Developing tissue specific antisense oligonucleotide-delivery to refine treatment for Duchenne muscular dystrophy

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Chapter 1

General introduction

1. Introduction

1.1.1 Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is a severe and progressive X-linked, muscle-wasting disorder affecting 1 in 5,000 newborn boys (1,2). DMD is caused by mutations in de *DMD* gene, leading to a truncated, non-functional dystrophin protein (figure 1). Dystrophin is an important muscle protein since it provides stability to the muscle fibers upon contraction (3). Without a functional dystrophin protein, muscle fibers are easily damaged during exercise and chronically damaged muscle fibers are eventually replaced by fibrotic and adipose tissues, resulting in a loss of muscle function (4). DMD has an early onset in childhood and patients are in general diagnosed before the age of five. Early symptoms are a delay in time at which children start to stand and walk, hypertrophy of the calf muscles, the need to use their hands to support their legs while rising from the floor (Gower's sign) and difficulties with running and stair climbing. For some patients a delay in cognitive development is seen. Overall the mean IQ is approximately one standard deviation below the mean and about 20-30% of the patients has an IQ of less than 70 (5,6). In general, DMD patients become wheel-chair dependent at around the age of 12 years, need assisted ventilation at around 20 years of age, first only at night and later throughout the day as well. Cardiomyopathy manifests around the age of 10 and is prevalent in most patients of 20 years of age (7). The life expectancy of DMD patients is, nowadays, around 30 years in the Western world, where respiratory and cardiac failure is the main cause of death for these patients (8).

1.1.2 Becker muscular dystrophy

In contrast to DMD, which is characterized by the presence of non-functional dystrophin proteins, Becker muscular dystrophy (BMD) is a muscle wasting disorder caused by mutations in the *DMD* gene that result in the production of in internally deleted, but partially functional dystrophin protein (figure 1). Symptoms in patients with BMD are generally milder, however phenotypical variation from mild to moderately severe is seen and within families asymptomatic patients have been identified (5,9). The age of onset of symptoms ranges from ~12 years to late midlife. Patients become wheelchair dependent 20-30 years after diagnosis, however, some patients remain ambulant throughout their life. The life expectancy for the severely affected patients is around 40-50 years of age whereas mildly affected patients have near normal life expectancies.

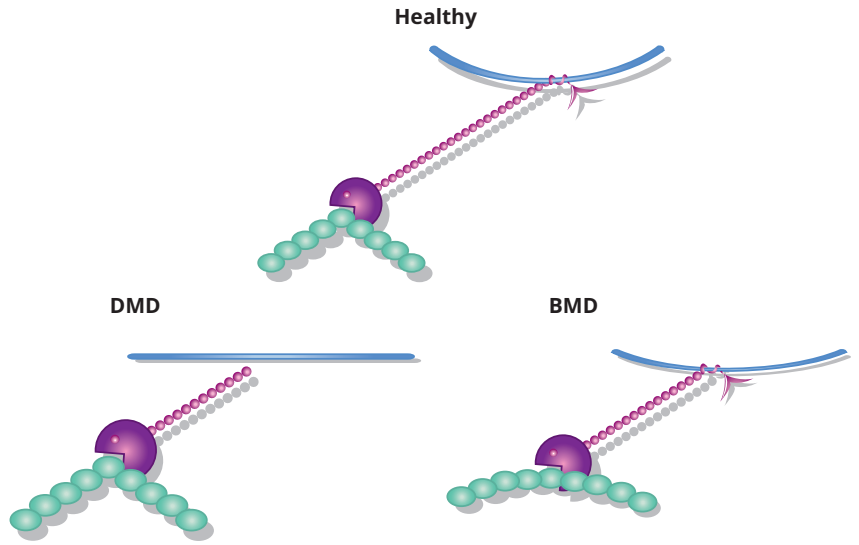

Figure 1. Illustration of dystrophin in healthy individuals versus DMD and BMD patients. Dystrophin is an important muscle protein. It provides stability to the muscle fibers upon contraction by connecting the internal cytoskeleton to the extracellular matrix. In healthy individuals the dystrophin protein is fully functional, however absent in DMD patients and impaired (due to a shorter dystrophin protein) in BMD patients.

1.2 *DMD* gene and protein

The *DMD* gene is the largest know gene of the human genome. It is located on the short arm of the X-chromosome at position 21.2 (Xp21.2) and consists of 2,241,764 base pairs (bp) (www.ensembl.org). The coding region for the full length protein is dispersed over 79 exons, transcribed into a 13,956 bp mRNA and coding for a full-length dystrophin protein of 3,658 amino acids with a molecular weight of 427 kDa (4,10,11). In DMD patients, mutations in the *DMD* gene cause a disruption of the reading frame (if the size of the deletion or insertion in base pairs is not divisible by three) or a premature stop codon. These mutations result in premature truncation of translation and an unstable protein leading to an absence of the dystrophin protein. Mutations can occur throughout the gene, but the region of exon 45-55 is known as a major hotspot for deletions and the region of exon 2-10 as minor hotspot for duplications (12,13).

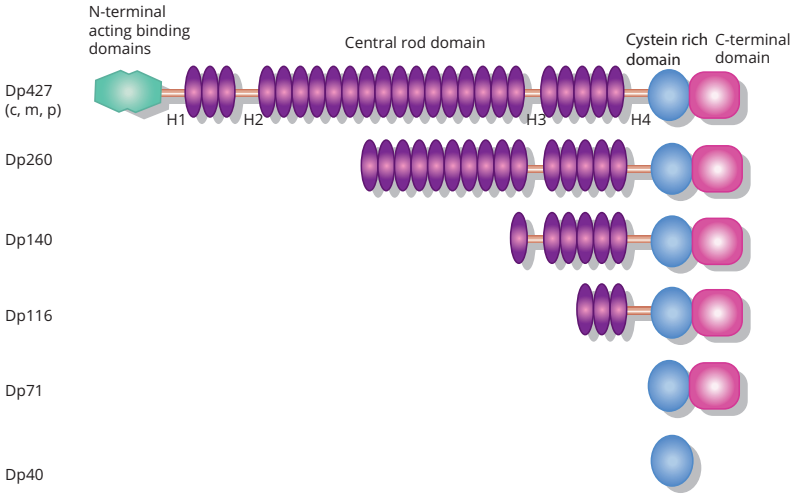

Figure 2. Illustration of dystrophin and its isoforms. Dystrophin contains four domains: N-terminal binding site (green), central rod domain (purple), cysteine rich domain (blue) and the C-terminal binding site (pink). Dystrophin has three full length isoforms regulated by three different promoters and each having unique first exons: Dp427m (skeletal and cardiac muscle), Dp427c (cortical neurons and hippocampus), Dp427p (cerebellar Purkinje cells). There are five shorter dystrophin proteins regulated by internal promoters: Dp260 (retina), Dp140 (central nervous system and kidney), Dp116 (Schwann cells), Dp71 and Dp40 (ubiquitous expressed but not in muscle).

The full length dystrophin protein contains two N-terminal actin-binding domains (encoded by exon 2-8) followed by a central rod domain (encoded by exon 9-63), a cysteine rich domain (encoded by exon 64-70) and the C-terminal domain (encoded by exon 71-79) (figure 2) (11,14,15). Within muscle, dystrophin connects the subsarcolemmal cytoskeleton to the extracellular matrix. With the N-terminal actin-binding domains it connects to the F-Actin in the cytoskeleton. A bridge is formed by binding of the C-terminal cysteine-rich binding domain to the β-dystroglycan protein of the dystrophin glycoprotein complex (DGC complex), which is in turn connected to the extracellular matrix protein laminin (figure 3). Within the muscle the dystrophin connection to the DGC complex provides stability to the muscle fibers upon contraction (3,16,17). Consequently, without a functional dystrophin protein, muscle fibers become vulnerable to exercise-induced damage. Chronically damaged muscle fibers lead to chronic inflammation and eventually these fibers are replaced by non-functional fibrotic and adipose tissues, resulting in muscle loss as seen in DMD patients.

