duchenne muscular dystrophy.* Ther Adv Neurol Disord; 3: 379-389.
271. Bönnemann C, Finkel R, Wong B, Flanigan K, Sampson J, Sweeney L *et al.* (2007) *G.P.3.05 Phase 2 study of PTC124 for nonsense mutation suppression therapy of Duchenne muscular dystrophy (DMD).* Neuromuscul Disord 17[9], 783.
272. Hirawat S, Welch EM, Elfring GL, Northcutt VJ, Paushkin S, Hwang S *et al.* (2007), *Safety, tolerability, and pharmacokinetics of PTC124, a nonaminoglycoside nonsense mutation suppressor, following single- and multiple-dose administration to healthy male and female adult volunteers.* J Clin Pharmacol; 47: 430-444.
273. Welch EM, Barton ER, Zhuo J, Tomizawa Y, Friesen WJ, Trifillis P *et al.* (2007), *PTC124 targets genetic disorders caused by nonsense mutations.* Nature; 447: 87-91.
274. Finkel RS (2010), *Read-through strategies for suppression of nonsense mutations in Duchenne/ Becker muscular dystrophy: aminoglycosides and ataluren (PTC124).* J Child Neurol; 25: 1158-1164.
275. Hoffman EP, Bronson A, Levin AA, Takeda S, Yokota T, Baudy AR *et al.* (2011), *Restoring dystrophin expression in duchenne muscular dystrophy muscle progress in exon skipping and stop codon read through.* Am J Pathol; 179: 12-22.
276. Auld DS, Thorne N, Maguire WF, Inglese J (2009), *Mechanism of PTC124 activity in cell-based luciferase assays of nonsense codon suppression.* Proc Natl Acad Sci USA; 106: 3585-3590.
277. Auld DS, Lovell S, Thorne N, Lea WA, Maloney DJ, Shen M *et al.* (2010), *Molecular basis for the high-affinity binding and stabilization of firefly luciferase by PTC124.* Proc Natl Acad Sci USA; 107: 4878-4883.
278. Du M, Liu X, Welch EM, Hirawat S, Peltz SW, Bedwell DM (2008), *PTC124 is an orally bioavailable compound that promotes suppression of the human CFTR-G542X nonsense allele in a CF mouse model.* Proc Natl Acad Sci USA; 105: 2064-2069.
279. Kayali R, Ku JM, Khitrov G, Jung ME, Prikhodko O, Bertoni C (2012), *Read-through compound 13 restores dystrophin expression and improves muscle function in the mdx mouse model for Duchenne muscular dystrophy.* Hum Mol Genet; 21: 4007-4020.
280. Linde L, Boelz S, Nissim-Rafinia M, Oren YS, Wilschanski M, Yaacov Y *et al.* (2007), *Nonsense-mediated mRNA decay affects nonsense transcript levels and governs response of cystic fibrosis patients to gentamicin.* J Clin Invest; 117: 683-692.
281. Puttaraju M, Jamison SF, Mansfield SG, Garcia-Blanco MA, Mitchell LG (1999), *Spliceosome-mediated RNA trans-splicing as a tool for gene therapy.* Nat Biotechnol; 17: 246-252.
282. Chao H, Mansfield SG, Bartel RC, Hiriyanna S, Mitchell LG, Garcia-Blanco MA *et al.* (2003), *Phenotype correction of hemophilia A mice by spliceosome-mediated RNA trans-splicing.* Nat Med; 9: 1015-1019.
283. Mansfield SG, Clark RH, Puttaraju M, Kole J, Cohn JA, Mitchell LG *et al.* (2003), *5'exon replacement and repair by spliceosome-mediated RNA trans-splicing.* RNA; 9: 1290-1297.
284. Lorain S, Peccate C, le HM, Garcia L (2010), *Exon exchange approach to repair Duchenne dystrophin transcripts.* PLoS One; 5: e10894.
285. Lorain S, Peccate C, le HM, Griffith G, Philippi S, Precigout G *et al.* (2013), *Dystrophin rescue by trans-splicing: a strategy for DMD genotypes not eligible for exon skipping approaches.* Nucleic Acids Res; 41: 8391-8402.
286. Romero NB, Braun S, Benveniste O, Leturcq F, Hogrel JY, Morris GE *et al.* (2004), *Phase I study of dystrophin plasmid-based gene therapy in Duchenne/Becker muscular dystrophy.* Hum Gene Ther; 15: 1065-1076.
287. van Deutekom JC, van Ommen GJ (2003), *Advances in Duchenne muscular dystrophy gene therapy.* Nat Rev Genet; 4: 774-783.
288. Harper SQ, Hauser MA, DelloRusso C, Duan D, Crawford RW, Phelps SF *et al.* (2002), *Modular flexibility of dystrophin: implications for gene therapy of Duchenne muscular dystrophy.* Nat Med; 8: 253-261.
289. Blankinship MJ, Gregorevic P, Chamberlain JS (2006), *Gene therapy strategies for Duchenne muscular dystrophy utilizing recombinant adeno-associated virus vectors.* Mol Ther; 13: 241-249.
290. England SB, Nicholson LV, Johnson MA, Forrest SM, Love DR, Zubrzycka-Gaarn EE *et al.* (1990), *Very*

*mild muscular dystrophy associated with the deletion of 46% of dystrophin.* Nature; 343: 180-182.
291. Wang B, Li J, Xiao X (2000), *Adeno-associated virus vector carrying human minidystrophin genes effectively ameliorates muscular dystrophy in mdx mouse model.* Proc Natl Acad Sci USA; 97: 13714-13719.
292. Wang B, Li J, Qiao C, Chen C, Hu P, Zhu X *et al.* (2008), *A canine minidystrophin is functional and therapeutic in mdx mice.* Gene Ther; 15: 1099-1106.
293. Fabb SA, Wells DJ, Serpente P, Dickson G (2002), *Adeno-associated virus vector gene transfer and sarcolemmal expression of a 144 kDa micro-dystrophin effectively restores the dystrophin-associated protein complex and inhibits myofibre degeneration in nude/mdx mice.* Hum Mol Genet; 11: 733-741.
294. Liu M, Yue Y, Harper SQ, Grange RW, Chamberlain JS, Duan D (2005), *Adeno-associated virus-mediated microdystrophin expression protects young mdx muscle from contraction-induced injury.* Mol Ther; 11: 245-256.
295. Townsend D, Yasuda S, Li S, Chamberlain JS, Metzger JM (2008), *Emergent dilated cardiomyopathy caused by targeted repair of dystrophic skeletal muscle.* Mol Ther; 16: 832-835.
296. Crisp A, Yin H, Goyenvalle A, Betts C, Moulton HM, Seow Y *et al.* (2011), *Diaphragm rescue alone prevents heart dysfunction in dystrophic mice.* Hum Mol Genet; 20: 413-421.
297. Wasala NB, Bostick B, Yue Y, Duan D (2013), *Exclusive skeletal muscle correction does not modulate dystrophic heart disease in the aged mdx model of Duchenne cardiomyopathy.* Hum Mol Genet; 22: 2634-2641.
298. Gregorevic P, Blankinship MJ, Allen JM, Crawford RW, Meuse L, Miller DG *et al.* (2004), *Systemic delivery of genes to striated muscles using adeno-associated viral vectors.* Nat Med; 10: 828-834.
299. Gregorevic P, Blankinship MJ, Allen JM, Chamberlain JS (2008), *Systemic microdystrophin gene delivery improves skeletal muscle structure and function in old dystrophic mdx mice.* Mol Ther; 16: 657-664.
300. Wang Z, Tapscott SJ, Chamberlain JS, Storb R (2011), *Immunity and AAV-mediated gene therapy for muscular dystrophies in large animal models and human trials.* Front Microbiol; 2: 201.
301. Wang Z, Allen JM, Riddell SR, Gregorevic P, Storb R, Tapscott SJ *et al.* (2007), *Immunity to adeno-associated virus-mediated gene transfer in a random-bred canine model of Duchenne muscular dystrophy.* Hum Gene Ther; 18: 18-26.
302. Wang Z, Storb R, Lee D, Kushmerick MJ, Chu B, Berger C *et al.* (2010), *Immune responses to AAV in canine muscle monitored by cellular assays and noninvasive imaging.* Mol Ther; 18: 617-624.
303. Yue Y, Ghosh A, Long C, Bostick B, Smith BF, Kornegay JN *et al.* (2008), *A single intravenous injection of adeno-associated virus serotype-9 leads to whole body skeletal muscle transduction in dogs.* Mol Ther; 16: 1944-1952.
304. Ohshima S, Shin JH, Yuasa K, Nishiyama A, Kira J, Okada T *et al.* (2009), *Transduction efficiency and immune response associated with the administration of AAV8 vector into dog skeletal muscle.* Mol Ther; 17: 73-80.
305. Gregorevic P, Schultz BR, Allen JM, Halldorson JB, Blankinship MJ, Meznarich NA *et al.* (2009), *Evaluation of vascular delivery methodologies to enhance rAAV6-mediated gene transfer to canine striated musculature.* Mol Ther; 17: 1427-1433.
306. Kornegay JN, Li J, Bogan JR, Bogan DJ, Chen C, Zheng H *et al.* (2010), *Widespread muscle expression of an AAV9 human mini-dystrophin vector after intravenous injection in neonatal dystrophin-deficient dogs.* Mol Ther; 18: 1501-1508.
307. Toromanoff A, Adjali O, Larcher T, Hill M, Guigand L, Chenuaud P *et al.* (2010), *Lack of immunotoxicity after regional intravenous (RI) delivery of rAAV to nonhuman primate skeletal muscle.* Mol Ther; 18: 151-160.
308. Rodino-Klapac LR, Montgomery CL, Bremer WG, Shontz KM, Malik V, Davis N *et al.* (2010), *Persistent expression of FLAG-tagged micro dystrophin in nonhuman primates following intramuscular and vascular delivery.* Mol Ther; 18: 109-117.
309. Bowles DE, McPhee SW, Li C, Gray SJ, Samulski JJ, Camp AS *et al.* (2012), *Phase 1 gene therapy for Duchenne muscular dystrophy using a translational optimized AAV vector.* Mol Ther; 20: 443-455.
310. Mendell JR, Campbell K, Rodino-Klapac L, Sahenk Z, Shilling C, Lewis S *et al.* (2010), *Dystrophin immunity in Duchenne's muscular dystrophy.* N Engl J Med; 363: 1429-1437.
311. Ghosh A, Yue Y, Shin JH, Duan D (2009), *Systemic Trans-splicing adeno-associated viral delivery efficiently transduces the heart of adult mdx mouse, a model for duchenne muscular dystrophy.* Hum Gene Ther; 20: 1319-1328.
312. Odom GL, Gregorevic P, Allen JM, Chamberlain JS (2011), *Gene therapy of mdx mice with large truncated dystrophins generated by recombination using rAAV6.* Mol Ther; 19: 36-45.
313. Zhang Y, Duan D (2012), *Novel mini-dystrophin gene dual adeno-associated virus vectors restore neuronal nitric oxide synthase expression at the sarcolemma.* Hum Gene Ther; 23: 98-103.
314. Ishizaki M, Maeda Y, Kawano R, Suga T, Uchida Y, Uchino K *et al.* (2011), *Rescue from respiratory*

*dysfunction by transduction of full-length dystrophin to diaphragm via the peritoneal cavity in utrophin/dystrophin double knockout mice.* Mol Ther; 19: 1230-1235.

315. Koo T, Okada T, Athanasopoulos T, Foster H, Takeda S, Dickson G (2011), *Long-term functional adeno-associated virus-microdystrophin expression in the dystrophic CXMDj dog.* J Gene Med; 13: 497-506.
316. Shin JH, Yue Y, Srivastava A, Smith B, Lai Y, Duan D (2012), *A simplified immune suppression scheme leads to persistent micro-dystrophin expression in duchenne muscular dystrophy dogs.* Hum Gene Ther; 23: 202-209.
317. Shin JH, Pan X, Hakim CH, Yang HT, Yue Y, Zhang K *et al.* (2013), *Microdystrophin ameliorates muscular dystrophy in the canine model of duchenne muscular dystrophy.* Mol Ther; 21: 750-757.
318. Evans VC, Graham IR, Athanasopoulos T, Galley DJ, Jackson CL, Simons JP *et al.* (2011), *Adeno-associated virus serotypes 7 and 8 outperform serotype 9 in expressing atheroprotective human apoE3 from mouse skeletal muscle.* Metabolism; 60: 491-498.
319. Gao G, Bish LT, Sleeper MM, Mu X, Sun L, Lou Y *et al.* (2011), *Transendocardial delivery of AAV6 results in highly efficient and global cardiac gene transfer in rhesus macaques.* Hum Gene Ther; 22: 979-984.
320. Zincarelli C, Soltys S, Rengo G, Koch WJ, Rabinowitz JE (2010), *Comparative cardiac gene delivery of adeno-associated virus serotypes 1-9 reveals that AAV6 mediates the most efficient transduction in mouse heart.* Clin Transl Sci; 3: 81-89.
321. Moulay G, Boutin S, Masurier C, Scherman D, Kichler A (2010), *Polymers for improving the in vivo transduction efficiency of AAV2 vectors.* PLoS One; 5: e15576.
322. Hegge JO, Wooddell CI, Zhang G, Hagstrom JE, Braun S, Huss T *et al.* (2010), *Evaluation of hydrodynamic limb vein injections in nonhuman primates.* Hum Gene Ther; 21: 829-842.
323. Qiao C, Li J, Zheng H, Bogan J, Li J, Yuan Z *et al.* (2009), *Hydrodynamic limb vein injection of adeno-associated virus serotype 8 vector carrying canine myostatin propeptide gene into normal dogs enhances muscle growth.* Hum Gene Ther; 20: 1-10.
324. Wooddell CI, Hegge JO, Zhang G, Sebestyen MG, Noble M, Griffin JB *et al.* (2011), *Dose response in rodents and nonhuman primates after hydrodynamic limb vein delivery of naked plasmid DNA.* Hum Gene Ther; 22: 889-903.
325. Zhang G, Wooddell CI, Hegge JO, Griffin JB, Huss T, Braun S *et al.* (2010), *Functional efficacy of dystrophin expression from plasmids delivered to mdx mice by hydrodynamic limb vein injection.* Hum Gene Ther; 21: 221-237.
326. Fan Z, Kocis K, Valley R, Howard JF, Chopra M, An H *et al.* (2012), *Safety and feasibility of high-pressure transvenous limb perfusion with 0.9% saline in human muscular dystrophy.* Mol Ther; 20: 456-461.
327. Bish LT, Sleeper MM, Forbes SC, Wang B, Reynolds C, Singletary GE *et al.* (2012), *Long-term restoration of cardiac dystrophin expression in golden retriever muscular dystrophy following rAAV6-mediated exon skipping.* Mol Ther; 20: 580-589.
328. Bostick B, Shin JH, Yue Y, Duan D (2011), *AAV-microdystrophin therapy improves cardiac performance in aged female mdx mice.* Mol Ther; 19: 1826-1832.
329. Schinkel S, Bauer R, Bekeredjian R, Stucka R, Rutschow D, Lochmuller H *et al.* (2012), *Long-term preservation of cardiac structure and function after adeno-associated virus serotype 9-mediated microdystrophin gene transfer in mdx mice.* Hum Gene Ther; 23: 566-575.
330. Shin JH, Nitahara-Kasahara Y, Hayashita-Kinoh H, Ohshima-Hosoyama S, Kinoshita K, Chiyo T *et al.* (2011), *Improvement of cardiac fibrosis in dystrophic mice by rAAV9-mediated microdystrophin transduction.* Gene Ther; 18: 910-919.
331. Peault B, Rudnicki M, Torrente Y, Cossu G, Tremblay JP, Partridge T *et al.* (2007), *Stem and progenitor cells in skeletal muscle development, maintenance, and therapy.* Mol Ther; 15: 867-877.
332. Skuk D, Roy B, Goulet M, Chapdelaine P, Bouchard JP, Roy R *et al.* (2004), *Dystrophin expression in myofibers of Duchenne muscular dystrophy patients following intramuscular injections of normal myogenic cells.* Mol Ther; 9: 475-482.
333. Skuk D, Tremblay JP (2011), *Intramuscular cell transplantation as a potential treatment of myopathies: clinical and preclinical relevant data.* Expert Opin Biol Ther; 11: 359-374.
334. Montarras D, Morgan J, Collins C, Relaix F, Zaffran S, Cumano A *et al.* (2005), *Direct isolation of satellite cells for skeletal muscle regeneration.* Science; 309: 2064-2067.
335. Urish K, Kanda Y, Huard J (2005), *Initial failure in myoblast transplantation therapy has led the way toward the isolation of muscle stem cells: potential for tissue regeneration.* Curr Top Dev Biol; 68: 263-280.
336. Tsao J, Vernet DA, Gelfand R, Kovanecz I, Nolazco G, Bruhn KW *et al.* (2013), *Myostatin genetic inactivation inhibits myogenesis by muscle derived stem cells in vitro but not when implanted in the mdx mouse muscle.* Stem Cell Res Ther; 4: 4.
337. Sampaolesi M, Blot S, D'Antona G, Granger N, Tonlorenzi R, Innocenzi A *et al.* (2006), *Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs.* Nature; 444: 574-579.

