FACULTY OF MEDICINE AND HEALTH SCIENCES

ADVANCEMENTS IN GENE THERAPY FOR DUCHENNE MUSCULAR DYSTROPHY

Elisa Dominicus
Student number: 00907402

Promotor: Dr. Sofie Symoens

A dissertation submitted to Ghent University in partial fulfillment of the requirements for the degree of Master of Medicine in Medicine.

Academic year: 2016-2018

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Acknowledgements

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I would also like to thank my mother, who has shown me how to live with integrity, determination, and compassion. Thank you for always having my back.

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The last two years I have learned how to analyze and write about experimental literature in a structured manner, how to distinguish essential from non-essential matters and to obtain a critical attitude towards scientific papers. My respect for people in the research field grew immensely as they often dedicate their life to science in the hope of helping others in the future.

My acknowledgments also extend to patients living with Duchenne and Becker muscular dystrophy. Reading and writing about boys who suffer from this appalling disease has put things in perspective for myself. The courage they have going through life carrying this immense burden is, to say the least, admirable.

Contents

List of abbreviations

AAV: adeno-associated virus

AO: antisense oligonucleotide

BMD: Becker muscular dystrophy

Cas9: CRISPR associated 9 endonuclease

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

crRNA: CRISPR RNA

DAPC: dystrophin-associated protein complex

DMD: Duchenne muscular dystrophy

DYS-HAC: human artificial chromosome that contains full-length dystrophin

EMA: the European Medicines Agency

ESC: embryogenic stem cell

EU: European Union

FDA: the Food and Drug Administration

HAC: human artificial chromosome

hMADS: human multipotent adipose derived stem cells

HR: homologous recombination

HSC: hematopoietic stem cell

IA: intra-arterial

IM: intramuscular

IMD: intermediate muscular dystrophy

indels: insertions and deletions

iPSC: induced pluripotent stem cell

ITR: inverted terminal repeat

IV: intravenously

LS means: least square means

m: meters

MABS: mesoangioblasts

MAPH: multiplex amplifiable probe hybridization

mdx:utrn(-/-): dystrophin and utrophin deficient mice

MLPA: multiplex ligation-dependent probe amplification

MMCT: microcell-mediated chromosome transfer technology

NHEJ: non-homologous end-joining

ORF: open reading frame

PAM: Protospacer Adjacent Motif

PB: piggybac transposon

PMO: phosphorodiamidate morpholino oligomer

PSC: pluripotent stem cell

PTC: premature termination codon

rAAV: recombinant adeno-associated virus

SB: sleeping beauty transposon

SC: satellite cell

sgRNA: single guide RNA

TALEN: transcription activator-like effector nuclease

TFTs: time function tests

TLR: Toll-like receptor

tracrRNA: transactivating RNA

UAPC: utrophin-associated protein complex

US: United States

6MWT: 6 minute walking test

Abstract (English)

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are two dystrophinopathies that affect 1/4000 boys worldwide. These conditions result from mutations in the X-linked dystrophin gene. Mutations that result in the absence of dystrophin show a severe phenotypical presentation, consistent with DMD. Mutations that result in diminished amounts of (functional) dystrophin result in a milder phenotype, consistent with BMD. Dystrophinopathies are primarily characterized by progressive muscle weakness and degeneration, low quality of life and premature death. Palliative treatment with corticosteroids is currently the only standard therapy in DMD boys but these drugs carry the risk of serious side effects. Up to this point, no curative treatment exists for DMD or BMD despite profound investigations on this subject. Increasing knowledge of the organization of the gene and the role of dystrophin in disease mechanisms provide insight in ways to manipulate the genome and downstream pathways. An intensive literature study was conducted with the aim to produce comprehensive literature on cell and gene therapy proceedings for DMD. We summarize recent progress and highlight advantages and limitations of *in vivo* gene therapy approaches which include: exon skipping, stop codon readthrough, adeno-associated virus mediated transfer, CRISPR-Cas9 and utrophin substitution. *Ex vivo* gene therapy is an approach which involves exterior modification of stem cells and was also addressed in this thesis.

Abstract (nederlands)

Duchenne musculaire dystrofie (DMD) en Becker musculaire dystrofie (BMD) zijn twee dystrofinopathieën die voorkomen met een prevalentie van 1/4000 jongens wereldwijd. Deze aandoeningen zijn het gevolg van mutaties op het dystrofine gen dat gelokaliseerd is op het X-chromosoom. Mutaties die resulteren in afwezigheid van het dystrofine eiwit resulteren in een ernstig verlopend ziektebeeld, passend bij DMD. Mutaties die aanleiding geven tot verminderde aanwezigheid van (functioneel) dystrofine veroorzaken een milder ziektebeeld, passend bij BMD. Dystrofinopathieën worden primair gekenmerkt door progressieve spierzwakte en spieratrofie, een verminderde levenskwaliteit en premature dood. Palliatieve therapie met corticosteroïden is momenteel de enige standaard behandeling in DMD jongens, maar deze medicatie brengt een groot risico van mogelijke serieuze bijwerkingen met zich mee. Tot nu toe bestaat er geen curatieve behandeling voor DMD en BMD ondanks uitgebreid onderzoek in dit gebied. De steeds groter wordende kennis over de organisatie van het gen en de rol van het dystrofine eiwit in ziektemechanismen heeft ons inzichten bezorgd in mogelijke manieren om het genoom of dystrofine pathways te manipuleren. Een intensieve literatuurstudie werd uitgevoerd met het doel een allesomvattend stuk te schrijven over de vorderingen binnen cel en gentherapie voor DMD. We vatten recente vorderingen samen en benadrukken voordelen en nadelen van *in vivo* gen therapie strategieën. Zo werden exon skipping, stop codon readthrough, adeno-associated virus gemedieerde transfer, CRISPR-Cas9 en utrofine substitutie besproken. *Ex vivo* gentherapie is een strategie die exterieure modificatie van stamcellen teweegbrengt en werd ook besproken in deze thesis.