In BMD patients, mutations do not lead to premature truncation of translation, but result in an internally deleted (shorter) but partly functional

dystrophin protein (figure 1). The connection between the subsarcolemmal cytoskeleton and the extracellular matrix is maintained, although with an altered connection and functionality, resulting in muscle fibers that are less vulnerable to exercise-induced damage.

Dystrophin is known to have eight isoforms, expressed from different promoters located throughout the gene (figure 2). Full-length dystrophin is regulated by three different promoters of the first exon. The full length Dp427m isoform is expressed in skeletal muscle and cardiomyocytes. Other two full-length isoforms are Dp427c, expressed in the brain (cortical neurons and hippocampus) and Dp427p expressed in cerebellar Purkinje cells. There are four internal promoters responsible for the expression of shorter proteins. Dp260 which is expressed in the retina. Dp140 which is expressed in the central nervous system and kidney. Dp116 which is expressed in Schwann cells and Dp71 and Dp40 (both expressed from the same promotor) are ubiquitous expressed but not in muscle tissue. DP71 is known to be highly expressed in brain compared to other tissues (3,18).

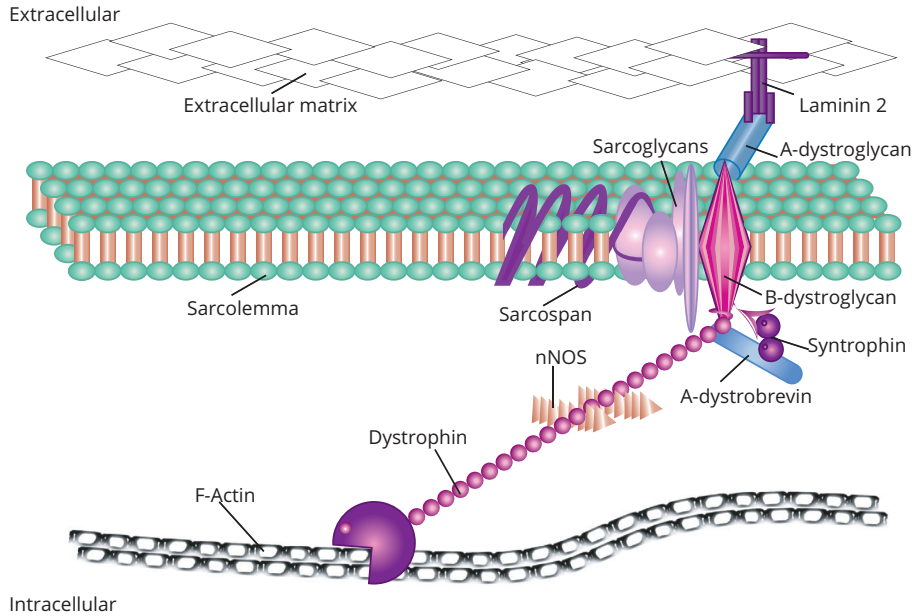

Figure 3. Location of dystrophin in muscle. Dystrophin binds with the N-terminal actin binding domains to F-actin and with the C-terminal cysteine rich binding domain to β-dystroglycan. A bridge is formed connecting the internal cytoskeleton to the extracellular matrix, providing stability upon muscle fiber contraction.

1.3 Therapeutic approaches

1.3.1 Multidisciplinary care

Currently there is no therapy for the majority of DMD patients. As part of the standard of care, patients receive symptomatic treatment (19,20). In general, corticosteroids (prednisone/prednisolone and deflazacort) are used and have shown, in various retrospective studies and clinical trials, to slow down disease progression (7,21-23). However, the use of steroids is associated with side effects like weight gain, loss of vertebral bone mass, growth inhibition and late onset of puberty.

In parallel, improved technology has led to the development of equipment like cough-assists and non-invasive mechanical ventilation devices and improved cardiac care. Furthermore, care standards involve multiple disciplines, e.g. neuromuscular, rehabilitation, orthopedic, pulmonary, cardiac, gastrointestinal and psychosocial management. Together this multidisciplinary approach has greatly improved the quality of life of Duchenne patients and nowadays resulted in a life expectancy into the fourth decade of life (8).

1.3.2 Dystrophin restoring therapeutic approaches

Various therapeutic approaches are currently under development aiming to restore the primary genetic defect i.e. absence of dystrophin protein. Other approaches aim to improve secondary pathology i.e. reduce fibrosis and inflammation, or improve muscle cell growth and strength. Focusing on dystrophin restoration, cell therapy as well as gene therapy based approaches are currently under development.

Cell therapy

Upon muscle damage, muscle stem cells (so called satellite cells or myoblast cells) are activated to repair the damaged muscle (24). By introducing healthy donor muscle stem cells with a functional copy of the *DMD* gene, one can in theory, gradually restore the whole muscle (25,26). This approach has been evaluated in human clinical trials with DMD patients in the early 1990s using myoblast cells. Disappointingly, no dystrophin restoration was observed (27-30). Failure of the trials was mainly caused by rapid cell death of the transplanted cells and their limited migration within the muscle upon intra muscular injections (myoblasts cannot cross blood vessel walls).

Currently, as an alternative to myoblasts, human pericyte-derived mesoangioblasts (MAB) are investigated. MABs are blood vessel-associated progenitor cells, which can be differentiated in various mesoderm cell types including muscle cells. They can cross blood vessel walls, which makes them applicable for systemic administration. Various preclinical animal studies with MABs have shown improvement of muscle pathology (31,32).

MABs from healthy HLA-identical siblings have been evaluated in a non-randomized open-label phase I-II clinical trial (EudraCT no2011-000176-33) (33). In total five DMD patients received four consecutive intra-arterial infusions of donor derived MABs in the limb arteries with a two month interval, under immunosuppressive therapy. Two months after the last infusion no dystrophin was observed in muscle biopsies nor were functional improvements seen by MRI. The authors concluded the study relatively safe. However one patient developed a thalamic stroke, and although the relation to the MAB infusions remained unclear, this is a serious safety concern.

When using donor cells, lifelong treatment with proper immune suppressants is needed to protect the donor cells from the recipients immune system, however these appear to be detrimental for the donor cells (34). To address this, alternatives are under development e.g. the use of $CD133^{+}$ donor derived cells or the use of genetically modified patient derived stem cells using human artificial chromosomes (35-37). The recently developed cluster regularly interspaced short palindromic repeat (CRISPR) associated nuclease nine (cas9) endonuclease system (CRISPR/Cas9) has shown promising results for genetic correction of patient derived stem cells as well. This system uses the cas9 nuclease to cleave the DNA at specific sequences targeted by guide RNAs (gRNA). Subsequently the loose ends are joined together by non-homologues end joining (NHEJ). For DMD, the CRISPR/Cas9 system is used to genetically correct the *DMD* gene by exon deletion. Using 2 gRNAs, designed to bind in the introns, one near the beginning and the other near the end of the exon of interest, Cas9 makes a cut at these sides. The lose-ends are then joined together by NHEJ (deleting the exon of interest), making the deletion larger but restoring the reading frame. In this way an internally deleted but party functional dystrophin protein is produced as seen in BMD (figure 4) (38-41). While minimal cellular toxicity is seen for CRISPR/Cas9 *in vitro*, off target effects (cutting elsewhere in the genome) do occur and this demands more research (42-45).

Although a lot of progress in developing cell therapy based approaches has been made in general, delivery and control of stem cells and their niche/microenvironment are remaining challenges today. For DMD body wide delivery to muscle is required but still not feasible. Little is known about the influence of fibrotic tissue and inflammation (present in de muscle of DMD patients) on the stem cell niche/microenvironment. In a healthy situation the cells present in this niche provide a specific microenvironment to maintain and promote cell differentiation of stem cells. Future research should focus on these challenges to make the approach successful.

Gene therapy

DNA based gene therapy

DNA based gene therapy aims to restore the expression of a gene from a patient by introducing specific DNA as treatment for the disease. An example for DMD is the introduction of the dystrophin gene in muscle cells, using adeno-associated virus derived delivery vectors (AAVs). AAVs are the only virus derived vectors that are capable of efficiently transducing muscle, however the cloning capacity of~4.5kb limits the use of the full *DMD* gene which has a coding sequence of around 11kb. Therefore mini- or micro-dystrophin genes are generated, mimicking a BMD like dystrophin protein (46-52). A different approach is the earlier described CRISPR/Cas9 system which, upon AAV delivery, can be used directly in muscle to restore the disrupted reading frame. Recently three papers in the journal Science were published using this approach; Nelson *et al.,* Tabebordbar *et al.* and Long *et al.* All showed dystrophin restoration in *mdx* mice after intramuscular and or intraperitoneal administration of CRISPR/Cas9 leading to a targeted exon 23 deletion. Dystrophin protein levels varied from 0.5% to 8% in skeletal muscle tissue and ~0.5% in cardiac muscle tissue (53-55).