338. Morgan J, Muntoni F (2007), *Mural cells paint a new picture of muscle stem cells.* Nat Cell Biol; 9: 249-251.
339. Vieira NM, Valadares M, Zucconi E, Secco M, Bueno Junior CR, Brandalise V *et al.* (2012), *Human adipose-derived mesenchymal stromal cells injected systemically into GRMD dogs without immunosuppression are able to reach the host muscle and express human dystrophin.* Cell Transplant; 21: 1407-1417.
340. Fakhfakh R, Michaud A, Tremblay JP (2011), *Blocking the myostatin signal with a dominant negative receptor improves the success of human myoblast transplantation in dystrophic mice.* Mol Ther; 19: 204-210.
341. Foster H, Popplewell L, Dickson G (2012), *Genetic therapeutic approaches for Duchenne muscular dystrophy.* Hum Gene Ther; 23: 676-687.
342. Chapdelaine P, Pichavant C, Rousseau J, Paques F, Tremblay JP (2010), *Meganucleases can restore the reading frame of a mutated dystrophin.* Gene Ther; 17: 846-858.
343. Rousseau J, Chapdelaine P, Boisvert S, Almeida LP, Corbeil J, Montpetit A *et al.* (2011), *Endonucleases: tools to correct the dystrophin gene.* J Gene Med; 13: 522-537.
344. Popplewell L, Koo T, Leclerc X, Duclert A, Mamchaoui K, Gouble A *et al.* (2013), *Gene correction of a duchenne muscular dystrophy mutation by meganuclease-enhanced exon knock-in.* Hum Gene Ther; 24: 692-701.
345. Ousterout DG, Perez-Pinera P, Thakore PI, Kabadi AM, Brown MT, Qin X *et al.* (2013), *Reading frame correction by targeted genome editing restores dystrophin expression in cells from duchenne muscular dystrophy patients.* Mol Ther; 21: 1718-1726.
346. Deconinck N, Tinsley J, De BF, Fisher R, Kahn D, Phelps S *et al.* (1997), *Expression of truncated utrophin leads to major functional improvements in dystrophin-deficient muscles of mice.* Nat Med; 3: 1216-1221.
347. Gillis JM (2002), *Multivariate evaluation of the functional recovery obtained by the overexpression of utrophin in skeletal muscles of the mdx mouse.* Neuromuscul Disord; 12 Suppl 1: S90-S94.
348. Tinsley J, Deconinck N, Fisher R, Kahn D, Phelps S, Gillis JM *et al.* (1998), *Expression of full-length utrophin prevents muscular dystrophy in mdx mice.* Nat Med; 4: 1441-1444.
349. Cerletti M, Negri T, Cozzi F, Colpo R, Andreetta F, Croci D *et al.* (2003), *Dystrophic phenotype of canine X-linked muscular dystrophy is mitigated by adenovirus-mediated utrophin gene transfer.* Gene Ther; 10: 750-757.
350. Moorwood C, Khurana TS (2013), *Duchenne muscular dystrophy drug discovery - the application of utrophin promoter activation screening.* Expert Opin Drug Discov; 8: 569-581.
351. Tinsley JM, Fairclough RJ, Storer R, Wilkes FJ, Potter AC, Squire SE *et al.* (2011), *Daily treatment with SMTC1100, a novel small molecule utrophin upregulator, dramatically reduces the dystrophic symptoms in the mdx mouse.* PLoS One; 6: e19189.
352. http://www.summitplc.com/programmes/publications/
353. Li D, Bareja A, Judge L, Yue Y, Lai Y, Fairclough R *et al.* (2010), *Sarcolemmal nNOS anchoring reveals a qualitative difference between dystrophin and utrophin.* J Cell Sci; 123: 2008-2013.
354. Bowe MA, Mendis DB, Fallon JR (2000), *The small leucine-rich repeat proteoglycan biglycan binds to alpha-dystroglycan and is upregulated in dystrophic muscle.* J Cell Biol; 148: 801-810.
355. Amenta AR, Yilmaz A, Bogdanovich S, McKechnie BA, Abedi M, Khurana TS *et al.* (2011), *Biglycan recruits utrophin to the sarcolemma and counters dystrophic pathology in mdx mice.* Proc Natl Acad Sci USA; 108: 762-767.
356. Amirouche A, Tadesse H, Lunde JA, Belanger G, Cote J, Jasmin BJ (2013), *Activation of p38 signaling increases utrophin A expression in skeletal muscle via the RNA-binding protein KSRP and inhibition of AU-rich element-mediated mRNA decay: implications for novel DMD therapeutics.* Hum Mol Genet; 22: 3093-3111.
357. Kharraz Y, Guerra J, Mann CJ, Serrano AL, Munoz-Canoves P (2013), *Macrophage plasticity and the role of inflammation in skeletal muscle repair.* Mediators Inflamm; 2013: 491497.
358. Spencer MJ, Tidball JG (2001), *Do immune cells promote the pathology of dystrophin-deficient myopathies?* Neuromuscul Disord; 11: 556-564.
359. Spencer MJ, Montecino-Rodriguez E, Dorshkind K, Tidball JG (2001), *Helper (CD4(+)) and cytotoxic (CD8(+)) T cells promote the pathology of dystrophin-deficient muscle.* Clin Immunol; 98: 235-243.
360. Wynn TA (2004), *Fibrotic disease and the T(H)1/T(H)2 paradigm.* Nat Rev Immunol; 4: 583-594.
361. Zhou L, Lu H (2010), *Targeting fibrosis in Duchenne muscular dystrophy.* J Neuropathol Exp Neurol; 69: 771-776.
362. Wehling M, Spencer MJ, Tidball JG (2001), *A nitric oxide synthase transgene ameliorates muscular dystrophy in mdx mice.* J Cell Biol; 155: 123-131.
363. Villalta SA, Nguyen HX, Deng B, Gotoh T, Tidball JG (2009), *Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy.*

Hum Mol Genet; 18: 482-496.
364. Wehling-Henricks M, Sokolow S, Lee JJ, Myung KH, Villalta SA, Tidball JG (2008), *Major basic protein-1 promotes fibrosis of dystrophic muscle and attenuates the cellular immune response in muscular dystrophy.* Hum Mol Genet; 17: 2280-2292.
365. Yoshizawa I, Nakajima T, Kawano Y, Noma T (1994), *Suppressive effect of oxatomide on the induction of IL2 responsiveness by DF-stimulated lymphocytes from patients with bronchial asthma.* Arerugi; 43: 535-543.
366. Gorospe JR, Tharp MD, Hinckley J, Kornegay JN, Hoffman EP (1994), *A role for mast cells in the progression of Duchenne muscular dystrophy? Correlations in dystrophin-deficient humans, dogs, and mice.* J Neurol Sci; 122: 44-56.
367. Gorospe JR, Tharp M, Demitsu T, Hoffman EP (1994), *Dystrophin-deficient myofibers are vulnerable to mast cell granule-induced necrosis.* Neuromuscul Disord; 4: 325-333.
368. Khan MA (1993), *Corticosteroid therapy in Duchenne muscular dystrophy.* J Neurol Sci; 120: 8-14.
369. Gaud A, Simon JM, Witzel T, Carre-Pierrat M, Wermuth CG, Segalat L (2004), *Prednisone reduces muscle degeneration in dystrophin-deficient Caenorhabditis elegans.* Neuromuscul Disord; 14: 365-370.
370. Wuebbles RD, Sarathy A, Kornegay JN, Burkin DJ (2013), *Levels of alpha7 integrin and laminin-alpha2 are increased following prednisone treatment in the mdx mouse and GRMD dog models of Duchenne muscular dystrophy.* Dis Model Mech; 6: 1175-1184.
371. Baltgalvis KA, Call JA, Nikas JB, Lowe DA (2009), *Effects of prednisolone on skeletal muscle contractility in mdx mice.* Muscle Nerve; 40: 443-454.
372. Tamma R, Annese T, Capogrosso RF, Cozzoli A, Benagiano V, Sblendorio V *et al.* (2013), *Effects of prednisolone on the dystrophin-associated proteins in the blood-brain barrier and skeletal muscle of dystrophic mdx mice.* Lab Invest; 93: 592-610.
373. Kolodziejczyk SM, Walsh GS, Balazsi K, Seale P, Sandoz J, Hierlihy AM *et al.* (2001), *Activation of JNK1 contributes to dystrophic muscle pathogenesis.* Curr Biol; 11: 1278-1282.
374. St-Pierre SJ, Chakkalakal JV, Kolodziejczyk SM, Knudson JC, Jasmin BJ, Megeney LA (2004), *Glucocorticoid treatment alleviates dystrophic myofiber pathology by activation of the calcineurin/NF-AT pathway.* FASEB J; 18: 1937-1939.
375. Fisher I, Abraham D, Bouri K, Hoffman EP, Muntoni F, Morgan J (2005), *Prednisolone-induced changes in dystrophic skeletal muscle.* FASEB J; 19: 834-836.
376. Jobling AI, Augusteyn RC (2002), *What causes steroid cataracts? A review of steroid-induced posterior subcapsular cataracts.* Clin Exp Optom; 85: 61-75.
377. Lo Cascio V, Kanis JA, Beneton MN, Bertoldo F, Adami S, Poggi G *et al.* (1995), *Acute effects of deflazacort and prednisone on rates of mineralization and bone formation.* Calcif Tissue Int; 56: 109-112.
378. Schakman O, Gilson H, Kalista S, Thissen JP (2009), *Mechanisms of muscle atrophy induced by glucocorticoids.* Horm Res; 72 Suppl 1: 36-41.
379. Guerron AD, Rawat R, Sali A, Spurney CF, Pistilli E, Cha HJ *et al.* (2010), *Functional and molecular effects of arginine butyrate and prednisone on muscle and heart in the mdx mouse model of Duchenne muscular dystrophy.* PLoS One; 5: e11220.
380. Sali A, Guerron AD, Gordish-Dressman H, Spurney CF, Iantorno M, Hoffman EP *et al.* (2012), *Glucocorticoid-treated mice are an inappropriate positive control for long-term preclinical studies in the mdx mouse.* PLoS One; 7: e34204.
381. Bauer R, Straub V, Blain A, Bushby K, MacGowan GA (2009), *Contrasting effects of steroids and angiotensin-converting-enzyme inhibitors in a mouse model of dystrophin-deficient cardiomyopathy.* Eur J Heart Fail; 11: 463-471.
382. Markham LW, Kinnett K, Wong BL, Woodrow BD, Cripe LH (2008), *Corticosteroid treatment retards development of ventricular dysfunction in Duchenne muscular dystrophy.* Neuromuscul Disord; 18: 365-370.
383. Silversides CK, Webb GD, Harris VA, Biggar DW (2003), *Effects of deflazacort on left ventricular function in patients with Duchenne muscular dystrophy.* Am J Cardiol; 91: 769-772.
384. Bonifati MD, Ruzza G, Bonometto P, Berardinelli A, Gorni K, Orcesi S *et al.* (2000), *A multicenter, double-blind, randomized trial of deflazacort versus prednisone in Duchenne muscular dystrophy.* Muscle Nerve; 23: 1344-1347.
385. Merlini L, Gennari M, Malaspina E, Cecconi I, Armaroli A, Gnudi S *et al.* (2012), *Early corticosteroid treatment in 4 Duchenne muscular dystrophy patients: 14-year follow-up.* Muscle Nerve; 45: 796-802.
386. Backman E, Henriksson KG (1995), *Low-dose prednisolone treatment in Duchenne and Becker muscular dystrophy.* Neuromuscul Disord; 5: 233-241.
387. Connolly AM, Schierbecker J, Renna R, Florence J (2002), *High dose weekly oral prednisone improves strength in boys with Duchenne muscular dystrophy.* Neuromuscul Disord; 12: 917-925.

388. Escolar DM, Hache LP, Clemens PR, Cnaan A, McDonald CM, Viswanathan V *et al.* (2011), *Randomized, blinded trial of weekend vs daily prednisone in Duchenne muscular dystrophy.* Neurology; 77: 444-452.
389. Fenichel GM, Mendell JR, Moxley RT, III, Griggs RC, Brooke MH, Miller JP *et al.* (1991), *A comparison of daily and alternate-day prednisone therapy in the treatment of Duchenne muscular dystrophy.* Arch Neurol; 48: 575-579.
390. Kinali M, Mercuri E, Main M, Muntoni F, Dubowitz V (2002), *An effective, low-dosage, intermittent schedule of prednisolone in the long-term treatment of early cases of Duchenne dystrophy.* Neuromuscul Disord; 12 Suppl 1: S169-S174.
391. Serra F, Quarta M, Canato M, Toniolo L, De A, V, Trotta A *et al.* (2012), *Inflammation in muscular dystrophy and the beneficial effects of non-steroidal anti-inflammatory drugs.* Muscle Nerve; 46: 773-784.
392. Pierno S, Nico B, Burdi R, Liantonio A, Didonna MP, Cippone V *et al.* (2007), *Role of tumour necrosis factor alpha, but not of cyclo-oxygenase-2-derived eicosanoids, on functional and morphological indices of dystrophic progression in mdx mice: a pharmacological approach.* Neuropathol Appl Neurobiol; 33: 344-359.
393. Brunelli S, Sciorati C, D'Antona G, Innocenzi A, Covarello D, Galvez BG *et al.* (2007), *Nitric oxide release combined with nonsteroidal antiinflammatory activity prevents muscular dystrophy pathology and enhances stem cell therapy.* Proc Natl Acad Sci USA; 104: 264-269.
394. Thomas GD, Ye J, De NC, Monopoli A, Ongini E, Victor RG (2012), *Treatment with a nitric oxide-donating NSAID alleviates functional muscle ischemia in the mouse model of Duchenne muscular dystrophy.* PLoS One; 7: e49350.
395. Pereira JA, Taniguti AP, Matsumura C, Marques MJ, Neto HS (2012), *Doxycycline ameliorates the dystrophic phenotype of skeletal and cardiac muscles in mdx mice.* Muscle Nerve; 46: 400-406.
396. Hori YS, Kuno A, Hosoda R, Tanno M, Miura T, Shimamoto K *et al.* (2011), *Resveratrol ameliorates muscular pathology in the dystrophic mdx mouse, a model for Duchenne muscular dystrophy.* J Pharmacol Exp Ther; 338: 784-794.
397. Kuno A, Hori YS, Hosoda R, Tanno M, Miura T, Shimamoto K *et al.* (2013), *Resveratrol improves cardiomyopathy in dystrophin-deficient mice through SIRT1 protein-mediated modulation of p300 protein.* J Biol Chem; 288: 5963-5972.
398. Gordon BS, Delgado Diaz DC, Kostek MC (2013), *Resveratrol decreases inflammation and increases utrophin gene expression in the mdx mouse model of Duchenne muscular dystrophy.* Clin Nutr; 32: 104-111.
399. Selsby JT, Morine KJ, Pendrak K, Barton ER, Sweeney HL (2012), *Rescue of dystrophic skeletal muscle by PGC-1alpha involves a fast to slow fiber type shift in the mdx mouse.* PLoS One; 7: e30063.
400. Patella V, de CG, Marino O, Spadaro G, Genovese A, Marone G (1996), *Oxatomide inhibits the release of proinflammatory mediators from human basophils and mast cells.* Int Arch Allergy Immunol; 111: 23-29.
401. Granchelli JA, Pollina C, Hudecki MS (2000), *Pre-clinical screening of drugs using the mdx mouse.* Neuromuscul Disord; 10: 235-239.
402. Buyse GM, Goemans N, Henricson E, Jara A, van den Hauwe M, Leshner R *et al.* (2007), *CINRG pilot trial of oxatomide in steroid-naive Duchenne muscular dystrophy.* Eur J Paediatr Neurol; 11: 337-340.
403. Lau KS, Grange RW, Chang WJ, Kamm KE, Sarelius I, Stull JT (1998), *Skeletal muscle contractions stimulate cGMP formation and attenuate vascular smooth muscle myosin phosphorylation via nitric oxide.* FEBS Lett; 431: 71-74.
404. Ennen JP, Verma M, Asakura A (2013), *Vascular-targeted therapies for Duchenne muscular dystrophy.* Skelet Muscle; 3: 9.
405. Cvelich RG, Roberts SC, Brown JN (2011), *Phosphodiesterase type 5 inhibitors as adjunctive therapy in the management of systolic heart failure.* Ann Pharmacother; 45: 1551-1558.
406. Dorsey P, Keel C, Klavens M, Hellstrom WJ (2010), *Phosphodiesterase type 5 (PDE5) inhibitors for the treatment of erectile dysfunction.* Expert Opin Pharmacother; 11: 1109-1122.
407. Kawahara G, Karpf JA, Myers JA, Alexander MS, Guyon JR, Kunkel LM (2011), *Drug screening in a zebrafish model of Duchenne muscular dystrophy.* Proc Natl Acad Sci USA; 108: 5331-5336.
408. Adamo CM, Dai DF, Percival JM, Minami E, Willis MS, Patrucco E *et al.* (2010), *Sildenafil reverses cardiac dysfunction in the mdx mouse model of Duchenne muscular dystrophy.* Proc Natl Acad Sci USA; 107: 19079-19083.
409. Percival JM, Whitehead NP, Adams ME, Adamo CM, Beavo JA, Froehner SC (2012), *Sildenafil reduces respiratory muscle weakness and fibrosis in the mdx mouse model of Duchenne muscular dystrophy.* J Pathol; 228: 77-87.
410. Percival JM, Siegel MP, Knowels G, Marcinek DJ (2013), *Defects in mitochondrial localization and ATP synthesis in the mdx mouse model of Duchenne muscular dystrophy are not alleviated by PDE5 inhibition.* Hum Mol Genet; 22: 153-167.
411. Koitabashi N, Aiba T, Hesketh GG, Rowell J, Zhang M, Takimoto E *et al.* (2010), *Cyclic GMP/PKG-de-*

*pendent inhibition of TRPC6 channel activity and expression negatively regulates cardiomyocyte NFAT activation Novel mechanism of cardiac stress modulation by PDE5 inhibition.* J Mol Cell Cardiol; 48: 713-724.