1. Introduction

Dystrophinopathies including Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) (and intermediate muscular dystrophy (IMD)), are devastating diseases characterized by progressive muscle weakness and degeneration that result in reduced life expectancy in affected boys. The incidence of dystrophinopathies is approximately 1 in 4000 male live births (1 in 5000 for DMD and 1 in 20000 for BMD) (1). Both DMD and BMD are caused by defects in the dystrophin gene which is the largest identified gene in humans to date. Located on the short arm of the X-chromosome (Xp21) (2) it spans approximately 2.4 Megabases of DNA within 79 exons. The gene accounts as a template for multiple dystrophin isoforms each with a different tissue-specificity, weight, and type of promotor (2). Because of its large size the gene is prone to mutations (3). DMD and BMD are inherited in a maternal recessive pattern consequently primarily affecting males whereas females are usually asymptomatic carriers of the mutated gene (1). Besides this maternal inheritance approximately 20-30% of dystrophinopathies is caused by *de novo* mutations due to germline mosaicism (1, 2, 4). In DMD patients, a mutation that disrupts the reading frame of the dystrophin gene results in the total absence of dystrophin; whereas BMD patients are affected by an in-frame mutation and produce a partially functional dystrophin protein. DMD and BMD patients show similar signs and symptoms but differ in their severity, age of onset, and progression rate making BMD the milder phenotype. Up till now, no curative treatment is found although intensive investigations show promising results.

1.1. Clinical presentation

Dystrophinopathies vary greatly in phenotypical presentation ranging from mild to very severe presentation and early death (2).

1.1.1. DMD

DMD boys usually do not manifest symptoms up until the age of 1-2 years, although delayed gross motor skills and muscle weakness can be noticed. Symptoms typically start getting noticed around the age of 2-5 years. One of the first and most dominant symptoms is skeletal muscle weakness, more prominent in the lower extremities and proximal muscles compared to the upper extremities and distal muscles. This weakness will manifest as difficulty in gait (running is difficult and a waddling gate develops), jumping, climbing stairs and arising from the floor (during which the typical Gower's maneuver is used (Figure 1)). Cranial and sphincter muscles are generally spared (2). Lumbar hyperlordosis and scoliosis frequently develop and may need orthopedic surgery (5). Although true muscle hypertrophy occurs at an early stage, pseudohypertrophy, caused by fibrous

and fatty tissue replacement, inevitably takes over and is most common in calf muscles but can also be seen in quadriceps muscle, gluteal muscle, deltoid muscle, and rarely in masseter muscle. Strength imbalance between ankle antagonists (plantar flexors are stronger than dorsal flexors) contributes to the typical toe gait and deformities (equinovarus) of the foot (2). DMD runs a predictable course. Strength may improve up until the 6th or 7th year through decompensation of motor development albeit at a slower pace. It is followed by a plateau faze of 1 to 2 years, with relative stable muscle strength, whereafter linear deterioration of muscle strength initiates (5). This so-called decline faze is characterized by a rapid deterioration in stair climbing, rise to superior position, and walking a short distance in chronological order around the age of 6-11 years. Muscle reflexes are diminished or difficult to provoke. Joint contractures of the heel cords, iliotibial band, hip, knee, elbow, and wrist are noticed and are correlated with loss of ambulation. In general long leg braces are needed around the age of 10 years and DMD boys become wheelchair bound before the age of 12 years (2).