There are a few major drawbacks of using the CRISPR/Cas9 system directly in patients. The first drawback is the delivery. Effective delivery of AAVs to all skeletal and cardiac muscle cells upon single administration is still challenging for humans and systemic administration requires high dosing of AAV vectors. Second but most important, upon muscle turnover the muscle transgenes are lost, thereby losing the mini- or micro-dystrophin genes. This is not the case when using the exon deletion strategy by CRISPR/cas9 technology, as this effect patients own genes (however in the case of muscle this is only true if satellite cells are targeted as these are responsible for generating new muscle cells). Repeated injections with AAVs are needed, this may lead to an immunological reaction of patients own immune system towards the vector. Third, restoring the dystrophin gene in fibrotic tissue will not lead to dystrophin restoration as there is no active dystrophin production in this tissue. Damaged tissue cannot be restored, making timing critical for this approach. Fourth, not a lot is known about the safety of the CRISPR/Cas9 system regarding specificity, as off target effects appear to be present (see cell therapy). So far only proof of principle has been shown, years of future work is needed assessing all of the issues before this approach can be evaluated for clinical use (56-59).

RNA targeting gene therapy

Ataluren

Around 14% of the DMD patients have a nonsense mutation *i.e.* a point mutation causing a premature stop codon, leading to premature truncation of translation and the absence of the dystrophin protein (60). Ataluren, PTC124 (Translarna™), is an orally available non-aminoglycoside drug with premature stop codon read-through activity, which in principle could restore dystrophin expression in this group of patients (61). In preclinical animal models PTC124 showed dystrophin restoration in skeletal and cardiac muscle and was well tolerated in healthy and DMD patients (62). In a phase 2a open-label clinical trial (38 DMD boys, treated for 28 days) 61% showed an increase of 11% in dystrophin levels assessed from muscle biopsies and treatment was well tolerated (63). In a follow up phase 2/3 clinical trial (174 randomized patients) two different doses (40 and 80 mg/kg) were evaluated for a year. Unfortunately, the primary end point (30 meter difference in the 6 minute walk test (6MWT) between treated and placebo groups) was not met (Δ29.7m). Nevertheless, patients treated with low dose (40 mg/kg/day) showed less (but not a significant) decline in the 6MWT than placebo and showed meaningful differences in secondary endpoints such as climbing and descending 4 stairs and walk or running 10 meters, thereby offering promise as treatment for DMD (64,65). After a post-hoc analyses PTC requested for conditional approval with the EMA, this was granted with the condition to perform a new study. By this means Ataluren, PTC124 (Translarna™), is the first conditionally approved drug by EMA for ambulant DMD patients age 5 years and older with a nonsense mutation causing premature stop codon (66). On October 15th, 2015 PTC announced their results from the phase 3 ACT-DMD clinical trial (40 mg/kg/day vs placebo for the duration of a year) showing a significant benefit of 47 meters in the 6MWT for patients who could walk 300-400 meters at baseline and a non-significant increase of 15 meters in the 6 MWT in the overall study population. In line they saw a benefit over placebo for secondary and tertiary endpoints, i.e. climbing and descending 4 stairs, walking or running 10 meters and North Star Ambulatory Assessment test (ir.ptcbio.com). In January 2016 PTC completed the New Drug Application (NDA) submission to FDA, but this was rejected. In parallel they submitted the data from the new phase 3 ACT DMD clinical trial to EMA, this is still pending.

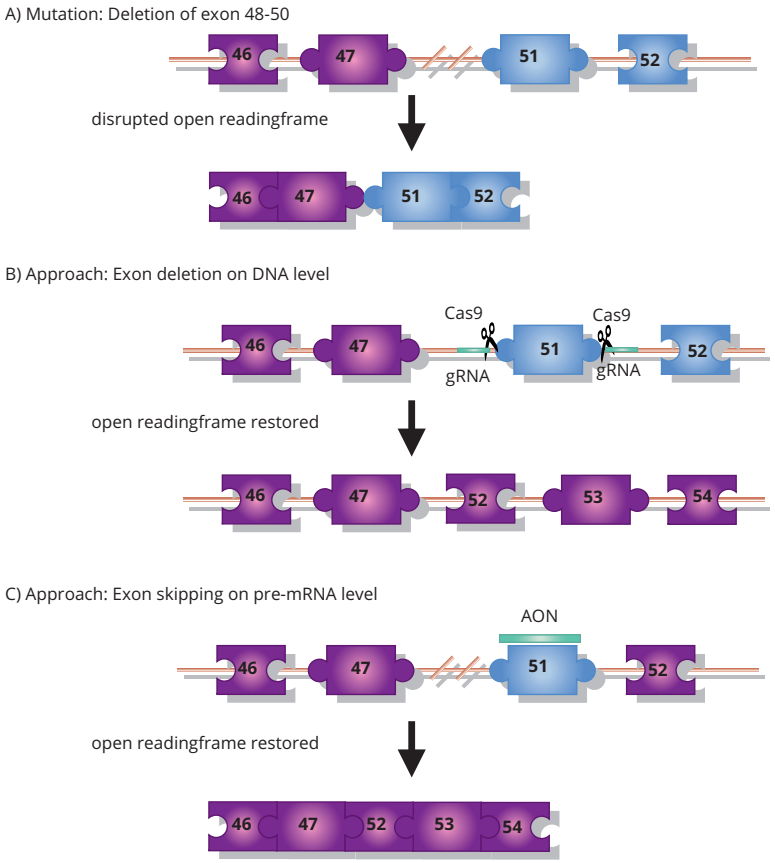

Figure 4. Targeted exon deletion versus exon skipping. Targeted exon deletion by genome editing as well as exon skipping are both applications to restore the disrupted reading frame of the DMD gene. CRISPR/cas9 technology is designed to delete an exon on DNA level. Exon skipping, using AON, is designed to target the pre-mRNA, interfere with pre-mRNA splicing. This results in a skip of the target exon. Both applications result in an internally deleted but partly functional dystrophin protein. A) an example of a disrupted reading frame, deletion of exon 48-50 in the DMD gene. B) restoration of the reading frame using CRISPR/cas9 technology. C) restoration of the reading frame using AON.

Exon skipping

The therapeutic approach that is the focus of this thesis is the restoration of the reading frame on RNA level in DMD patients using antisense oligonucleotides (AON). In theory this approach allows the production of a shorter but partly functional dystrophin protein as seen in patients with BMD (4,67). These AONs bind the target pre-mRNA in the nucleus in a sequence specific way. Here they interfere with splicing factors by binding the exonic splicing enhancer and/or exon inclusion sequences, preventing

exon recognition, and thereby cause the skipping of the target exon (68,69) (figure 4). In 1996 Pramono *et al*., showed it was feasible to induce exon skipping in an *in vitro* splicing assay (70). Later, Dunckley *et al*., and Wilton *et al*., showed the potential of exon skipping in cultured primary myotube cells of *mdx* mice (71,72). The feasibility of exon skipping upon intramuscular and systemic administration of AON, targeted to skip exon 23 in *mdx* mice, was shown by Mann *et al*., and Lu *et al*., respectively (73,74). Van Deutekom *et al*., showed for first time, the skipping of exon 46 in cultured myotube cells of DMD *patients* with a deletion of exon 45, thereby restoring the disrupted reading frame resulting in correctly localized dystrophin protein expression in 75% of the myotubes (75). Later Aartsma-Rus *et al*., designed AONs for 11 other human DMD exons and showed skipping of these exons in cultured human myotubes (76). In 2007 dystrophin restoration after a local injection of AON, targeting exon 51, in the tibialis anterior of DMD patients was shown by Deutekom *et al*., (77).