412. Zhao S, Zhang L, Lian G, Wang X, Zhang H, Yao X *et al.* (2011), *Sildenafil attenuates LPS-induced pro-inflammatory responses through down-regulation of intracellular ROS-related MAPK/NF-kappaB signaling pathways in N9 microglia.* Int Immunopharmacol; 11: 468-474.
413. Asai A, Sahani N, Kaneki M, Ouchi Y, Martyn JA, Yasuhara SE (2007), *Primary role of functional ischemia, quantitative evidence for the two-hit mechanism, and phosphodiesterase-5 inhibitor therapy in mouse muscular dystrophy.* PLoS One; 2: e806.
414. Martin EA, Barresi R, Byrne BJ, Tsimerinov EI, Scott BL, Walker AE *et al.* (2012), *Tadalafil alleviates muscle ischemia in patients with becker muscular dystrophy.* Sci Transl Med; 4: 162ra155.
415. Mizunoya W, Upadhaya R, Burczynski FJ, Wang G, Anderson JE (2011), *Nitric oxide donors improve prednisone effects on muscular dystrophy in the mdx mouse diaphragm.* Am J Physiol Cell Physiol; 300: C1065-C1077.
416. Kim JH, Kwak HB, Thompson LV, Lawler JM (2013), *Contribution of oxidative stress to pathology in diaphragm and limb muscles with Duchenne muscular dystrophy.* J Muscle Res Cell Motil; 34: 1-13.
417. McGeachie JK, Grounds MD, Partridge TA, Morgan JE (1993), *Age-related changes in replication of myogenic cells in mdx mice: quantitative autoradiographic studies.* J Neurol Sci; 119: 169-179.
418. Pastoret C, Sebille A (1995), *Age-related differences in regeneration of dystrophic (mdx) and normal muscle in the mouse.* Muscle Nerve; 18: 1147-1154.
419. Stedman HH, Sweeney HL, Shrager JB, Maguire HC, Panettieri RA, Petrof B *et al.* (1991), *The mdx mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy.* Nature; 352: 536-539.
420. Spurney CF, Rocha CT, Henricson E, Florence J, Mayhew J, Gorni K *et al.* (2011), *CINRG pilot trial of coenzyme Q10 in steroid-treated Duchenne muscular dystrophy.* Muscle Nerve; 44: 174-178.
421. Nagy I (1990), *Chemistry, toxicology, pharmacology and pharmacokinetics of idebenone: a review.* Arch Gerontol Geriatr; 11: 177-186.
422. Buyse GM, Van der MG, Erb M, D'hooge J, Herijgers P, Verbeken E *et al.* (2009), *Long-term blinded placebo-controlled study of SNT-MC17/idebenone in the dystrophin deficient mdx mouse: cardiac protection and improved exercise performance.* Eur Heart J; 30: 116-124.
423. Buyse GM, Goemans N, van den Hauwe M, Thijs D, de Groot IJ, Schara U *et al.* (2011), *Idebenone as a novel, therapeutic approach for Duchenne muscular dystrophy: results from a 12 month, double-blind, randomized placebo-controlled trial.* Neuromuscul Disord; 21: 396-405.
424. Buyse GM, Goemans N, van den Hauwe M, Meier T (2013), *Effects of glucocorticoids and idebenone on respiratory function in patients with duchenne muscular dystrophy.* Pediatr Pulmonol; 48: 912-920.
425. Evans NP, Misyak SA, Robertson JL, Bassaganya-Riera J, Grange RW (2009), *Immune-mediated mechanisms potentially regulate the disease time-course of duchenne muscular dystrophy and provide targets for therapeutic intervention.* PM R; 1: 755-768.
426. Porter JD, Khanna S, Kaminski HJ, Rao JS, Merriam AP, Richmonds CR *et al.* (2002), *A chronic inflammatory response dominates the skeletal muscle molecular signature in dystrophin-deficient mdx mice.* Hum Mol Genet; 11: 263-272.
427. Tidball JG (2005), *Inflammatory processes in muscle injury and repair.* Am J Physiol Regul Integr Comp Physiol; 288: R345-R353.
428. Acharyya S, Villalta SA, Bakkar N, Bupha-Intr T, Janssen PM, Carathers M *et al.* (2007), *Interplay of IKK/NF-kappaB signaling in macrophages and myofibers promotes muscle degeneration in Duchenne muscular dystrophy.* J Clin Invest; 117: 889-901.
429. Hayden MS, Ghosh S (2004), *Signaling to NF-kappaB.* Genes Dev; 18: 2195-2224.
430. Peterson JM, Guttridge DC (2008), *Skeletal muscle diseases, inflammation, and NF-kappaB signaling: insights and opportunities for therapeutic intervention.* Int Rev Immunol; 27: 375-387.
431. Kumar A, Boriek AM (2003), *Mechanical stress activates the nuclear factor-kappaB pathway in skeletal muscle fibers: a possible role in Duchenne muscular dystrophy.* FASEB J; 17: 386-396.
432. Grounds MD, Torrisi J (2004), *Anti-TNFalpha (Remicade) therapy protects dystrophic skeletal muscle from necrosis.* FASEB J; 18: 676-682.
433. Hodgetts S, Radley H, Davies M, Grounds MD (2006), *Reduced necrosis of dystrophic muscle by depletion of host neutrophils, or blocking TNFalpha function with Etanercept in mdx mice.* Neuromuscul Disord; 16: 591-602.
434. Messina S, Bitto A, Aguennouz M, Minutoli L, Monici MC, Altavilla D *et al.* (2006), *Nuclear factor kappa-B blockade reduces skeletal muscle degeneration and enhances muscle function in mdx mice.* Exp Neurol; 198: 234-241.
435. Carlson CG, Samadi A, Siegel A (2005), *Chronic treatment with agents that stabilize cytosolic IkappaB-*

*alpha enhances survival and improves resting membrane potential in MDX muscle fibers subjected to chronic passive stretch.* Neurobiol Dis; 20: 719-730.

436. Siegel AL, Bledsoe C, Lavin J, Gatti F, Berge J, Millman G *et al.* (2009), *Treatment with inhibitors of the NF-kappaB pathway improves whole body tension development in the mdx mouse.* Neuromuscul Disord; 19: 131-139.
437. Rangan GK, Wang Y, Tay YC, Harris DC (1999), *Inhibition of nuclear factor-kappaB activation reduces cortical tubulointerstitial injury in proteinuric rats.* Kidney Int; 56: 118-134.
438. Messina S, Bitto A, Aguennouz M, Mazzeo A, Migliorato A, Polito F *et al.* (2009), *Flavocoxid counteracts muscle necrosis and improves functional properties in mdx mice: a comparison study with methylprednisolone.* Exp Neurol; 220: 349-358.
439. Dorchies OM, Wagner S, Vuadens O, Waldhauser K, Buetler TM, Kucera P *et al.* (2006), *Green tea extract and its major polyphenol (-)-epigallocatechin gallate improve muscle function in a mouse model for Duchenne muscular dystrophy.* Am J Physiol Cell Physiol; 290: C616-C625.
440. Evans NP, Call JA, Bassaganya-Riera J, Robertson JL, Grange RW (2010), *Green tea extract decreases muscle pathology and NF-kappaB immunostaining in regenerating muscle fibers of mdx mice.* Clin Nutr; 29: 391-398.
441. Nakae Y, Dorchies OM, Stoward PJ, Zimmermann BF, Ritter C, Ruegg UT (2012), *Quantitative evaluation of the beneficial effects in the mdx mouse of epigallocatechin gallate, an antioxidant polyphenol from green tea.* Histochem Cell Biol; 137: 811-827.
442. Pan Y, Chen C, Shen Y, Zhu CH, Wang G, Wang XC *et al.* (2008), *Curcumin alleviates dystrophic muscle pathology in mdx mice.* Mol Cells; 25: 531-537.
443. Durham WJ, Arbogast S, Gerken E, Li YP, Reid MB (2006), *Progressive nuclear factor-kappaB activation resistant to inhibition by contraction and curcumin in mdx mice.* Muscle Nerve; 34: 298-303.
444. Yang KY, Lin LC, Tseng TY, Wang SC, Tsai TH (2007), *Oral bioavailability of curcumin in rat and the herbal analysis from Curcuma longa by LC-MS/MS.* J Chromatogr B Analyt Technol Biomed Life Sci; 853: 183-189.
445. Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, Gluud C (2012), *Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases.* Cochrane Database Syst Rev; 3: CD007176.
446. Terrill JR, Radley-Crabb HG, Iwasaki T, Lemckert FA, Arthur PG, Grounds MD (2013), *Oxidative stress and pathology in muscular dystrophies: focus on protein thiol oxidation and dysferlinopathies.* FEBS J; 280: 4149-4164.
447. de Senzi Moraes PR, Ferretti R, Moraes LH, Neto HS, Marques MJ, Minatel E (2013), *N-acetylcysteine treatment reduces TNF-alpha levels and myonecrosis in diaphragm muscle of mdx mice.* Clin Nutr; 32: 472-475.
448. Terrill JR, Radley-Crabb HG, Grounds MD, Arthur PG (2012), *N-Acetylcysteine treatment of dystrophic mdx mice results in protein thiol modifications and inhibition of exercise induced myofibre necrosis.* Neuromuscul Disord; 22: 427-434.
449. Whitehead NP, Pham C, Gervasio OL, Allen DG (2008), *N-Acetylcysteine ameliorates skeletal muscle pathophysiology in mdx mice.* J Physiol; 586: 2003-2014.
450. Davidson ZE, Truby H (2009), *A review of nutrition in Duchenne muscular dystrophy.* J Hum Nutr Diet; 22: 383-393.
451. Terrill JR, Boyatzis A, Grounds MD, Arthur PG (2013), *Treatment with the cysteine precursor l-2-oxothiazolidine-4-carboxylate (OTC) implicates taurine deficiency in severity of dystropathology in mdx mice.* Int J Biochem Cell Biol; 45: 2097-2108.
452. De Luca A, Pierno S, Liantonio A, Cetrone M, Camerino C, Fraysse B *et al.* (2003), *Enhanced dystrophic progression in mdx mice by exercise and beneficial effects of taurine and insulin-like growth factor-1.* J Pharmacol Exp Ther; 304: 453-463.
453. Cozzoli A, Rolland JF, Capogrosso RF, Sblendorio VT, Longo V, Simonetti S *et al.* (2011), *Evaluation of potential synergistic action of a combined treatment with alpha-methyl-prednisolone and taurine on the mdx mouse model of Duchenne muscular dystrophy.* Neuropathol Appl Neurobiol; 37: 243-256.
454. Terjung RL, Clarkson P, Eichner ER, Greenhaff PL, Hespel PJ, Israel RG *et al.* (2000), *American College of Sports Medicine roundtable. The physiological and health effects of oral creatine supplementation.* Med Sci Sports Exerc; 32: 706-717.
455. Pulido SM, Passaquin AC, Leijendekker WJ, Challet C, Wallimann T, Ruegg UT (1998), *Creatine supplementation improves intracellular Ca2+ handling and survival in mdx skeletal muscle cells.* FEBS Lett; 439: 357-362.
456. Passaquin AC, Renard M, Kay L, Challet C, Mokhtarian A, Wallimann T *et al.* (2002), *Creatine supplementation reduces skeletal muscle degeneration and enhances mitochondrial function in mdx mice.* Neu-

romuscul Disord; 12: 174-182.
457. Louis M, Raymackers JM, Debaix H, Lebacq J, Francaux M (2004), *Effect of creatine supplementation on skeletal muscle of mdx mice.* Muscle Nerve; 29: 687-692.
458. Pearlman JP, Fielding RA (2006), *Creatine monohydrate as a therapeutic aid in muscular dystrophy.* Nutr Rev; 64: 80-88.
459. Walter MC, Lochmuller H, Reilich P, Klopstock T, Huber R, Hartard M *et al.* (2000), *Creatine monohydrate in muscular dystrophies: A double-blind, placebo-controlled clinical study.* Neurology; 54: 1848-1850.
460. Walter MC, Reilich P, Lochmuller H, Kohnen R, Schlotter B, Hautmann H *et al.* (2002), *Creatine monohydrate in myotonic dystrophy: a double-blind, placebo-controlled clinical study.* J Neurol; 249: 1717-1722.
461. Louis M, Lebacq J, Poortmans JR, Belpaire-Dethiou MC, Devogelaer JP, Van HP *et al.* (2003), *Beneficial effects of creatine supplementation in dystrophic patients.* Muscle Nerve; 27: 604-610.
462. Tarnopolsky MA, Mahoney DJ, Vajsar J, Rodriguez C, Doherty TJ, Roy BD *et al.* (2004), *Creatine monohydrate enhances strength and body composition in Duchenne muscular dystrophy.* Neurology; 62: 1771-1777.
463. Banerjee B, Sharma U, Balasubramanian K, Kalaivani M, Kalra V, Jagannathan NR (2010), *Effect of creatine monohydrate in improving cellular energetics and muscle strength in ambulatory Duchenne muscular dystrophy patients: a randomized, placebo-controlled 31P MRS study.* Magn Reson Imaging; 28: 698-707.
464. Mok E, Constantin B, Favreau F, Neveux N, Magaud C, Delwail A *et al.* (2008), *l-Glutamine administration reduces oxidized glutathione and MAP kinase signaling in dystrophic muscle of mdx mice.* Pediatr Res; 63: 268-273.
465. Hankard RG, Hammond D, Haymond MW, Darmaun D (1998), *Oral glutamine slows down whole body protein breakdown in Duchenne muscular dystrophy.* Pediatr Res; 43: 222-226.
466. Mok E, Eleouet-Da VC, Daubrosse C, Gottrand F, Rigal O, Fontan JE *et al.* (2006), *Oral glutamine and amino acid supplementation inhibit whole-body protein degradation in children with Duchenne muscular dystrophy.* Am J Clin Nutr; 83: 823-828.
467. Mok E, Letellier G, Cuisset JM, Denjean A, Gottrand F, Alberti C *et al.* (2009), *Lack of functional benefit with glutamine versus placebo in Duchenne muscular dystrophy: a randomized crossover trial.* PLoS One; 4: e5448.
468. Letellier G, Mok E, Alberti C, De LA, Gottrand F, Cuisset JM *et al.* (2013), *Effect of glutamine on glucose metabolism in children with Duchenne muscular dystrophy.* Clin Nutr; 32: 386-390.
469. Escolar DM, Buyse G, Henricson E, Leshner R, Florence J, Mayhew J *et al.* (2005), *CINRG randomized controlled trial of creatine and glutamine in Duchenne muscular dystrophy.* Ann Neurol; 58: 151-155.
470. Chaubourt E, Fossier P, Baux G, Leprince C, Israel M, de la Porte S (1999), *Nitric oxide and l-arginine cause an accumulation of utrophin at the sarcolemma: a possible compensation for dystrophin loss in Duchenne muscular dystrophy.* Neurobiol Dis; 6: 499-507.
471. Hnia K, Gayraud J, Hugon G, Ramonatxo M, de la Porte S, Matecki S *et al.* (2008), *L-arginine decreases inflammation and modulates the nuclear factor-kappaB/matrix metalloproteinase cascade in mdx muscle fibers.* Am J Pathol; 172: 1509-1519.
472. Archer JD, Vargas CC, Anderson JE (2006), *Persistent and improved functional gain in mdx dystrophic mice after treatment with L-arginine and deflazacort.* FASEB J; 20: 738-740.
473. Marques MJ, Barbin IC, Taniguti AP, Oggian DS, Ferretti R, Santo NH (2010), *Myocardial fibrosis is unaltered by long-term administration of L-arginine in dystrophin deficient mdx mice: a histomorphometric analysis.* Acta Biol Hung; 61: 168-174.
474. Wehling-Henricks M, Jordan MC, Gotoh T, Grody WW, Roos KP, Tidball JG (2010), *Arginine metabolism by macrophages promotes cardiac and muscle fibrosis in mdx muscular dystrophy.* PLoS One; 5: e10763.
475. Vianello S, Yu H, Voisin V, Haddad H, He X, Foutz AS *et al.* (2013), *Arginine butyrate: a therapeutic candidate for Duchenne muscular dystrophy.* FASEB J; 27: 2256-2269.
476. Payne ET, Yasuda N, Bourgeois JM, Devries MC, Rodriguez MC, Yousuf J *et al.* (2006), *Nutritional therapy improves function and complements corticosteroid intervention in mdx mice.* Muscle Nerve; 33: 66-77.
477. Vandenburgh H, Shansky J, Benesch-Lee F, Skelly K, Spinazzola JM, Saponjian Y *et al.* (2009), *Automated drug screening with contractile muscle tissue engineered from dystrophic myoblasts.* FASEB J; 23: 3325-3334.
478. Kemaladewi DU, 't Hoen PA, ten Dijke P, van Ommen GJ, Hoogaars WM (2012), *TGF-β signaling in Duchenne muscular dystrophy.* Future Neurology; 7: 209-224.
479. Rahimi RA, Leof EB (2007), *TGF-beta signaling: a tale of two responses.* J Cell Biochem; 102: 593-608.
480. Zhang YE (2009), *Non-Smad pathways in TGF-beta signaling.* Cell Res; 19: 128-139.
481. Amthor H, Hoogaars WM (2012), *Interference with myostatin/ActRIIB signaling as a therapeutic strategy for Duchenne muscular dystrophy.* Curr Gene Ther; 12: 245-259.
482. Kemaladewi DU, de Gorter DJ, Aartsma-Rus A, van Ommen GJ, ten Dijke P, 't Hoen PA *et al.* (2012), *Cell-*