Exon skipping is a mutation specific approach. There is a high variation in mutations between DMD patients. Nonetheless some patients with different mutations can benefit from skipping the same exon, e.g. exon 51 skipping for a deletion of exon 45-50, 47-50, 48-50, 49-50, 50 and 52, making it applicable to the largest group of patients (13-14%) (60,78). Currently AON mediated exon skipping for DMD is evaluated in various clinical trials, *further described in paragraph 1.3.4*

1.3.3 Exon skipping antisense oligonucleotides

AON are single stranded, short pieces of chemically modified DNA or RNA, which can bind complementary to the target RNA (via Watson and Crick base paring) and modulate the function of the target RNA. In general AONs can be divided in two main groups based on their mechanism of action. The first group of AON promotes, upon binding, degradation or cleavage of the target RNA by the recruitment of endogenous enzymes. For example, RNase H or RNA-induced silencing complex (79-81). The second group consists of AONs which, upon binding, interfere with the function of the target RNA without degradation, for example by blocking translation, promoting alternative use of exons, exon skipping or exon inclusion (82). The difference between the groups after binding the target RNA depends on the chemistry of AON used, the binding location on the target RNA and the location of the target RNA in the cells.

Throughout the years different chemically modified AON have been developed to enhance nuclease resistance or improve thermodynamic stability, affinity for the target, bio-availability and tissue half-life (83,84). For DMD, the most studied exon skipping AON chemistries are the 2'-*O*-methyl phosphorothioate (2OMePS) and phosphorodiamidate morpholino oligomers (PMO) (Aartsma-Rus, 2014). 2OMePS AON is a second generation RNA-like AON. It has a 2'-*O*-methyl modification at the 2'OH position of the

ribose and a phosphorothioate (PS) backbone. It has a net negative charge, is almost completely nuclease resistant and has a high binding affinity for RNA. 2OMePS AONs have a prolonged half-life *in vivo* (plasma half-life ~ 4 weeks in patients) due to fact that PS- modification enables low affinity serum protein binding, thereby preventing acute renal clearance (85,86). It is estimated that 2OMePS AON have a tissue half-life of 10-33 days in skeletal muscle and 46 days in cardiac muscle (87). PMO AON is a third generation, DNA-like AON. The deoxy-ribose sugar ring is replaced by a morpholino ring and the phosphodiester bond by phorphorodiamadate linker. It is charge neutral has a high binding affinity and is completely nuclease resistant. Lacking the PS modification PMOs have a much shorter half-life *in vivo* due to fast renal clearance (plasma half-life ~3-4 hours in patients) (88).

Currently, a new DNA based AON, Tricyclo-DNA (Tc-DNA) is evaluated for exon skipping for DMD in animal models. This AON deviates from DNA by the presence of 3 additional carbon atoms between C5'and C3', is completely nuclease resistant, has a further improved binding affinity, thermodynamic stability, serum stability, and does not elicit RNase H activity (89,90). Serum levels of Tc-DNA rapidly decline in an hour after administration, but are still detectable after 90 minutes in mice (91). Compared to the 2OMePS AON, Tc-DNAs have improved tissue half-life, which is estimated to be 45 days on average and appears to be taken up by cardiac tissue more efficiently (91).

Modifying the 2' position of the sugar moiety of RNA is an attractive approach to improve, for example, affinity, stability and nuclease resistance. The 2OMe modification is an example of such modification. Another possible 2' modification is the substitution of a Fluoro (F) at the 2' position (2'-deoxy-2'-fluoro RNA modification (2F)). In a study from Rigo *et al.,* (Ionis pharmaceuticals) it was shown that 2F modified AONs recruit ILF2/3 proteins upon binding their target RNA. This led to additional steric hindrance, making the target RNA less accessible for splicing factors to bind and resulted in enhanced exon skipping in a model for spinal muscular atrophy (SMA) (92). This is an attractive feature for exon skipping AONs where blocking of the target RNA is essential. In this thesis, 2FPS and isosequential 2OMePS AON counterparts have been compared for DMD. While *in vitro* 2FPS AON resulted in increased exon skipping levels, the modification appeared less effective *in vivo* and was found to be toxic in *mdx* mice (a mouse model for DMD) (this thesis chapter 6).

It is also possible to combine different chemical modifications, making chimeric AONs. Studies from the group of Matsuo have shown increased exon skip levels targeting exon 19, 45 and 46 of the DMD gene, using an chimeric AON consisting of 2OMe RNA and ethylene bridged nucleic acids (ENA) for DMD (2OMe/ENA oligonucleotides)(93,94). In 2015 they showed

improved skipping of exon 45 in primary muscle cells from a patient with a deletion of exon 44 (95). Currently 2OMe/ENA AONs are evaluated in a clinical trial (see next paragraph).

1.3.4 AONs in clinical trials for DMD

AONs are a potential therapeutic approach for DMD and other neuromuscular disorders (e.g. spinal muscular atrophy, myotonic dystrophy). Currently they are evaluated in various pre-clinical and clinical trials. A complete overview of these AONs tested in clinical trials is given in chapter two and recently a nice overview is given by Fletcher *et al (96)*. Below you find the most important updates involving clinical trials at this moment.

Recent updates for drisapersen and eteplirsen (DMD)

Drisapersen

Drisapersen (Kyndrisa^{tm}), a 2OMePS AON targeting DMD exon 51, has been evaluated in nearly 300 DMD patients via subcutaneous administration and appeared safe. However, mild to moderate side effects have been observed e.g. injection side reaction, proteinuria and thrombocytopenia. In a phase II randomized, double-blind placebo controlled study involving 54 DMD patients (age 6-8), treated patients outperformed placebo treated patients in the 6MWT. Unfortunately in a phase III clinical trial involving 186 DMD patients (age 5-16) no significant difference in the 6MWT has been observed between treated patients and placebo-treated patients. Around the end of this phase III study natural history data came available and showed that it is very difficult to pick up treatment effect in a population with a broad age range. Younger patients respond different to treatment than older patients. By now 8 ambulant patients have been treated for 177 weeks and remained stable in their 6MWT. With new posthoc analyses in age matched groups BioMarin applied for accelerated approval of drisapersen to the Food and Drug Administration (FDA) in April 2015 and with the European Medicines Agency (EMA) in June 2015. On January 14th, 2016, BioMarin announced that the FDA considered the application not to be ready in its current form. On May 31st, 2016, BioMarin announced withdrawal of market authorization application of drisapersen with the EMA, because they anticipated a negative opinion from committee for Medicinal Product for human use. Also they announced to stop all clinical research for drisapersen. Anticipating similar problems for other AONs of the same chemistry, their clinical development was stopped as well. This includes three other first generation AON targeting exon 44, 45 and 53 which were in phase two of clinical development. Nonetheless, BioMarin will continue developing second generation AONs for DMD.

Eteplirsen

Eteplirsen, a PMO targeting DMD exon 51 has been evaluated in clinical trials and appeared safe. However, in some patients proteinuria was found. In the latest clinical trial involving 12 patients, dystrophin restoration has been observed in 30-60% of muscle fibers after 48 weeks of treatment. Moreover 6 patients treated from the onset of the study remained stable in their 6MWT for 120 weeks. Based on this data, Sarepta Therapeutics submitted an application for accelerated approval for eteplirsen, to the FDA on June 26th, 2015. On May 25th, 2016, Sarepta announced that the FDA is not able to complete their evaluation by the prescription drug user fee act goal date of May 26th, 2016, but will continue to complete their work in an as timely manner as possible. On June 6th 2016 Sarepta announced that, on request of the FDA, they will submit dystrophin protein data obtained from muscle biopsies (13 patients) to the ongoing evaluation. September 19th 2016, FDA granted accelerated approval to eteplirsen (EXONDYS 51tm) as treatment for DMD patients who benefit from exon 51 skipping (intravenous administration 30 mg/kg, once a week). However, this is based on the fact that dystrophin protein restauration is observed in a small group of patients (13) with a mean increase of just 0.2 to 0.3% of normal. http://jamanetwork.com/journals/jama/fullarticle/2572614). In the whole study population no clinical benefit has been shown and 38% of the patient treated (compared to placebo) experienced side reactions as vomiting and balance disorders with contusion. Other side reactions were excoriation, arthralgia, rash, catheter site pain and upper respiratory tract infection (≥ 10% compared to placebo). New, placebo controlled clinical trials need to be conducted to prove if eteplirsen is really working (www.sarepta.com).

Recent updates other AON for DMD

Recently two other clinical trials for DMD have been announced. On February 25th, 2016, Daiichi-Sankyo announced the start of a phase 1-2 clinical trial in Japan (now recruiting) with their DS-5141b drug, an chimeric AON (2OMe/ENA) targeting exon 45 of the DMD gene (https://clinicaltrials.gov/ct2/show/NCT02667483). On March 23rd 2016 NS-Pharma announced the start of a phase II, placebo controlled double-blind clinical trial in Japan (not yet recruiting) with NS-065/NCNP-01, a PMO targeting DMD exon 53. (https://clinicaltrials.gov/ct2/show/NCT02740972)

Recent updates for Nusinersen (SMA)

Nusinersen

On August 1st 2016, Ionis pharmaceuticals and BioGen announced that Nusinersen (Isis-SMN_{rx}, a 2'-O-methoxyethyl phosphorothioate (MOEPS) AON) met the primary endpoint pre-specified for interim analysis of ENDEAR, a broad phase 3 clinical trial for the treatment of infants and

children with spinal muscular atrophy (SMA). On November 7th 2016 they announced that Nusinersen met primary endpoint in a phase 3 clinical trial CHERISH with late-onset patients. Nusinersen is found to have an acceptable safety profile as well and now all participants are able to enrol in the open label extension study (SHINE) where all participants receive Nusinersen. BioGen is currently preparing marketing authorization applications in the USA and Europe.

1.3.5. AON delivery

In general, any drug must reach its target site in order to be effective. Without effective drug delivery the drug has no therapeutic activity and might cause side effects through non intended off target interactions. There are multiple routes of administration to effectively deliver drugs to humans e.g. oral, transdermal, transnasal, subcutaneous, intravenous, intrathecal or local (e.g. intramuscular for muscle diseases). Depending on the drug substance itself and the target tissue, the choice of the optimal route of delivery may vary. For DMD patients the target is skeletal and cardiac muscle tissue. Since the human body consists of 30-40% of muscle it is impossible to treat each muscle individually, a body wide approach is necessary. Oral delivery is most convenient for patients and for many drugs oral bioavailability is sufficient to obtain systemic effect. However the bioavailability of AONs after oral delivery is very poor, and uptake after intestinal administration is limited (97). Based on preclinical animal studies and clinical studies, body wide treatment is feasible using subcutaneous (SC) or intravenous (IV) injections. Today this is the most effective way to deliver AON systemically (98-101).

1.3.5.1 Challenges for AON delivery

Even though systemic administration of AON is feasible, the amount taken up by the target tissue is generally limited. For effective AON delivery, several hurdles need to be overcome (for more details see paragraph 1.3.5.2). In general AONs need to distribute throughout the body without degradation, need to be taken up adequately by the target cells in the target tissue and reach the nucleus where splicing takes place (figure 5). In DMD, patients' muscle fibers have, due to the absences of the dystrophin protein and muscle inflammation, leaky endothelium and therefore more easily take up AON than healthy muscle tissue. Pre-clinical animal studies have shown effective AON delivery. Nevertheless upon body wide treatment a great proportion of AONs end up in liver and or kidney and are lost for uptake by skeletal- and cardiac muscle. Furthermore, less (2OMePS) or minimal (PMO) exon skipping levels in cardiac muscle compared to skeletal muscle are seen (87,102). However, 6 months treatment with high dose of PMO (300 mg/kg and 1.5 g/kg) in *mdx* mice, did result in exon skipping and detectable levels of dystrophin protein in cardiac muscle (103).

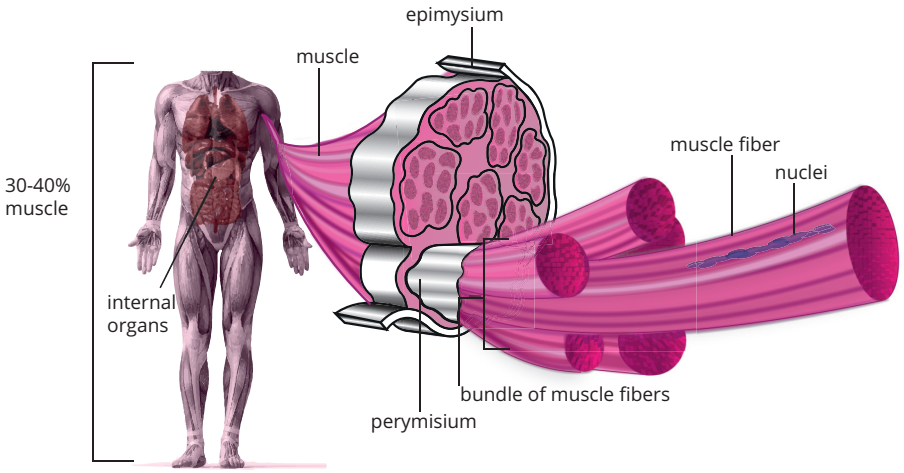

Figure 5. Human body and the location of muscle fiber nuclei. For DMD, the target of AON are muscle fiber nuclei. 30-40% of the human body consist of muscle making systemic treatment necessary. Muscle is built up from muscle fiber bundles surrounded by the epimysium. Each bundle is made from individual muscle fibers and is surrounded by the perimysium, which in turn are made by fusion of individual muscle cells which contain the nuclei.

1.3.5.2 Insight in AON delivery towards muscle

To improve the effective delivery of AON to skeletal and cardiac muscle, insight in the way AON behave *in vivo* is desirable. Upon systemic administration, AON distribute throughout the body via the blood (figure 6). Here, the first biological barrier to overcome is degradation by nucleases. To avoid this, AON are chemically modified making them nuclease resistant (see paragraph 1.3.3). Following SC injections PS modified AON reach peak plasma concentrations within 3-4 hours where after they rapidly decline. The second biological barrier is the endothelium of the blood vessel wall within the tissue. Molecules of 6 nm in diameter or smaller are mainly transported across the endothelium via the paracellular pathway, e.g. via the interendothelial junctions between the cells. Larger molecules, like albumin, are transported via the transcellular pathway, e.g. in vesicles via caveolar-mediated transcytosis. Transport across the endothelium of larger molecules (>100 nm is size) is limited. However, in some tissues like liver, gaps or fenestrations between the cells allow transport of larger molecules (104,105). As AON are small (<6 nm) they readily pass the endothelium of the vessel wall via the paracellular pathway (occurs mostly in kidney and intestinal tract). The uptake of PS modified AON by tissues from blood (but not the central nervous system) takes place within minutes to hours depending on the tissue type. Well perfused organs like liver, kidney and spleen, take up AON more rapidly than less perfused skeletal muscle tissue.

Acute clearance from the body predominantly takes place via the kidneys. However, AON with a PS modification bind serum proteins like albumin and are thereby protected from acute renal clearance (106). When AON have reached the target tissue, they need to overcome a third biological barrier, passing the cell membrane of the target tissue cells. The cell membrane has a dynamic structure, limiting free uptake of large and charged molecules which cannot pass the cell membrane by diffusion. There are various pathways known by which cells can take up larger molecules. In general it is assumed that AON are taken up via some form of endocytosis and are then trapped in cellular compartments like the endosomes. The fourth biological barrier to overcome is the escape from these cellular compartments and reach the nucleus where splicing takes place. It is good to bear in mind that tissues in general are made up of more than one cell type. This means that uptake by a certain tissues not necessarily means uptake by the target cells within that tissue.