*type specific regulation of myostatin signaling.* FASEB J; 26: 1462-1472.
483. McFarlane C, Hennebry A, Thomas M, Plummer E, Ling N, Sharma M *et al.* (2008), *Myostatin signals through Pax7 to regulate satellite cell self-renewal.* Exp Cell Res; 314: 317-329.
484. Sartori R, Milan G, Patron M, Mammucari C, Blaauw B, Abraham R *et al.* (2009), *Smad2 and 3 transcription factors control muscle mass in adulthood.* Am J Physiol Cell Physiol; 296: C1248-C1257.
485. Li ZB, Kollias HD, Wagner KR (2008), *Myostatin directly regulates skeletal muscle fibrosis.* J Biol Chem; 283: 19371-19378.
486. McLennan IS, Koishi K (2002), *The transforming growth factor-betas: multifaceted regulators of the development and maintenance of skeletal muscles, motoneurons and Schwann cells.* Int J Dev Biol; 46: 559-567.
487. Kingsley DM (1994), *The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms.* Genes Dev; 8: 133-146.
488. Lee SJ, Lee YS, Zimmers TA, Soleimani A, Matzuk MM, Tsuchida K *et al.* (2010), *Regulation of muscle mass by follistatin and activins.* Mol Endocrinol; 24: 1998-2008.
489. Shi S, Hoogaars WM, de Gorter DJ, van Heiningen SH, Lin HY, Hong CC *et al.* (2011), *BMP antagonists enhance myogenic differentiation and ameliorate the dystrophic phenotype in a DMD mouse model.* Neurobiol Dis; 41: 353-360.
490. Turk R, Sterrenburg E, de Meijer EJ, van Ommen GJ, Den Dunnen JT, 't Hoen PA (2005), *Muscle regeneration in dystrophin-deficient mdx mice studied by gene expression profiling.* BMC Genomics; 6: 98.
491. Sterrenburg E, van der Wees CG, White SJ, Turk R, de Menezes RX, van Ommen GJ *et al.* (2006), *Gene expression profiling highlights defective myogenesis in DMD patients and a possible role for bone morphogenetic protein 4*. Neurobiol Dis; 23: 228-236.
492. Ono Y, Calhabeu F, Morgan JE, Katagiri T, Amthor H, Zammit PS (2011), *BMP signalling permits population expansion by preventing premature myogenic differentiation in muscle satellite cells.* Cell Death Differ; 18: 222-234.
493. Wang H, Noulet F, Edom-Vovard F, Tozer S, Le GF, Duprez D (2010), *Bmp signaling at the tips of skeletal muscles regulates the number of fetal muscle progenitors and satellite cells during development.* Dev Cell; 18: 643-654.
494. Sartori R, Schirwis E, Blaauw B, Bortolanza S, Zhao J, Enzo E *et al.* (2013), *BMP signaling controls muscle mass.* Nat Genet; 45: 1309-1318.
495. Winbanks CE, Chen JL, Qian H, Liu Y, Bernardo BC, Beyer C *et al.* (2013), *The bone morphogenetic protein axis is a positive regulator of skeletal muscle mass.* J Cell Biol; 203: 345-357.
496. Grobet L, Martin LJ, Poncelet D, Pirottin D, Brouwers B, Riquet J *et al.* (1997), *A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle.* Nat Genet; 17: 71-74.
497. Kambadur R, Sharma M, Smith TP, Bass JJ (1997), *Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle.* Genome Res; 7: 910-916.
498. McPherron AC, Lee SJ (1997), *Double muscling in cattle due to mutations in the myostatin gene.* Proc Natl Acad Sci USA; 94: 12457-12461.
499. Schuelke M, Wagner KR, Stolz LE, Hubner C, Riebel T, Komen W *et al.* (2004), *Myostatin mutation associated with gross muscle hypertrophy in a child.* N Engl J Med; 350: 2682-2688.
500. Shelton GD, Engvall E (2007), *Gross muscle hypertrophy in whippet dogs is caused by a mutation in the myostatin gene.* Neuromuscul Disord; 17: 721-722.
501. Clop A, Marcq F, Takeda H, Pirottin D, Tordoir X, Bibe B *et al.* (2006), *A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep.* Nat Genet; 38: 813-818.
502. McPherron AC, Lawler AM, Lee SJ (1997), *Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member.* Nature; 387: 83-90.
503. Wagner KR, Liu X, Chang X, Allen RE (2005), *Muscle regeneration in the prolonged absence of myostatin.* Proc Natl Acad Sci USA; 102: 2519-2524.
504. Mosher DS, Quignon P, Bustamante CD, Sutter NB, Mellersh CS, Parker HG *et al.* (2007), *A mutation in the myostatin gene increases muscle mass and enhances racing performance in heterozygote dogs.* PLoS Genet; 3: e79.
505. Wagner KR, McPherron AC, Winik N, Lee SJ (2002), *Loss of myostatin attenuates severity of muscular dystrophy in mdx mice.* Ann Neurol; 52: 832-836.
506. Cohn RD, Liang HY, Shetty R, Abraham T, Wagner KR (2007), *Myostatin does not regulate cardiac hypertrophy or fibrosis.* Neuromuscul Disord; 17: 290-296.
507. Amthor H, Macharia R, Navarrete R, Schuelke M, Brown SC, Otto A *et al.* (2007), *Lack of myostatin results in excessive muscle growth but impaired force generation.* Proc Natl Acad Sci USA; 104: 1835-1840.
508. Welle S, Bhatt K, Pinkert CA, Tawil R, Thornton CA (2007), *Muscle growth after postdevelopmental myostatin gene knockout.* Am J Physiol Endocrinol Metab; 292: E985-E991.

509. Bogdanovich S, Krag TO, Barton ER, Morris LD, Whittemore LA, Ahima RS *et al.* (2002), *Functional improvement of dystrophic muscle by myostatin blockade.* Nature; 420: 418-421.
510. Murphy KT, Ryall JG, Snell SM, Nair L, Koopman R, Krasney PA *et al.* (2010), *Antibody-directed myostatin inhibition improves diaphragm pathology in young but not adult dystrophic mdx mice.* Am J Pathol; 176: 2425-2434.
511. Wagner KR, Fleckenstein JL, Amato AA, Barohn RJ, Bushby K, Escolar DM *et al.* (2008), *A phase I/IItrial of MYO-029 in adult subjects with muscular dystrophy.* Ann Neurol; 63: 561-571.
512. Cadena SM, Tomkinson KN, Monnell TE, Spaits MS, Kumar R, Underwood KW *et al.* (2010), *Administration of a soluble activin type IIB receptor promotes skeletal muscle growth independent of fiber type.* J Appl Physiol; 109: 635-642.
513. Carlson CG, Bruemmer K, Sesti J, Stefanski C, Curtis H, Ucran J *et al.* (2011), *Soluble activin receptor type IIB increases forward pulling tension in the mdx mouse.* Muscle Nerve; 43: 694-699.
514. Pistilli EE, Bogdanovich S, Goncalves MD, Ahima RS, Lachey J, Seehra J *et al.* (2011), *Targeting the activin type IIB receptor to improve muscle mass and function in the mdx mouse model of Duchenne muscular dystrophy.* Am J Pathol; 178: 1287-1297.
515. Attie KM, Borgstein NG, Yang Y, Condon CH, Wilson DM, Pearsall AE *et al.* (2013), *A single ascending-dose study of muscle regulator ACE-031 in healthy volunteers.* Muscle Nerve; 47: 416-423.
516. Hoogaars WM, Mouisel E, Pasternack A, Hulmi JJ, Relizani K, Schuelke M *et al.* (2012), *Combined effect of AAV-U7-induced dystrophin exon skipping and soluble activin Type IIB receptor in mdx mice.* Hum Gene Ther; 23: 1269-1279.
517. Dumonceaux J, Marie S, Beley C, Trollet C, Vignaud A, Ferry A *et al.* (2010), *Combination of myostatin pathway interference and dystrophin rescue enhances tetanic and specific force in dystrophic mdx mice.* Mol Ther; 18: 881-887.
518. Bogdanovich S, Perkins KJ, Krag TO, Whittemore LA, Khurana TS (2005), *Myostatin propeptide-mediated amelioration of dystrophic pathophysiology.* FASEB J; 19: 543-549.
519. Qiao C, Li J, Jiang J, Zhu X, Wang B, Li J *et al.* (2008), *Myostatin propeptide gene delivery by adeno-associated virus serotype 8 vectors enhances muscle growth and ameliorates dystrophic phenotypes in mdx mice.* Hum Gene Ther; 19: 241-254.
520. Morine KJ, Bish LT, Pendrak K, Sleeper MM, Barton ER, Sweeney HL (2010), *Systemic myostatin inhibition via liver-targeted gene transfer in normal and dystrophic mice.* PLoS One; 5: e9176.
521. Bish LT, Sleeper MM, Forbes SC, Morine KJ, Reynolds C, Singletary GE *et al.* (2011), *Long-term systemic myostatin inhibition via liver-targeted gene transfer in golden retriever muscular dystrophy.* Hum Gene Ther; 22: 1499-1509.
522. Zhu J, Li Y, Lu A, Gharaibeh B, Ma J, Kobayashi T *et al.* (2011), *Follistatin improves skeletal muscle healing after injury and disease through an interaction with muscle regeneration, angiogenesis, and fibrosis.* Am J Pathol; 179: 915-930.
523. Lee SJ, McPherron AC (2001), *Regulation of myostatin activity and muscle growth.* Proc Natl Acad Sci USA; 98: 9306-9311.
524. Lee SJ (2007), *Quadrupling muscle mass in mice by targeting TGF-beta signaling pathways.* PLoS One; 2: e789.
525. Haidet AM, Rizo L, Handy C, Umapathi P, Eagle A, Shilling C *et al.* (2008), *Long-term enhancement of skeletal muscle mass and strength by single gene administration of myostatin inhibitors.* Proc Natl Acad Sci USA; 105: 4318-4322.
526. Nakatani M, Takehara Y, Sugino H, Matsumoto M, Hashimoto O, Hasegawa Y *et al.* (2008), *Transgenic expression of a myostatin inhibitor derived from follistatin increases skeletal muscle mass and ameliorates dystrophic pathology in mdx mice.* FASEB J; 22: 477-487.
527. Kota J, Handy CR, Haidet AM, Montgomery CL, Eagle A, Rodino-Klapac LR *et al.* (2009), *Follistatin gene delivery enhances muscle growth and strength in nonhuman primates.* Sci Transl Med; 1: 6ra15.
528. Rodino-Klapac LR, Janssen PM, Shontz KM, Canan B, Montgomery CL, Griffin D *et al.* (2013), *Micro-dystrophin and follistatin co-delivery restores muscle function in aged DMD model.* Hum Mol Genet; 22: 4929-4937.
529. Kemaladewi DU, Hoogaars WM, van Heiningen SH, Terlouw S, de Gorter DJ, den Dunnen JT *et al.* (2011), *Dual exon skipping in myostatin and dystrophin for Duchenne muscular dystrophy.* BMC Med Genomics; 4: 36.
530. Kang JK, Malerba A, Popplewell L, Foster K, Dickson G (2011), *Antisense-induced myostatin exon skipping leads to muscle hypertrophy in mice following octa-guanidine morpholino oligomer treatment.* Mol Ther; 19: 159-164.
531. Malerba A, Kang JK, McClorey G, Saleh AF, Popplewell L, Gait MJ *et al.* (2012), *Dual myostatin and dystrophin exon skipping by morpholino nucleic acid oligomers conjugated to a cell-penetrating peptide*

*is a promising therapeutic strategy for the treatment of Duchenne muscular dystrophy.* Mol Ther Nucleic Acids; 1: e62.