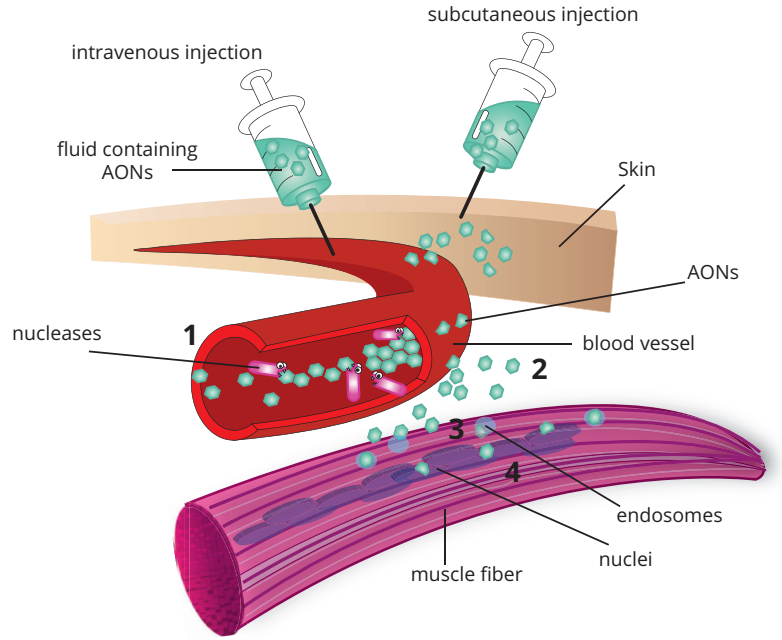

Figure 6. AON delivery, hurdles to overcome. In order to be effective, several hurdles need to be overcome before AONs reach the muscle fiber nuclei. 1) Degradation by nucleases. 2) Escape from endothelium of the blood vessel wall within the tissue. 3) Passing cell membrane of tissue cells. 4) Escape from the endosomes and reach the nucleus.

1.3.5.3 Endocytosis

Endocytosis is the term used for the transport of molecules into cells. Using this pathway cells take up nutrients and communicate with each

other and the environment. There are multiple endocytic pathways known and in general they are classified in two groups: phagocytosis (restricted to specialized cells like macrophages and dendritic cells) and pinocytosis (occurs in all cells)(107). Pinocytosis can be further subdivided in (i) macropinocytosis, (ii) clathrin-dependent endocytosis, (iii) caveolin-dependent endocytosis (iv) clathrin and caveolin independent endocytosis. They differ in coat proteins (when present), vesicle size and intracellular fate (table 1).

Phagocytosis (cell eating), is the process where phagocyte cells take up and clear the body of solids such as parasites, bacteria, death cells, cellular debris etc. It is a commonly known process used by macrophages defending the body against infection, invasion of foreign substances.

Macropinocytosis (cell drinking), shares many features with phagocytosis, but is used by non-phagocytic cells. It is a process of cell membrane ruffling, forming external macropinocytotic vesicles, up to 5 μM in diameter, by which the cell can internalize extracellular fluids containing various molecules (108).

Table 1. Endocytic pathways

Endocytic pathway	Process	Cell type involved
Phagocytosis	Uptake (cell eating) of parasites, bacteria, death cells, cellular debris	Mainly macrophages
Macropinocytosis	Uptake (cell drinking) of extracellular fluids containing molecules	Non-phagocytic cells
Clathrin-dependent endocytosis	Receptor mediated uptake in clathrin coated pits	All cells
Caveolin-dependent endocytosis	Cholesterol dependent uptake in caveolin coated vesicles	Endothelial cells, adipocytes, fibroblast, smooth muscle cells
Clathrin and Caveolin independent	All non-clathrin or caveolin dependent uptake	Various cells

Clathrin-dependent endocytosis, is a process where molecules are taken up by binding a specific receptor in clathrin-coated pits of the cell membrane. After clustering of the ligand-receptor complexes, the clathrin-coated pits invaginate and pinch of the cell membrane forming a clathrin-coated vesicle (100-150 nm in diameter). From these vesicles molecules are further transported through other cellular compartments like early and late endosomes, the golgi apparatus, nucleus and lysosomes. Hereafter

the molecules are released in the cytoplasm or transported back to the cell membrane (107,109,110).

Caveolin-dependent endocytosis is the most commonly reported non clathrin-mediated form of endocytosis. Caveolae are flask-shaped plasma membrane domains formed by the assembly of the caveolin membrane proteins 1, 2 and 3 (60-80 nm in diameter) (111). Caveolae are rich in cholesterol like proteins, and formation of caveolae is cholesterol dependent. Molecules that interact with cholesterol are invaginated and transported from caveolae-coated vesicles directly to other cellular compartment like golgi- or endoplasmatic reticulum and often circumvent degradation by lysosomes. Caveolae are especially abundant in endothelial cells, adipocytes, fibroblasts and smooth muscle cells (107,112,113).

Clathrin and Caveolin independent endocytosis is a process that is less understood and basically consists of all uptake that is not clathrin- or caveolin-mediated. Examples are the uptake of molecules depending on DNM2/Dynamin-two, small GTPases or tyrosine kinase for example. What is known is that bacteria and viruses sometimes hijack one of these pathways to enter host cells (114,115).

1.3.6 Strategies taken to improve delivery of AON

Various strategies are being explored to improve the delivery to and uptake by muscle for AON. Examples are, different chemical modifications of the AON, the use of nanoparticles as delivery vehicles or conjugation or co-administration of additive compounds to enhance cell uptake. For delivery of AON to muscle, the ideal delivery system should first of all not interfere with the function of the AON, should have a good safety profile and good biostability. Secondly, the delivery system should be small in size, efficiently taken up by muscle cells and promote endosomal escape. Third, preferably the delivery system is muscle specific thereby limiting or preventing uptake by e.g. liver, kidney and spleen. Finding or developing the perfect delivery system is a major challenge. Throughout the years many approaches have been evaluated. However some are only evaluated after intramuscular administration whereas for DMD systemic administration is the goal.

1.3.6.1 Formulation of AON

To enhance the delivery of 2OMePS AONs, poly(ethylene imine) (PEI) and poly(ethylene glycol) (PEG) copolymers alone, combined with the cell penetrating TAT peptide (GRKKRRQRRRPQ), adsorbed colloidal gold (CG) or a combination have been investigated using AONs targeting mouse *Dmd* exon 23 in the *mdx* mouse model. The PEI-PEG copolymer combined with TAT was most potent and resulted, in 6-fold increased dystrophin positive fibers and up to 30% of dystrophin expression compared to wild type levels upon intramuscular administration in tibialis anterior muscle of *mdx* mice (116). However these polyplexes have a cationic charge which

limits their biodistribution due to nonspecific binding to target unrelated components, limiting systemic administration. Later the encapsulation of these polyplexes in biodegradable PLGA nanospheres has been investigated to improve the strategy. Nevertheless, upon intramuscular administration no improvement in dystrophin levels were observed compared to the unencapsulated polyplexes (117). 2OMePS AONs adsorbed onto PMMA/N-isopropil-acrylamide+ (NIPAM) nanoparticles (ZM2) have been investigated as well. Intraperitoneal administration of these nanoparticles resulted in 20% exon 23 skipping levels and up to 40% dystrophin positive muscle fibers in *mdx* mice (118). Later Bassi *et al.,* showed that this was persistent for over 90 days (119). A next generation of this approach involves ZM4 nanoparticles. Intraperitoneally injected fluorescently labelled ZM4 nanoparticles resulted in detectable fluorescence (analysed with odyssey imaging system) in skeletal muscle and heart. Higher levels of fluorescence were found in liver kidney, spleen and lymph nodes but not in brain (120). Whether ZM4 nanoparticles effectively deliver AON to muscle remains to be evaluated.

1.3.6.2 Co-administration of additive compounds

In addition to formulating AONs, it is also possible to combine AON treatment with compounds that increase exon skipping levels or enhance the uptake of AON. Kendall *et al.,* reported that co-administration of Dantrolene increased exon skipping levels *in vitro* of 2OMePS AONs in cultured cells of *mdx* mice and in reprogrammed myotubes from DMD patients. Systemic administration in *mdx* mice, Dantrolene (intraperitoneal administration) combined with a PMO (intravenous administration), resulted in increased exon 23 skipping levels and dystrophin protein levels in various muscle but not triceps and cardiac muscle (121). However other researchers were not able to reproduce these results in myotube cultures (Aartsma-Rus and van Vliet, personal communication).