532. Rodgers BD, Interlichia JP, Garikipati DK, Mamidi R, Chandra M, Nelson OL *et al.* (2009), *Myostatin represses physiological hypertrophy of the heart and excitation-contraction coupling.* J Physiol; 587: 4873-4886.
533. Andreetta F, Bernasconi P, Baggi F, Ferro P, Oliva L, Arnoldi E *et al.* (2006), *Immunomodulation of TGF-beta 1 in mdx mouse inhibits connective tissue proliferation in diaphragm but increases inflammatory response: implications for antifibrotic therapy.* J Neuroimmunol; 175: 77-86.
534. Huebner KD, Jassal DS, Halevy O, Pines M, Anderson JE (2008), *Functional resolution of fibrosis in mdx mouse dystrophic heart and skeletal muscle by halofuginone.* Am J Physiol Heart Circ Physiol; 294: H1550-H1561.
535. Turgeman T, Hagai Y, Huebner K, Jassal DS, Anderson JE, Genin O *et al.* (2008), *Prevention of muscle fibrosis and improvement in muscle performance in the mdx mouse by halofuginone.* Neuromuscul Disord; 18: 857-868.
536. Spector I, Zilberstein Y, Lavy A, Genin O, Barzilai-Tutsch H, Bodanovsky A *et al.* (2013), *The involvement of collagen triple helix repeat containing 1 in muscular dystrophies.* Am J Pathol; 182: 905-916.
537. Zanotti S, Negri T, Cappelletti C, Bernasconi P, Canioni E, Di BC *et al.* (2005), *Decorin and biglycan expression is differentially altered in several muscular dystrophies.* Brain; 128: 2546-2555.
538. Zanotti S, Saredi S, Ruggieri A, Fabbri M, Blasevich F, Romaggi S *et al.* (2007), *Altered extracellular matrix transcript expression and protein modulation in primary Duchenne muscular dystrophy myotubes.* Matrix Biol; 26: 615-624.
539. Fadic R, Mezzano V, Alvarez K, Cabrera D, Holmgren J, Brandan E (2006), *Increase in decorin and biglycan in Duchenne muscular dystrophy: role of fibroblasts as cell source of these proteoglycans in the disease.* J Cell Mol Med; 10: 758-769.
540. Gosselin LE, Williams JE, Deering M, Brazeau D, Koury S, Martinez DA (2004), *Localization and early time course of TGF-beta 1 mRNA expression in dystrophic muscle.* Muscle Nerve; 30: 645-653.
541. Li Y, Foster W, Deasy BM, Chan Y, Prisk V, Tang Y *et al.* (2004), *Transforming growth factor-beta1 induces the differentiation of myogenic cells into fibrotic cells in injured skeletal muscle: a key event in muscle fibrogenesis.* Am J Pathol; 164: 1007-1019.
542. Li Y, Li J, Zhu J, Sun B, Branca M, Tang Y *et al.* (2007), *Decorin gene transfer promotes muscle cell differentiation and muscle regeneration.* Mol Ther; 15: 1616-1622.
543. Taniguti AP, Pertille A, Matsumura CY, Santo NH, Marques MJ (2011), *Prevention of muscle fibrosis and myonecrosis in mdx mice by suramin, a TGF-beta1 blocker.* Muscle Nerve; 43: 82-87.
544. de Oliveira Moreira D, Pereira JA, Taniguti AP, Matsumura CY, Ramos LA, Areas MA *et al.* (2013), *Suramin attenuates dystrophin-deficient cardiomyopathy in the mdx mouse model of duchenne muscular dystrophy.* Muscle Nerve; 48: 911-919.
545. Taniguti AP, Matsumura CY, Rodrigues-Simioni L, Santo NH, Marques MJ (2012), *Suramin affects metalloproteinase-9 activity and increases beta-dystroglycan levels in the diaphragm of the dystrophin-deficient mdx mouse.* Muscle Nerve; 46: 810-813.
546. Garcia-Schurmann JM, Schulze H, Haupt G, Pastor J, Allolio B, Senge T (1999), *Suramin treatment in hormone- and chemotherapy-refractory prostate cancer.* Urology; 53: 535-541.
547. Vidal B, Serrano AL, Tjwa M, Suelves M, Ardite E, De MR *et al.* (2008), *Fibrinogen drives dystrophic muscle fibrosis via a TGFbeta/alternative macrophage activation pathway.* Genes Dev; 22: 1747-1752.
548. Mosesson MW (2005), *Fibrinogen and fibrin structure and functions.* J Thromb Haemost; 3: 1894-1904.
549. Vidal B, Ardite E, Suelves M, Ruiz-Bonilla V, Janue A, Flick MJ *et al.* (2012), *Amelioration of Duchenne muscular dystrophy in mdx mice by elimination of matrix-associated fibrin-driven inflammation coupled to the alphaMbeta2 leukocyte integrin receptor.* Hum Mol Genet; 21: 1989-2004.
550. Huang P, Zhao XS, Fields M, Ransohoff RM, Zhou L (2009), *Imatinib attenuates skeletal muscle dystrophy in mdx mice.* FASEB J; 23: 2539-2548.
551. Bizario JC, Cerri DG, Rodrigues LC, Oliveira GL, Nomizo A, de Araujo DD *et al.* (2009), *Imatinib mesylate ameliorates the dystrophic phenotype in exercised mdx mice.* J Neuroimmunol; 212: 93-101.
552. Ito T, Ogawa R, Uezumi A, Ohtani T, Watanabe Y, Tsujikawa K *et al.* (2013), *Imatinib attenuates severe mouse dystrophy and inhibits proliferation and fibrosis-marker expression in muscle mesenchymal progenitors.* Neuromuscul Disord; 23: 349-356.
553. Morris CA, Selsby JT, Morris LD, Pendrak K, Sweeney HL (2010), *Bowman-Birk inhibitor attenuates dystrophic pathology in mdx mice.* J Appl Physiol; 109: 1492-1499.
554. Cozzoli A, Nico B, Sblendorio VT, Capogrosso RF, Dinardo MM, Longo V *et al.* (2011), *Enalapril treatment discloses an early role of angiotensin II in inflammation- and oxidative stress-related muscle damage in dystrophic mdx mice.* Pharmacol Res; 64: 482-492.

555. Sun G, Haginoya K, Dai H, Chiba Y, Uematsu M, Hino-Fukuyo N *et al.* (2009), *Intramuscular renin-angiotensin system is activated in human muscular dystrophy.* J Neurol Sci; 280: 40-48.
556. Seshiah PN, Weber DS, Rocic P, Valppu L, Taniyama Y, Griendling KK (2002), *Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators.* Circ Res; 91: 406-413.
557. Wei Y, Sowers JR, Clark SE, Li W, Ferrario CM, Stump CS (2008), *Angiotensin II-induced skeletal muscle insulin resistance mediated by NF-kappaB activation via NADPH oxidase.* Am J Physiol Endocrinol Metab; 294: E345-E351.
558. Rosenkranz S (2004), *TGF-beta1 and angiotensin networking in cardiac remodeling.* Cardiovasc Res; 63: 423-432.
559. Yang F, Chung AC, Huang XR, Lan HY (2009), *Angiotensin II induces connective tissue growth factor and collagen I expression via transforming growth factor-beta-dependent and -independent Smad pathways: the role of Smad3.* Hypertension; 54: 877-884.
560. Rodriguez-Vita J, Sanchez-Lopez E, Esteban V, Ruperez M, Egido J, Ruiz-Ortega M (2005), *Angiotensin II activates the Smad pathway in vascular smooth muscle cells by a transforming growth factor-beta-independent mechanism.* Circulation; 111: 2509-2517.
561. Burks TN, Andres-Mateos E, Marx R, Mejias R, van EC, Simmers JL *et al.* (2011), *Losartan restores skeletal muscle remodeling and protects against disuse atrophy in sarcopenia.* Sci Transl Med; 3: 82ra37.
562. Kasper SO, Phillips EE, Castle SM, Daley BJ, Enderson BL, Karlstad MD (2011), *Blockade of the Renin-Angiotensin system improves insulin receptor signaling and insulin-stimulated skeletal muscle glucose transport in burn injury.* Shock; 35: 80-85.
563. Jugdutt BI (2004), *Apoptosis after reperfused myocardial infarction: Role of angiotensin II.* Exp Clin Cardiol; 9: 219-228.
564. Reid JL (1997), *From kinetics to dynamics: are there differences between ACE inhibitors?* Eur Heart J; 18 Suppl E: E14-E18.
565. Bushby K, Muntoni F, Bourke JP (2003), *107th ENMC international workshop: the management of cardiac involvement in muscular dystrophy and myotonic dystrophy.* 7th-9th June 2002, Naarden, the Netherlands. Neuromuscul Disord; 13: 166-172.
566. Morales MG, Cabrera D, Cespedes C, Vio CP, Vazquez Y, Brandan E *et al.* (2013), *Inhibition of the angiotensin-converting enzyme decreases skeletal muscle fibrosis in dystrophic mice by a diminution in the expression and activity of connective tissue growth factor (CTGF/CCN-2).* Cell Tissue Res; 353: 173-187.
567. Rafael-Fortney JA, Chimanji NS, Schill KE, Martin CD, Murray JD, Ganguly R *et al.* (2011), *Early treatment with lisinopril and spironolactone preserves cardiac and skeletal muscle in Duchenne muscular dystrophy mice.* Circulation; 124: 582-588.
568. Krueger C, Hoffmann FM (2010), *Identification of retinoic acid in a high content screen for agents that overcome the anti-myogenic effect of TGF-beta-1.* PLoS One; 5: e15511.
569. Ogata H, Ishikawa Y, Ishikawa Y, Minami R (2009), *Beneficial effects of beta-blockers and angiotensin-converting enzyme inhibitors in Duchenne muscular dystrophy.* J Cardiol; 53: 72-78.
570. Viollet L, Thrush PT, Flanigan KM, Mendell JR, Allen HD (2012), *Effects of angiotensin-converting enzyme inhibitors and/or beta blockers on the cardiomyopathy in Duchenne muscular dystrophy.* Am J Cardiol; 110: 98-102.
571. Jefferies JL, Eidem BW, Belmont JW, Craigen WJ, Ware SM, Fernbach SD *et al.* (2005), *Genetic predictors and remodeling of dilated cardiomyopathy in muscular dystrophy.* Circulation; 112: 2799-2804.
572. Duboc D, Meune C, Lerebours G, Devaux JY, Vaksmann G, Becane HM (2005), *Effect of perindopril on the onset and progression of left ventricular dysfunction in Duchenne muscular dystrophy.* J Am Coll Cardiol; 45: 855-857.
573. Duboc D, Meune C, Pierre B, Wahbi K, Eymard B, Toutain A *et al.* (2007), *Perindopril preventive treatment on mortality in Duchenne muscular dystrophy: 10 years' follow-up.* Am Heart J; 154: 596-602.
574. Glass DJ (2003), *Molecular mechanisms modulating muscle mass.* Trends Mol Med; 9: 344-350.
575. Zhang P, Chen X, Fan M (2007), *Signaling mechanisms involved in disuse muscle atrophy.* Med Hypotheses; 69: 310-321.
576. Clemmons DR (2009), *Role of IGF-I in skeletal muscle mass maintenance.* Trends Endocrinol Metab; 20: 349-356.
577. Florini JR, Ewton DZ, Coolican SA (1996), *Growth hormone and the insulin-like growth factor system in myogenesis.* Endocr Rev; 17: 481-517.
578. Gregorevic P, Plant DR, Leeding KS, Bach LA, Lynch GS (2002), *Improved contractile function of the mdx dystrophic mouse diaphragm muscle after insulin-like growth factor-I administration.* Am J Pathol; 161: 2263-2272.
579. Gregorevic P, Plant DR, Lynch GS (2004), *Administration of insulin-like growth factor-I improves fatigue resistance of skeletal muscles from dystrophic mdx mice.* Muscle Nerve; 30: 295-304.

580. Barton ER, Morris L, Musaro A, Rosenthal N, Sweeney HL (2002), *Muscle-specific expression of insulin-like growth factor I counters muscle decline in mdx mice.* J Cell Biol; 157: 137-148.
581. Schertzer JD, Ryall JG, Lynch GS (2006), *Systemic administration of IGF-I enhances oxidative status and reduces contraction-induced injury in skeletal muscles of mdx dystrophic mice.* Am J Physiol Endocrinol Metab; 291: E499-E505.
582. Schertzer JD, Gehrig SM, Ryall JG, Lynch GS (2007), *Modulation of insulin-like growth factor (IGF)-I and IGF-binding protein interactions enhances skeletal muscle regeneration and ameliorates the dystrophic pathology in mdx mice.* Am J Pathol; 171: 1180-1188.
583. Gehrig SM, van der Poel C, Hoeflich A, Naim T, Lynch GS, Metzger F (2012), *Therapeutic potential of PEGylated insulin-like growth factor I for skeletal muscle disease evaluated in two murine models of muscular dystrophy.* Growth Horm IGF Res; 22: 69-75.
584. Cittadini A, Ines CL, Longobardi S, Rocco P, V, Casaburi C, Passamano L *et al.* (2003), *A preliminary randomized study of growth hormone administration in Becker and Duchenne muscular dystrophies.* Eur Heart J; 24: 664-672.
585. Lynch GS (2001), *Therapies for improving muscle function in neuromuscular disorders.* Exerc Sport Sci Rev; 29: 141-148.
586. Lynch GS, Ryall JG (2008), *Role of beta-adrenoceptor signaling in skeletal muscle: implications for muscle wasting and disease.* Physiol Rev; 88: 729-767.
587. Lynch GS, Hinkle RT, Faulkner JA (2000), *Power output of fast and slow skeletal muscles of mdx (dystrophic) and control mice after clenbuterol treatment.* Exp Physiol; 85: 295-299.
588. Zeman RJ, Peng H, Danon MJ, Etlinger JD (2000), *Clenbuterol reduces degeneration of exercised or aged dystrophic (mdx) muscle.* Muscle Nerve; 23: 521-528.
589. Lynch GS, Hinkle RT, Faulkner JA (2001), *Force and power output of diaphragm muscle strips from mdx and control mice after clenbuterol treatment.* Neuromuscul Disord; 11: 192-196.
590. Fowler EG, Graves MC, Wetzel GT, Spencer MJ (2004), *Pilot trial of albuterol in Duchenne and Becker muscular dystrophy.* Neurology; 62: 1006-1008.
591. Skura CL, Fowler EG, Wetzel GT, Graves M, Spencer MJ (2008), *Albuterol increases lean body mass in ambulatory boys with Duchenne or Becker muscular dystrophy.* Neurology; 70: 137-143.
592. Kissel JT, McDermott MP, Natarajan R, Mendell JR, Pandya S, King WM *et al.* (1998), *Pilot trial of albuterol in facioscapulohumeral muscular dystrophy. FSH-DY Group.* Neurology; 50: 1402-1406.
593. Kissel JT, McDermott MP, Mendell JR, King WM, Pandya S, Griggs RC *et al.* (2001), *Randomized, double-blind, placebo-controlled trial of albuterol in facioscapulohumeral dystrophy.* Neurology; 57: 1434-1440.
594. Payan CA, Hogrel JY, Hammouda EH, Lacomblez L, Ollivier G, Doppler V *et al.* (2009), *Periodic salbutamol in facioscapulohumeral muscular dystrophy: a randomized controlled trial.* Arch Phys Med Rehabil; 90: 1094-1101.
595. Oya Y, Ogawa M, Kawai M (2001), *Therapeutic trial of beta 2-adrenergic agonist clenbuterol in muscular dystrophies.* Rinsho Shinkeigaku; 41: 698-700.
596. Harcourt LJ, Schertzer JD, Ryall JG, Lynch GS (2007), *Low dose formoterol administration improves muscle function in dystrophic mdx mice without increasing fatigue.* Neuromuscul Disord; 17: 47-55.
597. Gehrig SM, Koopman R, Naim T, Tjoakarfa C, Lynch GS (2010), *Making fast-twitch dystrophic muscles bigger protects them from contraction injury and attenuates the dystrophic pathology.* Am J Pathol; 176: 29-33.
598. Leger B, Koopman R, Walrand S, Gehrig SM, Murphy KT, Lynch GS (2011), *Chronic formoterol administration reduces cardiac mitochondrial protein synthesis and oxidative capacity in mice.* Int J Cardiol; 146: 270-272.
599. Ryall JG, Schertzer JD, Murphy KT, Allen AM, Lynch GS (2008), *Chronic beta2-adrenoceptor stimulation impairs cardiac relaxation via reduced SR Ca2+-ATPase protein and activity.* Am J Physiol Heart Circ Physiol; 294: H2587-H2595.
600. Orr R, Fiatarone SM (2004), *The anabolic androgenic steroid oxandrolone in the treatment of wasting and catabolic disorders: review of efficacy and safety.* Drugs; 64: 725-750.
601. Fenichel G, Pestronk A, Florence J, Robison V, Hemelt V (1997), *A beneficial effect of oxandrolone in the treatment of Duchenne muscular dystrophy: a pilot study.* Neurology; 48: 1225-1226.
602. Fenichel GM, Griggs RC, Kissel J, Kramer TI, Mendell JR, Moxley RT *et al.* (2001), *A randomized efficacy and safety trial of oxandrolone in the treatment of Duchenne dystrophy.* Neurology; 56: 1075-1079.
603. Balagopal P, Olney R, Darmaun D, Mougey E, Dokler M, Sieck G *et al.* (2006), *Oxandrolone enhances skeletal muscle myosin synthesis and alters global gene expression profile in Duchenne muscular dystrophy.* Am J Physiol Endocrinol Metab; 290: E530-E539.
604. Krahn MJ, Anderson JE (1994), *Anabolic steroid treatment increases myofiber damage in mdx mouse*

*muscular dystrophy.* J Neurol Sci; 125: 138-146.
605. Guidance for Industry (2005), http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm078932.pdf.
606. Cozzoli A, Capogrosso RF, Sblendorio VT, Dinardo MM, Jagerschmidt C, Namour F *et al.* (2013), *GLPG0492, a novel selective androgen receptor modulator, improves muscle performance in the exercised-mdx mouse model of muscular dystrophy.* Pharmacol Res; 72: 9-24.
607. Dorchies OM, Reutenauer-Patte J, Dahmane E, Ismail HM, Petermann O, Vuadens O *et al.* (2013), *The anticancer drug tamoxifen counteracts the pathology in a mouse model of duchenne muscular dystrophy.* Am J Pathol; 182: 485-504.
608. Selsby J, Pendrak K, Zadel M, Tian Z, Pham J, Carver T *et al.* (2010), *Leupeptin-based inhibitors do not improve the mdx phenotype.* Am J Physiol Regul Integr Comp Physiol; 299: R1192-R1201.
609. Consalvi S, Saccone V, Giordani L, Minetti G, Mozzetta C, Puri PL (2011), *Histone deacetylase inhibitors in the treatment of muscular dystrophies: epigenetic drugs for genetic diseases.* Mol Med; 17: 457-465.
610. Minetti GC, Colussi C, Adami R, Serra C, Mozzetta C, Parente V *et al.* (2006), *Functional and morphological recovery of dystrophic muscles in mice treated with deacetylase inhibitors.* Nat Med; 12: 1147-1150.
611. Consalvi S, Mozzetta C, Bettica P, Germani M, Fiorentini F, Del BF *et al.* (2013), *Preclinical studies in the mdx mouse model of duchenne muscular dystrophy with the histone deacetylase inhibitor givinostat.* Mol Med; 19: 79-87.
612. Burkin DJ, Wallace GQ, Nicol KJ, Kaufman DJ, Kaufman SJ (2001), *Enhanced expression of the alpha 7 beta 1 integrin reduces muscular dystrophy and restores viability in dystrophic mice.* J Cell Biol; 152: 1207-1218.
613. Gurpur PB, Liu J, Burkin DJ, Kaufman SJ (2009), *Valproic acid activates the PI3K/Akt/mTOR pathway in muscle and ameliorates pathology in a mouse model of Duchenne muscular dystrophy.* Am J Pathol; 174: 999-1008.
614. Rommel C, Clarke BA, Zimmermann S, Nunez L, Rossman R, Reid K *et al.* (1999), *Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt.* Science; 286: 1738-1741.
615. Grounds MD (2008), *Two-tiered hypotheses for Duchenne muscular dystrophy.* Cell Mol Life Sci; 65: 1621-1625.
616. Deconinck N, Dan B (2007), *Pathophysiology of duchenne muscular dystrophy: current hypotheses.* Pediatr Neurol; 36: 1-7.
617. Allen DG, Gervasio OL, Yeung EW, Whitehead NP (2010), *Calcium and the damage pathways in muscular dystrophy.* Can J Physiol Pharmacol; 88: 83-91.
618. Iwata Y, Katanosaka Y, Arai Y, Shigekawa M, Wakabayashi S (2009), *Dominant-negative inhibition of Ca2+ influx via TRPV2 ameliorates muscular dystrophy in animal models.* Hum Mol Genet; 18: 824-834.
619. Harisseh R, Chatelier A, Magaud C, Deliot N, Constantin B (2013), *Involvement of TRPV2 and SOCE in calcium influx disorder in DMD primary human myotubes with a specific contribution of alpha1-syntrophin and PLC/PKC in SOCE regulation.* Am J Physiol Cell Physiol; 304: C881-C894.
620. Gehrig SM, van der Poel C, Sayer TA, Schertzer JD, Henstridge DC, Church JE *et al.* (2012), *Hsp72 preserves muscle function and slows progression of severe muscular dystrophy.* Nature; 484: 394-398.
621. Millay DP, Sargent MA, Osinska H, Baines CP, Barton ER, Vuagniaux G *et al.* (2008), *Genetic and pharmacologic inhibition of mitochondrial-dependent necrosis attenuates muscular dystrophy.* Nat Med; 14: 442-447.
622. Chen YW, Zhao P, Borup R, Hoffman EP (2000), *Expression profiling in the muscular dystrophies: identification of novel aspects of molecular pathophysiology.* J Cell Biol; 151: 1321-1336.
623. Berman B, Duncan MR (1989), *Pentoxifylline inhibits normal human dermal fibroblast in vitro proliferation, collagen, glycosaminoglycan, and fibronectin production, and increases collagenase activity.* J Invest Dermatol; 92: 605-610.
624. Berman B, Wietzerbin J, Sanceau J, Merlin G, Duncan MR (1992), *Pentoxifylline inhibits certain constitutive and tumor necrosis factor-alpha-induced activities of human normal dermal fibroblasts.* J Invest Dermatol; 98: 706-712.
625. Rolland JF, De LA, Burdi R, Andreetta F, Confalonieri P, Conte CD (2006), *Overactivity of exercise-sensitive cation channels and their impaired modulation by IGF-1 in mdx native muscle fibers: beneficial effect of pentoxifylline.* Neurobiol Dis; 24: 466-474.
626. Gosselin LE, Williams JE (2006), *Pentoxifylline fails to attenuate fibrosis in dystrophic (mdx) diaphragm muscle.* Muscle Nerve; 33: 820-823.
627. Zimmerman A, Clemens PR, Tesi-Rocha C, Connolly A, Iannaccone ST, Kuntz N *et al.* (2011), *Liquid formulation of pentoxifylline is a poorly tolerated treatment for duchenne dystrophy.* Muscle Nerve; 44: 170-173.
628. Escolar DM, Zimmerman A, Bertorini T, Clemens PR, Connolly AM, Mesa L *et al.* (2012), *Pentoxifylline*

*as a rescue treatment for DMD: a randomized double-blind clinical trial.* Neurology; 78: 904-913.
629. Matsumura CY, Pertille A, Albuquerque TC, Santo NH, Marques MJ (2009), *Diltiazem and verapamil protect dystrophin-deficient muscle fibers of MDX mice from degeneration: a potential role in calcium buffering and sarcolemmal stability.* Muscle Nerve; 39: 167-176.
630. Pernice W, Beckmann R, Ketelsen UP, Frey M, Schmidt-Redemann B, Haap KP *et al.* (1988), *A double-blind placebo controlled trial of diltiazem in Duchenne dystrophy.* Klin Wochenschr; 66: 565-570.
631. Bertorini TE, Palmieri GM, Griffin JW, Igarashi M, McGee J, Brown R *et al.* (1988), *Effect of chronic treatment with the calcium antagonist diltiazem in Duchenne muscular dystrophy.* Neurology; 38: 609-613.
632. Phillips MF, Quinlivan R (2008), *Calcium antagonists for Duchenne muscular dystrophy.* Cochrane Database Syst Rev; CD004571.
633. Gentil C, Leturcq F, Ben YR, Kaplan JC, Laforet P, Penisson-Besnier I *et al.* (2012), V*ariable phenotype of del45-55 Becker patients correlated with nNOSmu mislocalization and RYR1 hypernitrosylation.* Hum Mol Genet; 21: 3449-3460.
634. Weigl L, Zidar A, Gscheidlinger R, Karel A, Hohenegger M (2003), *Store operated Ca2+ influx by selective depletion of ryanodine sensitive Ca2+ pools in primary human skeletal muscle cells.* Naunyn Schmiedebergs Arch Pharmacol; 367: 353-363.
635. Richard I, Broux O, Allamand V, Fougerousse F, Chiannilkulchai N, Bourg N *et al.* (1995), *Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A.* Cell; 81: 27-40.
636. Briguet A, Erb M, Courdier-Fruh I, Barzaghi P, Santos G, Herzner H *et al.* (2008), *Effect of calpain and proteasome inhibition on Ca2+-dependent proteolysis and muscle histopathology in the mdx mouse.* FASEB J; 22: 4190-4200.
637. Spencer MJ, Mellgren RL (2002), *Overexpression of a calpastatin transgene in mdx muscle reduces dystrophic pathology.* Hum Mol Genet; 11: 2645-2655.
638. Badalamente MA, Stracher A (2000), *Delay of muscle degeneration and necrosis in mdx mice by calpain inhibition.* Muscle Nerve; 23: 106-111.
639. Childers MK, Bogan JR, Bogan DJ, Greiner H, Holder M, Grange RW *et al.* (2011), *Chronic administration of a leupeptin-derived calpain inhibitor fails to ameliorate severe muscle pathology in a canine model of duchenne muscular dystrophy.* Front Pharmacol; 2: 89.
640. Burdi R, Didonna MP, Pignol B, Nico B, Mangieri D, Rolland JF *et al.* (2006), *First evaluation of the potential effectiveness in muscular dystrophy of a novel chimeric compound, BN 82270, acting as calpain-inhibitor and anti-oxidant.* Neuromuscul Disord; 16: 237-248.
641. von Moers A, Zwirner A, Reinhold A, Bruckmann O, van LF, Stoltenburg-Didinger G *et al.* (2005), *Increased mRNA expression of tissue inhibitors of metalloproteinase-1 and -2 in Duchenne muscular dystrophy.* Acta Neuropathol; 109: 285-293.
642. Nadarajah VD, van PM, Chaouch A, Garrood P, Straub V, Lochmuller H *et al.* (2011), *Serum matrix metalloproteinase-9 (MMP-9) as a biomarker for monitoring disease progression in Duchenne muscular dystrophy (DMD).* Neuromuscul Disord; 21: 569-578.
643. Kumar A, Bhatnagar S, Kumar A (2010), *Matrix metalloproteinase inhibitor batimastat alleviates pathology and improves skeletal muscle function in dystrophin-deficient mdx mice.* Am J Pathol; 177: 248-260.
644. Li H, Mittal A, Makonchuk DY, Bhatnagar S, Kumar A (2009), *Matrix metalloproteinase-9 inhibition ameliorates pathogenesis and improves skeletal muscle regeneration in muscular dystrophy.* Hum Mol Genet; 18: 2584-2598.
645. Miyazaki D, Nakamura A, Fukushima K, Yoshida K, Takeda S, Ikeda S (2011), *Matrix metalloproteinase-2 ablation in dystrophin-deficient mdx muscles reduces angiogenesis resulting in impaired growth of regenerated muscle fibers.* Hum Mol Genet; 20: 1787-1799.
646. De Palma C, Morisi F, Cheli S, Pambianco S, Cappello V, Vezzoli M *et al.* (2012), *Autophagy as a new therapeutic target in Duchenne muscular dystrophy.* Cell Death Dis; 3: e418.
647. Mammucari C, Milan G, Romanello V, Masiero E, Rudolf R, Del PP *et al.* (2007), *FoxO3 controls autophagy in skeletal muscle in vivo.* Cell Metab; 6: 458-471.
648. Mammucari C, Schiaffino S, Sandri M (2008), *Downstream of Akt: FoxO3 and mTOR in the regulation of autophagy in skeletal muscle.* Autophagy; 4: 524-526.
649. Pauly M, Daussin F, Burelle Y, Li T, Godin R, Fauconnier J *et al.* (2012), *AMPK activation stimulates autophagy and ameliorates muscular dystrophy in the mdx mouse diaphragm.* Am J Pathol; 181: 583-592.
650. Chatterjee S, Moeller C, Shah N, Bolorunduro O, Lichstein E, Moskovits N *et al.* (2012), *Eplerenone is not superior to older and less expensive aldosterone antagonists.* Am J Med; 125: 817-825.
651. Miller AB (2007), *Aldosterone antagonism in heart failure.* Vasc Health Risk Manag; 3: 605-609.
652. Weber MA, Nagel AM, Wolf MB, Jurkat-Rott K, Kauczor HU, Semmler W *et al.* (2012), *Permanent muscular sodium overload and persistent muscle edema in Duchenne muscular dystrophy: a possible contributor of progressive muscle degeneration.* J Neurol; 259: 2385-2392.

653. Lehmann-Horn F, Weber MA, Nagel AM, Meinck HM, Breitenbach S, Scharrer J *et al.* (2012), *Rationale for treating oedema in Duchenne muscular dystrophy with eplerenone.* Acta Myol; 31: 31-39.
654. Frishman WH (1998), *Carvedilol.* N Engl J Med; 339: 1759-1765.
655. Saito T, Matsumura T, Miyai I, Nozaki S, Shinno S (2001), *Carvedilol effectiveness for left ventricular-insufficient patients with Duchenne muscular dystrophy.* Rinsho Shinkeigaku; 41: 691-694.
656. Matsumura T, Tamura T, Kuru S, Kikuchi Y, Kawai M (2010), *Carvedilol can prevent cardiac events in Duchenne muscular dystrophy.* Intern Med; 49: 1357-1363.
657. Kajimoto H, Ishigaki K, Okumura K, Tomimatsu H, Nakazawa M, Saito K *et al.* (2006), *Beta-blocker therapy for cardiac dysfunction in patients with muscular dystrophy.* Circ J; 70: 991-994.
658. http://www.treat-nmd.eu
659. Willmann R, De LA, Benatar M, Grounds M, Dubach J, Raymackers JM *et al.* (2012), *Enhancing translation: guidelines for standard pre-clinical experiments in mdx mice.* Neuromuscul Disord; 22: 43-49.
660. Mendell JR, Shilling C, Leslie ND, Flanigan KM, al-Dahhak R, Gastier-Foster J *et al.* (2012), *Evidence-based path to newborn screening for Duchenne muscular dystrophy.* Ann Neurol; 71: 304-313.
661. Aartsma-Rus A (2010), *Antisense-mediated modulation of splicing: therapeutic implications for Duchenne muscular dystrophy.* RNA Biol; 7: 453-461.
662. Tennyson CN, Shi Q, Worton RG (1996), *Stability of the human dystrophin transcript in muscle.* Nucleic Acids Res; 24: 3059-3064.
663. Spitali P, Heemskerk H, Vossen RH, Ferlini A, den Dunnen JT, 't Hoen PA *et al.* (2010), *Accurate quantification of dystrophin mRNA and exon skipping levels in duchenne muscular dystrophy.* Lab Invest; 90: 1396-1402.
664. Yu RZ, Baker B, Chappell A, Geary RS, Cheung E, Levin AA (2002), *Development of an ultrasensitive noncompetitive hybridization-ligation enzyme-linked immunosorbent assay for the determination of phosphorothioate oligodeoxynucleotide in plasma.* Anal Biochem; 304: 19-25.
665. R Development Core Team (2009), *R: A Language and Environment for Statistical Computing.* Vienna, Austria: R Foundation for Statistical Computing.
666. Bates D, Maechler M (2010), *lme4: Linear mixed-effects models using S4 classes.* R package version 0.999375-35.
667. Spitali P, Aartsma-Rus A (2012), *Splice modulating therapies for human disease.* Cell; 148: 1085-1088.
668. Brown DA, Kang SH, Gryaznov SM, DeDionisio L, Heidenreich O, Sullivan S *et al.* (1994), *Effect of phosphorothioate modification of oligodeoxynucleotides on specific protein binding.* J Biol Chem; 269: 26801-26805.
669. Yu RZ, Kim TW, Hong A, Watanabe TA, Gaus HJ, Geary RS (2007), *Cross-species pharmacokinetic comparison from mouse to man of a second-generation antisense oligonucleotide, ISIS 301012, targeting human apolipoprotein B-100.* Drug Metab Dispos; 35: 460-468.
670. Blake DJ, Weir A, Newey SE, Davies KE (2002), *Function and genetics of dystrophin and dystrophin-related proteins in muscle.* Physiol Rev; 82: 291-329.
671. Torres LF, Duchen LW (1987), *The mutant mdx: inherited myopathy in the mouse. Morphological studies of nerves, muscles and end-plates.* Brain; 110 ( Pt 2): 269-299.
672. Banks GB, Chamberlain JS (2008), *The value of mammalian models for duchenne muscular dystrophy in developing therapeutic strategies.* Curr Top Dev Biol; 84: 431-453.
673. Verhaart IE, Aartsma-Rus A (2012), *Gene therapy for Duchenne muscular dystrophy.* Curr Opin Neurol; 25: 588-596.
674. Spitali P, van den Bergen JC, Verhaart IE, Wokke B, Janson AA, van den Eijnde R *et al.* (2013), *DMD transcript imbalance determines dystrophin levels.* FASEB J; 27: 4909-4916.
675. Yazaki M, Yoshida K, Nakamura A, Koyama J, Nanba T, Ohori N *et al.* (1999), *Clinical characteristics of aged Becker muscular dystrophy patients with onset after 30 years.* Eur Neurol; 42: 145-149.
676. Aartsma-Rus A (2012), *Overview on AON design.* Methods Mol Biol; 867: 117-129.
677. Yin H, Moulton HM, Seow Y, Boyd C, Boutilier J, Iverson P *et al.* (2008), *Cell-penetrating peptide-conjugated antisense oligonucleotides restore systemic muscle and cardiac dystrophin expression and function.* Hum Mol Genet; 17: 3909-3918.
678. Elion GB (1989), *The purine path to chemotherapy.* Science; 244: 41-47.
679. Zhu CH, Mouly V, Cooper RN, Mamchaoui K, Bigot A, Shay JW *et al.* (2007), *Cellular senescence in human myoblasts is overcome by human telomerase reverse transcriptase and cyclin-dependent kinase 4: consequences in aging muscle and therapeutic strategies for muscular dystrophies.* Aging Cell; 6: 515-523.
680. Amantana A, Iversen PL (2005), *Pharmacokinetics and biodistribution of phosphorodiamidate morpholino antisense oligomers.* Curr Opin Pharmacol; 5: 550-555.
681. Swann PF, Waters TR, Moulton DC, Xu YZ, Zheng Q, Edwards M *et al.* (1996), *Role of postreplicative DNA mismatch repair in the cytotoxic action of thioguanine.* Science; 273: 1109-1111.