Hu *et al.,* showed that PMOs combined with guanine analogues (particularly 6-thioguanine) resulted in improved exon 23 skipping levels (~2-fold) *in vitro* as well as *in vivo* upon intramuscular administration in tibialis anterior muscle of *mdx* mice (122). Verhaart *et al.,* confirmed that 6-thioguanine improved exon skipping levels *in vitro* but, in contradiction, not *in vivo* for 2OMePS and PMO AONs after intramuscular administration in *mdx* mice (123).

Small-sized polyethylenimine (PEI)-conjugated pluronic copolymers (PCMs) have been evaluated to improve the uptake of PMOs. Systemic administration (intravenous) resulted in increased exon 23 skipping levels and on average 15% dystrophin positive muscle fibers (particularly in cardiac muscle tissue) when combining the PMO with PCMs compared to the PMO alone (<5%) in *mdx* mice (124). Nonetheless the overall percentages of exon skipping and dystrophin positive fibers remained low.

1.3.6.3 Conjugation of additive compounds

Cell penetrating peptides (CPPs) are most studied for PMOs. CPPs are short cationic peptides designed to transport drug into cells. They have a long history consisting of a broad range of peptides that have been investigated e.g. Poly-L-lysine, Hiv-1 TAT protein, penetratin and the arginine rich peptides used today (125-128). Moulton *et al.,* was one of the first to describe the use of arginine rich peptides to enhance the delivery and uptake of PMOs in muscle for DMD (so called PPMOs)(128). However these arginine rich peptides resulted in acute toxicity in monkeys (129). Throughout the years various improvements have been made regarding arginine rich CPPs; RXR_4, B-peptide $(RXRRBR)_2$ and the more recently developed Pip peptides (130-134). The most potent Pip peptides come from the Pip5 and Pip6 series. Conjugates resulted *in vivo,* upon systemic administration, in high levels of exon 23 skipping and dystrophin protein production in skeletal and cardiac muscle. Further evaluation of these conjugates showed improvement of muscle strength and cardiac function in exercised *mdx* mice (131,135). Lehto *et al.,* studied the mechanism by which CPP Pip6a-PMO is taken up. Results showed an energy dependent uptake via caveolin-dependent endocytosis in skeletal muscle with nuclear localization in myotubes but not in myoblast cell cultures (cytoplasm). For cardiac muscle tissue clathrin-dependent endocytosis was found to be the most prominent uptake pathway. However, here Pip6a-PMO was mainly found in the cytoplasm and to a less extent in the nucleus where splicing takes place (133). The newly developed CPPs appear to be well tolerated in *mdx* mice. Nevertheless they contain many arginine residues making it questionable if they are not toxic in higher animals then mice as shown in the early studies of Moulton *et al.*

Despite the fact that the well-studied CPPs have the potential to improve de delivery of PMOs, they are not suitable for delivery of 2OMePS. The cationic nature of CPPs have the tendency to strongly form aggregates when combined with the anionic 2OMePS AON backbone. A different approach is needed: e.g. the use of tissue specific homing peptides selected from phage display experiments (see paragraph 1.5).

1.4 Phage display

1.4.1. Phage display

Phage display is a well described, powerful technique to identify peptides, antibodies or other proteins with target specific binding properties from phage libraries. Phage display technology was first introduced by Smith in 1985 (136,137) who later introduced the concept of affinity selection using phage display peptide or antibody libraries. From here the technology has spread rapidly (136,138-140). Nowadays phage display technology has been modified and is used in for example, vaccine development (vaccines consisting of phages that display a disease specific antigen), studying protein-protein interactions (different domains of a protein is displayed on a phage and panned against a specific protein), identification of substrates or inhibitors (analyses of enzyme activity and specificity or identification of enzyme inhibitors) and epitope mapping using antibody libraries (141-143). Phages (or so called bacteriophages), are viruses that consist of DNA or RNA within a protein coat. A phage library is constructed by fusing a foreign peptide or protein with one of the protein coat genes in such a way that these are expressed on the surface of the phage. Phage libraries consist of millions or billions of uniquely constructed phages from which affinity selection takes place, a process called bio-panning. Nowadays the identity of the peptides or proteins of interest can be easily detected using various high throughput sequencing approaches.

1.4.2 Biopanning

Biopanning selections using phage libraries can be undertaken for various targets like specific molecules (e.g. proteins, enzymes, receptors), cultured cells *in vitro* or tissues, or cells within a tissue *in vivo*. The basic principles are the same for all targets. First, the phage library is exposed to the target for binding. Secondly, all non-binders are removed. Third, binding phages are recovered by elution. Fourth, binding phages are amplified and prepared for a next bio-panning round. Fifth, after several rounds of biopanning, binding phages are identified by for example sequencing of candidate phage DNA (figure 7).

1.4.3 Phage display vectors

Different phages have been used for the development of phage display vectors. The M13 filamentous phage is the most used worldwide, the T4, T7 and lambda phages are also often used. Each phage display vector has its own benefits and limitations (table 2).

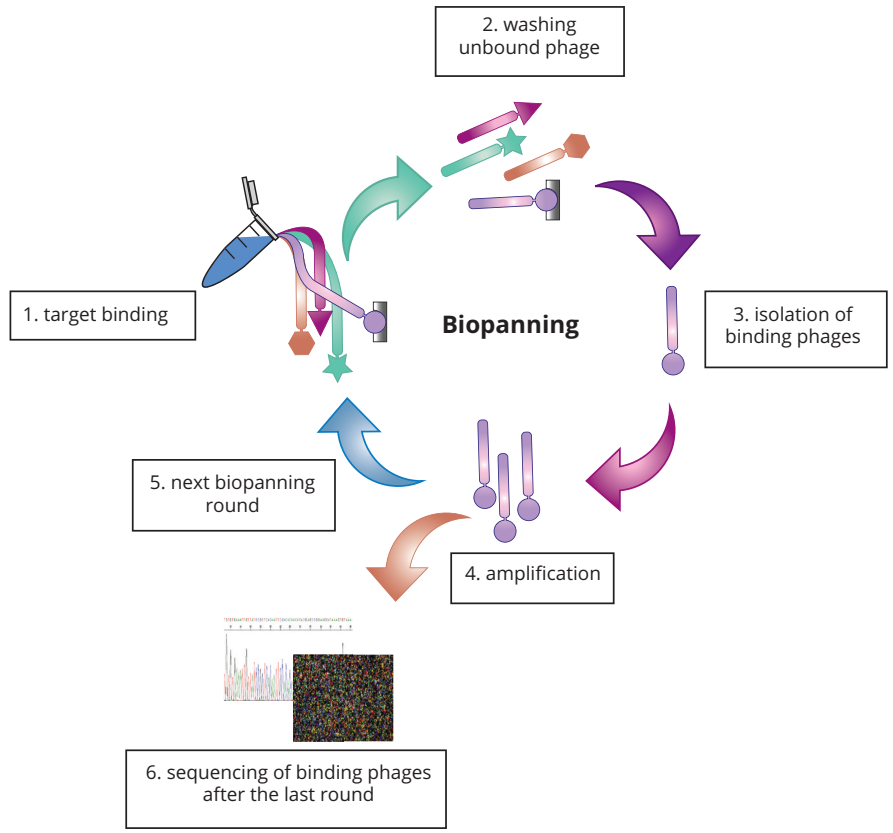

Figure 7. Phage display biopanning. Phage display biopanning is a selection technique to identify high affinity binders for a selected target. Step one, exposing the phage library to the target. Step two, wash away unbound phage. Step 3, isolation of binding phages. Step 4, amplification of isolated phages. Step 5, preparing the amplified isolated phages for another screening round. Step 6, identification of binding phages by sequencing.