682. Waters TR, Swann PF (1997), *Cytotoxic mechanism of 6-thioguanine: hMutSalpha, the human mismatch binding heterodimer, binds to DNA containing S6-methylthioguanine.* Biochemistry; 36: 2501-2506.
683. Somerville L, Krynetski EY, Krynetskaia NF, Beger RD, Zhang W, Marhefka CA *et al.* (2003), *Structure and dynamics of thioguanine-modified duplex DNA.* J Biol Chem; 278: 1005-1011.
684. Ling YH, Chan JY, Beattie KL, Nelson JA (1992), *Consequences of 6-thioguanine incorporation into DNA on polymerase, ligase, and endonuclease reactions.* Mol Pharmacol; 42: 802-807.
685. Marathias VM, Sawicki MJ, Bolton PH (1999), *6-Thioguanine alters the structure and stability of duplex DNA and inhibits quadruplex DNA formation.* Nucleic Acids Res; 27: 2860-2867.
686. Uribe-Luna S, Quintana-Hau JD, Maldonado-Rodriguez R, Espinosa-Lara M, Beattie KL, Farquhar D *et al.* (1997), *Mutagenic consequences of the incorporation of 6-thioguanine into DNA.* Biochem Pharmacol; 54: 419-424.
687. Hann IM, Stevens RF, Goldstone AH, Rees JK, Wheatley K, Gray RG *et al.* (1997), *Randomized comparison of DAT versus ADE as induction chemotherapy in children and younger adults with acute myeloid leukemia. Results of the Medical Research Council's 10th AML trial (MRC AML10). Adult and Childhood Leukaemia Working Parties of the Medical Research Council.* Blood; 89: 2311-2318.
688. Preisler H, Davis RB, Kirshner J, Dupre E, Richards F, III, Hoagland HC *et al.* (1987), *Comparison of three remission induction regimens and two postinduction strategies for the treatment of acute nonlymphocytic leukemia: a cancer and leukemia group B study.* Blood; 69: 1441-1449.
689. Pinkel D (1993), *Intravenous mercaptopurine: life begins at 40.* J Clin Oncol; 11: 1826-1831.
690. Sheu CJ, Moreland FM (1983), *Detection of dominant lethal mutation in mice after repeated low-dose administration of 6-mercaptopurine.* Drug Chem Toxicol; 6: 83-92.
691. Koenig M, Monaco AP, Kunkel LM (1988), *The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein.* Cell; 53: 219-228.
692. Blake DJ, Weir A, Newey SE, Davies KE (2002), *Function and genetics of dystrophin and dystrophin-related proteins in muscle.* Physiol Rev; 82: 291-329.
693. Alexakis C, Partridge T, Bou-Gharios G (2007), *Implication of the satellite cell in dystrophic muscle fibrosis: a self-perpetuating mechanism of collagen overproduction.* Am J Physiol Cell Physiol; 293: C661-C669.
694. Mezzano V, Cabrera D, Vial C, Brandan E (2007), *Constitutively activated dystrophic muscle fibroblasts show a paradoxical response to TGF-beta and CTGF/CCN2.* J Cell Commun Signal; 1: 205-217.
695. Desguerre I, Mayer M, Leturcq F, Barbet JP, Gherardi RK, Christov C (2009), *Endomysial fibrosis in Duchenne muscular dystrophy: a marker of poor outcome associated with macrophage alternative activation.* J Neuropathol Exp Neurol; 68: 762-773.
696. Chen YW, Nagaraju K, Bakay M, McIntyre O, Rawat R, Shi R *et al.* (2005), *Early onset of inflammation and later involvement of TGFbeta in Duchenne muscular dystrophy.* Neurology; 65: 826-834.
697. Aartsma-Rus A, van Ommen GJ (2007), *Antisense-mediated exon skipping: a versatile tool with therapeutic and research applications.* RNA; 13: 1609-1624.
698. Payne ET, Yasuda N, Bourgeois JM, Devries MC, Rodriguez MC, Yousuf J *et al.* (2006), *Nutritional therapy improves function and complements corticosteroid intervention in mdx mice.* Muscle Nerve; 33: 66-77.
699. Anderson LV, Davison K (1999), *Multiplex Western blotting system for the analysis of muscular dystrophy proteins.* Am J Pathol; 154: 1017-1022.
700. Ruijter JM, Ramakers C, Hoogaars WM, Karlen Y, Bakker O, van den Hoff MJ *et al.* (2009), *Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data.* Nucleic Acids Res; 37: e45.
701. Mendell JR, Moxley RT, Griggs RC, Brooke MH, Fenichel GM, Miller JP *et al.* (1989), *Randomized, double-blind six-month trial of prednisone in Duchenne's muscular dystrophy.* N Engl J Med; 320: 1592-1597.
702. Tanabe Y, Esaki K, Nomura T (1986), *Skeletal muscle pathology in X chromosome-linked muscular dystrophy (mdx) mouse.* Acta Neuropathol; 69: 91-95.
703. Doran P, Wilton SD, Fletcher S, Ohlendieck K (2009), *Proteomic profiling of antisense-induced exon skipping reveals reversal of pathobiochemical abnormalities in dystrophic mdx diaphragm.* Proteomics; 9: 671-685.
704. Yao W, Cheng Z, Pham A, Busse C, Zimmermann EA, Ritchie RO *et al.* (2008), *Glucocorticoid-induced bone loss in mice can be reversed by the actions of parathyroid hormone and risedronate on different pathways for bone formation and mineralization.* Arthritis Rheum; 58: 3485-3497.
705. Keeling RM, Golumbek PT, Streif EM, Connolly AM (2007), *Weekly oral prednisolone improves survival and strength in male mdx mice.* Muscle Nerve; 35: 43-48.
706. 't Hoen PA, van der Wees CG, Aartsma-Rus A, Turk R, Goyenvalle A, Danos O *et al.* (2006), *Gene expression profiling to monitor therapeutic and adverse effects of antisense therapies for Duchenne muscular dystrophy.* Pharmacogenomics; 7: 281-297.
707. Spurney CF, Knoblach S, Pistilli EE, Nagaraju K, Martin GR, Hoffman EP (2008), *Dystrophin-deficient cardiomyopathy in mouse: expression of Nox4 and Lox are associated with fibrosis and altered functional*

*parameters in the heart.* Neuromuscul Disord; 18: 371-381.
708. Jin Y, Murakami N, Saito Y, Goto Y, Koishi K, Nonaka I (2000), *Expression of MyoD and myogenin in dystrophic mice, mdx and dy, during regeneration.* Acta Neuropathol; 99: 619-627.
709. te Pas MF, de Jong PR, Verburg FJ (2000), *Glucocorticoid inhibition of C2C12 proliferation rate and differentiation capacity in relation to mRNA levels of the MRF gene family.* Mol Biol Rep; 27: 87-98.
710. van Putten M, Aartsma-Rus A (2011), *Opportunities and challenges for the development of antisense treatment in neuromuscular disorders.* Expert Opin Biol Ther; 11: 1025-1037.
711. Almon RR, Dubois DC, Jusko WJ (2007), *A microarray analysis of the temporal response of liver to methylprednisolone: a comparative analysis of two dosing regimens.* Endocrinology; 148: 2209-2225.
712. Marotta M, Sarria Y, Ruiz-Roig C, Munell F, Roig-Quilis M (2007), *Laser microdissection-based expression analysis of key genes involved in muscle regeneration in mdx mice.* Neuromuscul Disord; 17: 707-718.
713. Cacchiarelli D, Incitti T, Martone J, Cesana M, Cazzella V, Santini T *et al.* (2011), *miR-31 modulates dystrophin expression: new implications for Duchenne muscular dystrophy therapy.* EMBO Rep; 12: 136-141.
714. Paul M, Poyan MA, Kreutz R (2006), *Physiology of local renin-angiotensin systems.* Physiol Rev; 86: 747-803.
715. Zhou L, Porter JD, Cheng G, Gong B, Hatala DA, Merriam AP *et al.* (2006), *Temporal and spatial mRNA expression patterns of TGF-beta1, 2, 3 and TbetaRI, II, III in skeletal muscles of mdx mice.* Neuromuscul Disord; 16: 32-38.
716. Bernasconi P, Torchiana E, Confalonieri P, Brugnoni R, Barresi R, Mora M *et al.* (1995), *Expression of transforming growth factor-beta 1 in dystrophic patient muscles correlates with fibrosis. Pathogenetic role of a fibrogenic cytokine.* J Clin Invest; 96: 1137-1144.
717. van Putten M, de Winter C, van Roon-Mom W, van Ommen GJ, 't Hoen PA, Aartsma-Rus A (2010), *A 3 months mild functional test regime does not affect disease parameters in young mdx mice.* Neuromuscul Disord; 20: 273-280.

Summary
Samenvatting voor niet-ingewijden
List of abbreviations
Curriculum Vitae
List of publications
Dankwoord

Appendix

## Summary

Duchenne muscular dystrophy (DMD) is a severe, progressive neuromuscular disorder, nowadays affecting around 1 in 5 000 newborn boys worldwide. First symptoms of muscle weakness appear around the age of two or three years, reflected by difficulties with running, standing upright and climbing stairs. Progressive loss of muscle function leads to wheelchair-dependency around the age of ten to fifteen. Involvement of respiratory and cardiac muscles will lead to premature death, often before the age of thirty. Becker muscular dystrophy (BMD) is a related disease, but symptoms are often much milder and life expectancies are nearly normal.

DMD is caused by the absence of the dystrophin protein, encoded by the *DMD* gene. Dystrophin forms a bridge between the actin cytoskeleton inside the muscle fibres and the extracellular matrix surrounding the fibres. Hereby it provides mechanical stability to the muscle fibres during contractions. In the absence of dystrophin, fibres easily get damaged during use. Normally, muscles are capable of repairing this damage. When this becomes chronic, the repair system gets exhausted and eventually muscle tissue will be replaced by fat and fibrotic tissue, leading to a loss of muscle function.

Three base pairs of a gene encode one amino acid of a protein (genetic code). Translation occurs by first transcribing the DNA-code in an RNA-code and thereafter translating the RNA into protein. Translation starts at the 'start signal' and ends at the 'stop signal' of the transcript. The genetic code is dispersed over exons, divided by introns (non-coding parts), which are removed at RNA-level during splicing. In DMD patients mutations in the *DMD* gene cause a disruption of the genetic code, *i.e.* by insertion or deletion of (an) exon(s) consisting of a number of base pairs not divisible by three, or by introduction of a premature stop signal. This leads to premature termination of protein synthesis and complete absence of a functional protein, since one of the anchoring parts is missing. BMD is also caused by mutations in the *DMD* gene, but the reading frame stays intact, *i.e.* the number of base pairs inserted or deleted is divisible by three. This allows the production of a largely functional protein, containing both anchoring parts, which only is shorter or longer in the middle.

At the moment no treatments addressing the underlying genetic cause are available for DMD. A limited number of therapies targets one or a few of the symptoms, delaying disease progression, but not preventing it. Corticosteroid treatment has shown to be effective and ACE-inhibitors can delay the onset of cardiomyopathy. Many more compounds are tested pre-clinically, some showing positive effects on some of the mechanisms deregulated by the disease.

Antisense oligonucleotide (AON)-mediated exon skipping aims to restore the disrupted open reading frame in DMD patients, by removing ('skipping') an exon, thereby allowing the production of shorter, but largely functional Becker-like dystrophin proteins. The exon is hidden during splicing by covering it with an AON specific for the targeted exon. Proof-of-principle for this approach has been shown in cell cultures and animal models for DMD and is currently tested in clinical trials.

The efficiency of AON-mediated exon skipping relies on several aspects. First of all the presence of the target tissue. Dystrophin is only expressed by muscle tissue and not by adipose and fibrotic tissue, which replaces the muscle tissue during disease progression. Better preservation and improvement of quality of muscle tissue would increase the amount of target for AON-mediated exon skipping. Secondly it depends on the amount of AON that

reaches this target. One way to influence this is optimisation of dosing regimen. In this thesis both aspects have been studied in (DMD) cell cultures and the *mdx* mouse model (a mouse model with a mutation in the *DMD* gene leading to the absence of a functional dystrophin protein).

In **chapter 3** different dosage treatment regimens were compared in *mdx* mice. This revealed that if the same total amount of AON compound is given in multiple smaller portions, it leads to more uptake of the compound by the muscles, where it is needed for therapeutic effects. This in turn led to higher exon skipping percentages and restoration of dystrophin production. On the other hand, a higher uptake of AONs by other organs such as the liver and the kidney was also observed.

AONs do not lead to a permanent change in exon skipping or dystrophin restoration. Due to turn-over of the compound, chronic treatment will be required. Comparison of different maintenance schemes, after initial treatment, showed that more frequent repetition of treatment led to higher maintenance of the effects in muscle, but again also increased the AON level in the other organs (**chapter 3**). To search for a balance between these effects, the turnover of effects at AON, RNA and protein levels were studied in more detailed (**chapter 4**). This showed, unfortunately, that for AONs this was comparable between muscle and other organs. However the dystrophin protein was still detectable six months after treatment, long after the exon skipping or AONs could be detected. These results indicate that the use of an intermittent dosing schedule might be useful to maintain treatment effects, but reduce side effects.

Small molecules could be used to enhance exon skipping efficiency. We tested one of these compounds (called 6TG), described by others to enhance skipping efficiency (**chapter 5**). However, we discovered that, although it indeed enhanced exon skipping mainly in DMD cell cultures, also various other skipping events were observed next to the skipping of the targeted exon. This increases the chance of off-target effects and therefore we do not consider 6TG as a suitable candidate for further use.

As mentioned, dystrophin is only expressed by muscle tissue and not by adipose and fibrotic tissue. Therefore less targets remain when disease progresses. Improving muscle quality with pharmaceutical compounds is another way to enhance treatment outcomes. Nowadays most patients are already treated with corticosteroids. Prednisolone (the most used corticosteroid) did not negatively influence the AON treatment itself or its functional outcomes in *mdx* mice (**chapter 6**). For prednisolone this is important to know, since patients in clinical trials are often treated with corticosteroids.

While searching for muscle quality improving compounds, losartan was tested (**chapter 7**), as literature described it as a promising candidate. However, no positive effects on muscle quality of losartan itself or on AON treatment were observed. Furthermore, during experiments more literature became available doubting its positive effects. Therefore these experiments were stopped. Unfortunately no other candidate could be found that enhanced therapeutic outcomes by dual treatment with AONs.

## Samenvatting voor niet-ingewijden

Duchenne spierdystrofie (DMD) is een ernstige, X-gebonden spierziekte, die voorkomt bij ongeveer 1 op de 5 000 pasgeboren jongetjes. De ziekte wordt gekenmerkt door progressieve spierzwakte. Bij deze patiënten worden de eerste symptomen zichtbaar rond hun tweede of derde levensjaar: ze hebben moeite met rennen, opstaan en traplopen. Toename van spierzwakte zorgt ervoor dat ze meestal tussen hun tiende en vijftiende in een rolstoel belanden en uiteindelijk door ademhalings- en tegenwoordig met name hartproblemen sterven voor het dertigste levensjaar. Becker spierdystrofie (BMD) is een verwante ziekte. In BMD zijn de symptomen echter over het algemeen een stuk milder en de levensverwachting is vrijwel normaal.

Duchenne wordt veroorzaakt door mutaties in het *DMD*-gen dat codeert voor het dystrofine eiwit. Dit eiwit vormt een brug tussen het actine cytoskelet in spiervezels en de extracellulaire matrix rondom de spiervezels, noodzakelijk voor de stabiliteit. Door de afwezigheid van dystrofine raken de spiervezels gemakkelijk beschadigd tijdens bewegingen. Normaal gesproken kunnen de spieren deze schade herstellen, maar als de beschadiging chronisch wordt, raakt het herstelsysteem uitgeput en wordt steeds meer spierweefsel vervangen door bind- en vetweefsel. Dit leidt uiteindelijk tot een verlies van spierfunctie.

Genen (DNA) bevatten de genetische code voor eiwitten. Hierbij coderen drie baseparen in een gen voor één aminozuur van een eiwit. Vertaling van de genetische code vindt plaats door eerst de DNA-code over te schrijven naar een RNA-code en vervolgens deze te vertalen in een eiwit. De vertaling begint bij een 'start signaal' en eindigt bij een 'stop signaal' van een transcript. De genetische code is verdeeld over exonen, waartussen zich intronen (niet-coderende delen) bevinden. Deze intronen worden er op RNA-niveau uitgeknipt tijdens een proces genaamd 'splicing'. Bij Duchenne patiënten zorgen mutaties in het *DMD*-gen voor verstoringen in de genetische code; bijvoorbeeld door de insertie of deletie van één of meerdere exonen waarvan het aantal baseparen niet deelbaar is door drie, of door de introductie van een vroegtijdig stop signaal. Dit zorgt ervoor dat de aanmaak van eiwit vroegtijdig gestopt wordt. Een functioneel eiwit ontbreekt dan, aangezien het één van de verbindingsdelen mist. Becker wordt ook veroorzaakt door mutaties in het *DMD*-gen. In dit geval blijft echter het leesraam behouden, d.w.z. het aantal toegevoegde of verwijderde baseparen is deelbaar door drie. Hierdoor kan een grotendeels functioneel eiwit gevormd worden dat beide verbindingsdelen bevat en alleen in het midden een stukje korter of langer is.

Op dit moment zijn er voor Duchenne geen therapieën beschikbaar die de onderliggende genetische oorzaak aanpakken. Er bestaan slechts een klein aantal therapieën gericht op één of een paar symptomen van de ziekte. Deze kunnen hooguit de progressie van de ziekte vertragen, maar niet voorkomen. Corticosteroïden zijn één van de weinige middelen die bewezen hebben effectief te zijn (hierdoor belanden patiënten gemiddeld drie jaar later in een rolstoel) Deze worden dan ook door de meeste patiënten gebruikt. Verder kunnen ACE-inhibitoren de eerste symptomen van hartfalen vertragen. Verder worden momenteel veel middelen preklinisch getest, waarvan enkele positieve effecten laten zien op sommige mechanismen die door de ziekte verstoord zijn.

Exon skippen door middel van antisense oligonucleotides (AONs) probeert het verstoorde leesraam te herstellen door een extra exon te verwijderen ('skippen'), waardoor een korter, maar grotendeels functioneel Becker-achtig dystrofine eiwit gevormd kan worden. Het wordt gedaan met behulp van AONs die binden aan een specifiek exon waardoor dit

tijdens het splicen niet meer herkend wordt als exon. Het eerste bewijs dat dit daadwerkelijk mogelijk was, is aangetoond in celkweken en diermodellen voor Duchenne. De aanpak wordt momenteel getest in klinische trials.

Verschillende aspecten beïnvloeden de effectiviteit van het exon skippen m.b.v. AONs. Allereerst de aanwezigheid van spierweefsel waarin de skip plaats moet vinden. Dystrofine wordt namelijk alleen tot expressie gebracht in spierweefsel en niet in het bind- en vetweefsel, dat het spierweefsel in latere stadia van de ziekte vervangt. Als het spierweefsel beter behouden zou blijven en van betere kwaliteit zou zijn, zou er meer beschikbaar zijn voor de AONs. Een andere manier om de effectiviteit te verbeteren, is door het verhogen van de hoeveelheid AON die daadwerkelijk in de spiercellen terecht komt. Dit kan onder andere beïnvloed worden door de toediening te optimaliseren. In dit proefschrift is naar beide aspecten onderzoek gedaan in (patiënten) cellen en in de *mdx* muis (een muismodel met een mutatie in het *DMD*-gen waardoor geen functioneel dystrofine eiwit aanwezig is). Er is onderzocht of het kon leiden tot een verhoogde effectiviteit van de AON therapie.

In **hoofdstuk 3** zijn verschillende doseringsschema's vergeleken in de *mdx* muis. Dit liet zien dat als dezelfde totale hoeveelheid AON toegediend werd in meerdere, kleinere porties, meer AON opgenomen werd door de spieren (de plek waar het nodig is voor de therapeutische effecten). Dit leidde tot hogere exon skip percentages en meer herstel van dystrofine productie. Het leidde echter ook tot een hoger opname van AONs door andere organen zoals de lever en de nieren.

Het exon skippen en het herstel van dystrofine eiwit productie door AONs is niet permanent. Omdat de AONs afgebroken en/of uitgescheiden worden door het lichaam, zal chronische behandeling nodig zijn. Vergelijking van verschillende onderhoudsdoseringen na initiële behandeling, liet zien dat vaker herhalen van de injecties ervoor zorgde dat de effecten in de spier beter behouden bleven, maar dat eveneens dit leidde tot hogere AON niveaus in andere organen (**hoofdstuk 3**). Om hier een evenwicht tussen te vinden, is het verloop van de effecten op AON, RNA en eiwit niveau in meer detail bestudeerd in **hoofdstuk 4**. Hieruit bleek, helaas, dat het verloop van de niveaus van de AONs vergelijkbaar was voor spieren en andere organen. Dystrofine eiwit was echter zes maanden na de behandeling nog steeds detecteerbaar. Dit is lang nadat exon skippen en AONs gedetecteerd konden worden. Deze resultaten geven aan dat een doseringsschema met onderbrekingen mogelijk gebruik zou kunnen worden om de therapeutische effecten te behouden, maar de bijwerkingen te verminderen.

Kleine moleculen kunnen gebruikt worden om de effectiviteit van het exon skippen te verhogen. Wij hebben één van deze moleculen (6TG genaamd) getest. In de literatuur is beschreven dat dit de exon skip effectiviteit verhoogt (**hoofdstuk 5**). Uit ons onderzoek, dat hoofdzakelijk in celkweken werd uitgevoerd, bleek echter dat, hoewel de exon skip percentages inderdaad hoger werden, ook verschillende andere exonen onbedoeld geskipt werden. Dit verhoogt de kans op bijwerkingen en daardoor is 6TG naar onze mening geen goede kandidaat voor verdere experimenten.

Zoals eerder genoemd wordt dystrofine alleen tot expressie gebracht in spierweefsel en niet in bind- en vetweefsel, waardoor minder aangrijpingspunten voor de therapie overblijven naarmate de ziekte vordert. Daarom is het verbeteren van de spierkwaliteit met farmaceutica een andere manier om therapeutische effecten te verhogen. Tegenwoordig worden de meeste

patiënten behandeld met corticosteroïden, voornamelijk prednisolone. Prednisolone had geen negatieve effecten op de AON behandeling of de functionele effecten daarvan in *mdx* muizen (**hoofdstuk 6**). Dit is van belang voor klinische trials, aangezien de meeste patiënten die daaraan deel nemen, behandeld zullen worden met corticosteroïden.

Van de verschillende middelen die volgens beschrijvingen in de literatuur de spierkwaliteit verhogen, was losartan één van de meest belovende. Daarom hebben wij losartan ook getest, alleen en in combinatie met AONs (**hoofdstuk 7**). Hierbij werden echter geen positieve effecten van losartan op de spierkwaliteit of op de effectiviteit van de AONs gezien. Daar kwam bij dat tijdens de experimenten, meer literatuur naar buiten kwam die de eerder beschreven resultaten in twijfel trokken. Dit heeft ons doen besluiten de experimenten te stoppen. Helaas hebben we tot op heden geen andere kandidaat kunnen vinden die tot verbeterde therapeutische effecten leidde bij combinatiebehandeling met AONs.

## List of Abbreviations

| | |
|---|---|
| 2OMe | 2'-*O*-methyl |
| 2OMOE | 2'-*O*-(2-methoxy)ethyl |
| 6TG | 6-thioguanine |
| AAS | Anabolic androgenic steroids |
| AAV | Adeno-associated virus |
| ABD | Actin-binding domain |
| ACE | Angiotensin-converting enzyme |
| Acvr | Activin receptor |
| AMPK | AMP-activated protein kinase |
| ANP | Atrial natriuretic peptide |
| AON | Antisense oligonucleotide |
| ASC | Adipose-derived stromal cell |
| AT | Angiotensin II receptor |
| BMD | Becker muscular dystrophy |
| BMP | Bone morphogenic protein |
| BNP | Brain natriuretic peptide |
| CK | Creatine kinase |
| CMV | Cytomegalo virus |
| CNS | Central nervous system |
| CXMD | Canine X-linked muscular dystrophy |
| DGC | Dystrophin-associated glycoprotein complex |
| DMD | Duchenne muscular dystrophy |
| ECM | Extracellular matrix |
| EGCG | Epigallocatechin gallate |
| EIS | Exon inclusion sequence |
| ENA | Ethylene bridged nucleic acid |
| ESC | Embryonic stem cell |
| ESE | Exonic splicing enhancer |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GRMD | Golden retriever muscular dystrophy |
| HDAC | Histone deacetylase |
| HFMD | Hypertrophic feline muscular dystrophy |
| IGF | Insulin-like growth factor |
| IFN | Interferon |
| IKK | IκB kinase |
| Lgals3 | Lectin, galactoside binding, soluble, 3 |
| LGMD | Limb girdle muscular dystrophy |
| LNA | Locked nucleic acid |
| LVEF | Left ventricular ejection fraction |
| MDSC | Muscle-derived stem cell |
| MHC | Major histocompatibility complex |
| MMP | Matrix metalloproteinase |
| MNF | Myocyte nuclear factor |
| MRI | Magnetic resonance imaging |
| MSP | Muscle-targeting heptapeptide |

| | |
|---|---|
| NBD | NEMO-binding domain |
| NF-AT | Nuclear transcription factor of activated T-cell |
| NMD | Nonsense-mediated decay |
| nNOS | Neuronal nitric oxide synthase |
| NP | Nanoparticle |
| NSAID | Non-steroidal anti-inflammatory drug |
| PD | Pharmacodynamic |
| PDE | Phosphodiesterase |
| PDGF | Platelet-derived growth factor |
| PDTC | Pyrrolidine dithiocarbamate |
| Pip | PMO or PNA internalisation peptide |
| PK | Pharmacokinetic |
| PMMA | Polymethylmethacrylate |
| PMO | Phosphorodiamidate morpholino oligomer |
| PNA | Peptide nucleic acid |
| PO | Phosphodiester |
| pPMO | Peptide-conjugated phosphorodiamidate morpholino oligomer |
| PS | Phosphorothioate |
| PTM | Pre-*trans*-splicing molecule |
| PV | Parvalbumin |
| RA(A)S | Renin-anigiotensin(-aldosterone) system |
| ROS | Reactive oxygen species |
| RyR | Ryanodine receptor |
| SACNSC | Stretch-activated channels for non-specific cations |
| snRNP | Small nuclear ribonucleoprotein |
| SOCE | Store-operated calcium entry |
| SR | Serine-and arginine-rich |
| TALEN | Transcription activator-like effector nuclease |
| TGF | Tranforming growth factor |
| TNF | Tumour necrosis factor |
| Utrn | Utrophin |
| XCI | X-chromosome inactivation |

## Curriculum Vitae

Ingrid Verhaart was born in Eindhoven on the 19th of March 1986. After finishing gymnasium at the Christiaan Huygens College in Eindhoven, she moved to Leiden in 2004 to study Biomedical Sciences. During her bachelors, she participated in an exchange program with the Karolinska Institute in Stockholm, Sweden. She did her internship in the group of Prof. dr. Ferry Ossendorp at the department of Immunohematology and Blood Transfusion. The subject was "The use of antigen-antibody immune complexes for CTL mediated immunotherapy of melanoma" and was supervised by Nadine van Montfoort. Thereafter, in 2007, she continued with her masters Biomedical Sciences in Leiden. Her first internship, entitled "Study of the effects of the flavonoid silibinin on glucose uptake in insulin signalling in muscle cells", was conducted at the department of Molecular Cell Biology, guided by Dr. Bruno Guigas. She did her final internship and wrote her master's thesis at the department of Human Genetics under supervision of Hans Heemskerk in the group of Dr. Annemieke Aartsma-Rus. Here she studied the effect of combining prednisolone and antisense oligonucleotide-mediated exon skipping for Duchenne muscular dystrophy in the *mdx* mouse. After obtaining her master's degree, she continued this research in the same group from September 2009 until 2014 as a PhD student of Prof. dr. Gertjan van Ommen. This resulted in this thesis "Optimising antisense oligonucleotide-mediated exon skipping for Duchenne muscular dystrophy".

## List of Publications


1. Verhaart IEC, van Vliet-van den Dool L, Sipkens JA, de Kimpe SJ, Kolfschoten IG, van Deutekom JC, Liefaard L, Ridings JE, Hood SR and Aartsma-Rus A, *The dynamics of compound, transcript, and protein effects after treatment with 2OMePS antisense oligonucleotides in mdx mice.* Mol Ther Nucleic Acids 2014; 3:e148.
2. Verhaart IEC, van Vliet-van den Dool L, Sipkens JA,de Kimpe SJ, Kolfschoten IG, van Deutekom JC, Liefaard L, Ridings JE, Hood SR, Aartsma-Rus A (2014), *The dynamics of 2OMePS antisense oligonucleotides, transcripts, and protein effects in mdx mice.* Mol Ther Nucleic Acids; 3: e148.
3. Verhaart IEC, Tanganyika-de Winter CL, Karnaoukh TG, Kolfschoten IG, de Kimpe SJ, van Deutekom JC, Aartsma-Rus A (2013), *Dose-dependent pharmacokinetic profiles of 2'-O-methyl phosphorothioate antisense oligonucleotides in mdx mice.* Nucleic Acid Ther; 23: 228-237.
4. Verhaart IEC, Aartsma-Rus A (2012), *The effect of 6-thioguanine on alternative splicing and antisense-mediated exon skipping treatment for duchenne muscular dystrophy.* PLoS Curr MD; 4.
5. Verhaart IEC, Aartsma-Rus A (2012), *Gene therapy for Duchenne muscular dystrophy.* Curr Opin Neurol; 25: 588-596.
6. Verhaart IEC, Aartsma-Rus A (2012), *AON-mediated exon skipping for Duchenne muscular dystrophy.* In: Zaher A, editor, Neuromuscular Disorders. ISBN 978-953-51-0696-8, InTech.
7. Verhaart IEC, van Duijn RJ, den Adel B, Roest AA, Verschuuren JJ, Aartsma-Rus A , van der Weerd L (2012), *Assessment of cardiac function in three mouse dystrophinopathies by magnetic resonance imaging.* Neuromuscul Disord; 22: 418-426.
8. Verhaart IEC, Heemskerk H, Karnaoukh TG, Kolfschoten IG, Vroon A, van Ommen GJ, van Deutekom JC, Aartsma-Rus A (2012), *Prednisolone treatment does not interfere with 2'-O-methyl phosphorothioate antisense-mediated exon skipping in Duchenne muscular dystrophy.* Hum Gene Ther; 23: 262-273.
9. Verhaart IEC, Aartsma-Rus A (2011), *Therapeutic approaches in development for Duchenne muscular dystrophy.* Journal for Neurology and Psychiatry of Child and Adolescent in Romania; 4.
10. van Putten M, van der Pijl EM, Hulsker M, Verhaart IEC, Nadarajah VD, van der Weerd L, Aartsma-Rus A (2014), *Low dystrophin levels in heart can delay heart failure in mdx mice.* J Mol Cell Cardiol.
11. Spitali P, van den Bergen JC, Verhaart IEC, Wokke B, Janson AA, van den Eijnde R, den Dunnen JT, Laros JF, Verschuuren JJ, 't Hoen PA, Aartsma-Rus A (2013), *DMD transcript imbalance determines dystrophin levels.* FASEB J; 27: 4909-4916.
12. de Brouwer AP, Nabuurs SB, Verhaart IEC, Oudakker AR, Hordijk R, Yntema HG, Hordijk-Hos JM, Voesenek K, de Vries BB, van Essen T, Chen W, Hu H, Chelly J, den Dunnen JT, Kalscheuer VM, Aartsma-Rus AM, Hamel BC, van Bokhoven H, Kleefstra T (2013), *A 3-base pair deletion, c.9711_9713del, in DMD results in intellectual disability without muscular dystrophy.* Eur J Hum Genet.
13. van Montfoort N, Mangsbo SM, Camps MG, van Maren WW, Verhaart IEC, Waisman A, Drijfhout JW, Melief CJ, Verbeek JS, Ossendorp F (2012), *Circulating specific antibodies enhance systemic cross-priming by delivery of complexed antigen to dendritic cells in vivo.* Eur J Immunol; 42: 598-606.

# Dankwoord

Uiteraard wil ik hier op de laatste, maar zeker niet onbelangrijke, bladzijde van mijn proefschrift iedereen bedanken die mij tijdens mijn promotieperiode praktisch geholpen heeft dan wel op een andere manier ondersteund heeft.

Allereerst mijn promotor Gertjan van Ommen en copromotor Annemieke Aartsma-Rus voor het mogelijk maken van mijn promotie en ook voor de inhoudelijke ondersteuning, goede suggesties en feedback.

Daarnaast iedereen van het lab die een bijdrage aan mijn verschillende projecten en artikelen geleverd heeft. Hans, met wie ik tijdens mijn stage en het begin van mijn promotie samengewerkt heb aan de prednisolone-experimenten. Christa, voor de hulp bij de muizenproeven en Laura bij de western blots. Maaike, voor alle hulp met de kleuringen en de samenwerking tijdens de MRI-studies. Dwi, for all your help with the TGF-β related experiments and the western blots, especially those for pSmad2. Pietro, for working together on the western blots for the wild type mice. Maarten, voor de statistische ondersteuning. Melvin, voor de tips over Illustrator en InDesign, zeker belangrijk voor de totstandkoming van het boekje. Verder, mijn kamergenoot van de eerste jaren: Ivo, voor alle keren dat er weer een computerprobleem opgelost moest worden. Furthermore, all the other members of the exon skip group, labJ and the Human Genetics department, who I have not mentioned and who have helped me during the past years.

Bovendien wil ik ook mijn familie en vrienden bedanken die me naast mijn werk altijd gesteund hebben.