M13 filamentous phage display vectors consist of rod shaped linear filamentous phage particles containing single-stranded looped DNA (6.4kb). They can only infect *E-coli* bacteria which express the F-pilus. After infection the F-pilus is depolymerized, preventing multiple phage infections. The advantage of this unique characteristic is that each bacterial clone represents a specific phage with a unique foreign peptide or protein (144). Upon infection, phages are assembled in the periplasmic environment and, after replication, leave the bacterial host via the membrane without killing its host. Another unique characteristic of this phage is the resistance against extreme conditions such as acidic pH, high temperature and enzymatic cleavage, making the phages very convenient to use *in vitro* as well as *in vivo* (144,145). The M13 phage consists of a major PVIII coat protein (2.700

copies) and the minor PIII and PVII coat proteins on one end and PVII, PIX coat proteins on the other end (3-5 copies). In most cases the phages are constructed to express foreign peptides or proteins on the minor PIII (used to infect cells) or major PVIII coat protein, but all 5 coat proteins have been used (136,146). Fusion to the PIII protein can be used for peptides or proteins taking up to 100kb in insertion size. Fusion to the PVIII protein is limited to 6-8 amino acids in length because of the large copy numbers expressed on the phage.

The T4 phage display system has relatively large phages containing double stranded linear DNA (160 kb) packed in a capsid head, which is attached to a contractible tail and tail fibers. Phages infect *E-coli* bacteria upon binding the host with their tail fibers and injecting the phage DNA via their contractible tail directly in the cytoplasm. The phage DNA will be replicated, new phage particles will form and eventually leave the host by cell lysis. The T4 capsid head consist of 3 essential proteins, gp23, gp24 and gp20, and 2 non-essential proteins Hoc (highly antigenic outer capsid protein) and Soc (small outer capsid protein). The Hoc and Soc proteins are used most frequently to express a foreign peptide or protein on the surface. The advantage of using T4 phages over M13 phages is the possibility of fusions with complicated, high molecular weight proteins on the phages surface. T4 phages are most used in vaccine technology by fusion of pathogen antigens to the N- or C-terminus of Hoc and Soc (147,148).

The T7 phage display system is similar to the T4. It consists of a capsid head, a tail, tail fibers and has dsDNA (39kb). T7 differs from the T4 in that the capsid head consists mainly of various combinations of gp10 proteins, and that the tail is not contractible (upon infection the phage builds a protein channel towards the bacterial cytoplasm)(149,150). Foreign proteins can be expressed by fusion with the C-terminus of gp10 protein in high copy numbers for small peptides, or with low or intermediate copy numbers for larger peptides or proteins. The main advantage of T7 phages is their short lytic life cycle, which significantly shortens the screening process. Another advantage is their extreme resistance to various environmental conditions.

Lambda page display vectors contain double stranded linear DNA (48 kb), which is similar to theT4 and T7 phage display systems capsids in a head, and contains tail and tail filaments. The capsids head consists of 2 major coat proteins, pgE and gpD and the major tail protein is gpV. A unique feature of the lambda genome is the 12 nucleotides long GC-rich cos sites (sticky ends) at both 5'ends, which upon infection are used by the host DNA ligase to make the DNA circular (151). Lambda phages can either be lytic or lysogenic (when integrated in the host genome) and are used to express complicated high molecular weight proteins fused to the D head protein (high copy number) or pV tail protein (low copy number).

Table 2. Phage display vectors

Phage vector	Genome size	Size foreign insert	Advantage	Lytic or Lysogenic
M13	6.4kb	Up to 100kb on PIII (3-5 copies) 3-8 amino acids (2.700 copies) on PVIII coat protein	F-Pilus is depolymerized up on infection preventing multiple phage infection	Lysogenic
T4	160kb	High molecular weight proteins to Hoc or Soc coat proteins	Fusion of complicated high molecular weight proteins	Lytic
T7	39kb	Low molecular weight in high copy number. Intermediate to high molecular weight in low copy number to gp10 protein	Short life cycle significantly shortens the screening process	Lytic
lambda	48kb	Complicated high molecular weight to D head protein (high copy number) or pV tail protein (low copy number)	Expression of high molecular weight proteins in high copy number	Lytic and Lysogenic

1.5 Using phage display peptide library screens to improve delivery of AON towards skeletal and cardiac muscle tissue

The advantage of using tissue specific homing peptides over CCPs is that short peptides in general have a better safety profile, do not or minimally provoke an immune reaction and their tissue specificity limits off target effects. The use of tissue specific homing peptides is applicable to 2OMePS and PMO AONs, as well as for other drugs. Various random combinatorial phage display peptide libraries are commercially available ranging from 6 amino acids in length (6-mer) up to 24 amino acids in length (24-mer) in general. Random 7-mer, 9-mer and 12-mer peptide libraries are studied most. These peptides are expressed on the phage coat protein either in linear or circular conformation (e.g. the substitution of cysteines at either end of the random peptide sequence, joint by a disulfide bond, makes the peptide have a circular conformation), and in high or low copy numbers. Using phage display peptide libraries to search for tissue specific homing peptides is not without challenges: Phage display peptide libraries are

known to be prone to parasite sequences i.e. phages with a growth advantage or phages binding plastic or other experimental related but not target related compounds (152). Peptides as therapeutics by themselves have limitations as well. Peptides are known to be aggregation prone, have solubility issues and a pore bioavailability. Nonetheless when peptides are conjugated to an AON most of these issues can be overcome.

In the last couple of years, technology used to analyze phage display outcomes rapidly improved. Various high throughput platforms were integrated in the analyses, resulting in new insights and improved biopanning selections (153,154). This thesis is focused on 7-mer phage display peptide libraries (Ph.D.-7). We integrated for the first time Illumina next generation sequencing (NGS) to analyze phage display biopanning selections (this thesis chapter 4). We showed that high throughput sequencing of the naïve library after one round of bacterial amplification is a powerful tool to identify parasite sequences with a growth advantage. We also showed that by using NGS a single biopanning round is enough to identify candidate peptides. A few months later, a comparable story was published by Matochko *et al*,. (155)

Back in 1999 Samoylova *et al*., described a phage, expressing a 7-mer peptide ASSLNIA, showing increased uptake (judged on fluorescent intensity) in murine tissues compared to control phages. Later studies showed that the peptide alone was unable to increase delivery or exon skipping levels in *mdx* mice, when conjugated to a PMO (Samoylova and Smith, 1999; Yin et al., 2009). Seow *et al.*, identified after several rounds of *in vivo* biopanning towards heart and quadriceps muscle, peptide T9 (12-mer: SKTFNTHPQSTP). Despite that this peptide appeared to preferentially bind skeletal muscle, they observed limited internalization of muscle cells. In this thesis the first muscle homing peptide for 2OMePS AON is described, peptide P4 (7-mer: LGAQSNF) identified after *in vivo* biopanning towards heart and quadriceps muscle (this thesis chapter 3). P4 conjugated 2OMePS AONs significantly increased exon skipping levels in diaphragm and cardiac muscle tissue, compared to unconjugated AONs, after subcutaneous administration in *mdx* mice.

In search for better muscle specific homing peptides to enhance the delivery of PMOs, Gao et al., identified the M12 peptide RRQPPRSISSHP (Gao *et al.*, 2014), which resulted in improved but variable exon skipping levels in skeletal muscle but not cardiac muscle when conjugated to a PMO. Searching for better muscle specific homing peptides to enhance the delivery 2OMePS AON, a phage display peptide library expressing cyclic 7-mer peptides combined with NGS analyses has been investigated (this thesis chapter 5). The lead peptide CyPep10 (CQLFPLFRC), resulted upon conjugation to 2OMePS AONs, in a significant 2-fold increase in exon skipping levels in all muscle tissues analyzed. All together these results

demonstrate the value of tissue specific homing peptides for the delivery of AON.

Outline of this thesis

The work described in this thesis aimed to optimize delivery of AON towards skeletal and cardiac muscle for DMD. In Chapter 2 an update on RNA-targeting therapies using AON for neuromuscular disorders is given. With emerging new possibilities there is a growing need for improved delivery of AON towards muscle. Conjugation of muscle specific homing peptides is a strategy to accomplish this. In Chapter 3 the first muscle homing peptide for 2OMePS AON is described. This peptide has been identified from a phage display peptide library. Phage display peptide libraries are prone to parasite sequences that dominate the selection. In chapter 4 it is described how NGS improves phage display selections by increased sequencing depth and identification of parasite sequences with a growth advantage. Chapter 5 describes how these improvements have been integrated in new phage display selection experiments towards muscle, which led to the identification of a more potent peptide candidate. In Chapter 6, 2FPS AON have been evaluated as exon skipping AON for DMD. A general discussion where results are put in a broader context is given in chapter 7.

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