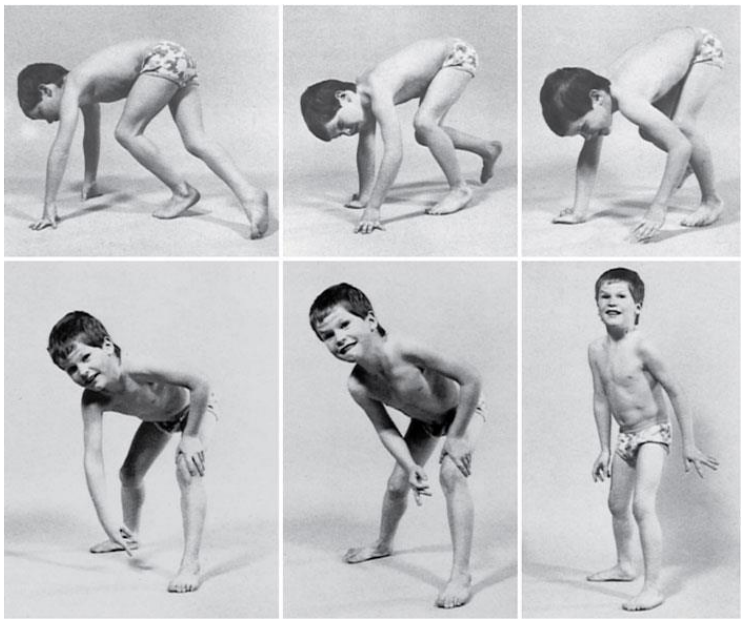

Figure 1: DMD boy demonstrating the Gower's sign. While he tries to rise from the floor, his legs are spread, his buttock is raised and he uses his hands to climb up his thighs as a compensation for the lack of proximal muscle strength in his legs (7).

Loss of secondary respiratory muscle strength, scoliosis and decline in vital capacity (as a consequence of confinement to a wheelchair) manifests in ventilatory insufficiency especially at night and can even cause respiratory failure (2, 5). Cardiac symptoms are present in 1 in 3 cases at the age of 14 years while approximately all boys above the age of 18 years suffer from cardiomyopathy. Presentation at an older age is probably due to their inability to exercise, which may mask cardiac symptoms. This hypothesis is empowered by the constatation of abnormal ECGs before the age of 6 years in 76% of all cases. Myocardial fibrosis dominantly occurs in the left ventricle and occasionally in the septa whereas atria and right ventricle are seldomly affected.

The conducting system, sinoatrial, and atrioventricular node can also be affected. As a result congestive heart failure and arrhythmias are prevalent and even more so during infections (2). Intestinal pseudo-obstruction, due to smooth muscle degeneration in the bowel wall, can be present. This manifests in vomiting, abdominal pain, acute gastric dilatation and distention. Gastric smooth muscle degeneration has also been described (2). Osteoporosis develops in the early stages of disease and primarily affects the lower extremities. DMD boys with osteoporosis have increased risk at fractures which would subsequently constrain their ambulation. Treatment with corticosteroids also increases the risk of vertebral compression fractures (2).

Progressive cognitive impairment has been described. The IQ of DMD boys is reduced with 1 standard deviation from the normal population (2). Although language development is typically delayed, verbal IQ tends to ameliorate with age (5). Additionally, DMD boys suffer from reduced praxis and executive functioning. Studies suggest a causal role for the dystrophin isoform that is highly represented in the brain as its expression is inversely correlated with the severity of the cognitive deficit (2). Respiratory failure and cardiomyopathy are the most common causes of death in DMD boys who generally do not live beyond the age of 30 years (6). Without ventilatory intervention and corticosteroid therapy death occurs at the age of 20 years (5).

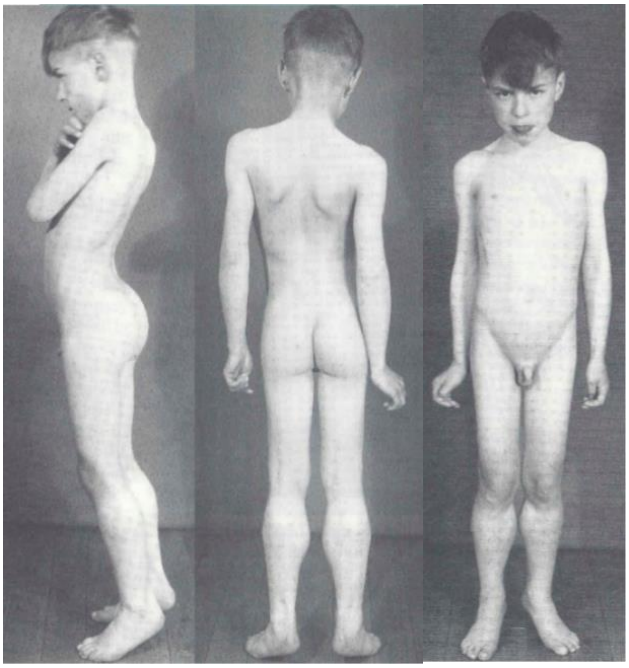

Figure 2: Boy with a text-book DMD posture. Pseudohypertrophy of the calve muscles, tip toe gate, forward tilted pelvis with compensatory hyperlordosis, scoliosis, protruding belly due to poor abdominal muscle strength, thin and weak thigh and gluteal muscles and shoulders and hands that are held backwards are typically seen (7).

1.1.2. BMD

BMD manifests as a milder and less progressive dystrophinopathy. It covers a wide spectrum of clinical presentations (2). Typically symptoms initiate at a later age compared to DMD boys, with some patients being diagnosed at childhood (50% of BMD boys develop muscle weakness at the age of 10 years) and others being diagnosed in mid- or even late adulthood (5). Pelvic and thigh muscles are most commonly affected first and calf pseudohypertrophy occurs at an early age (2). As helpful feature in differential diagnosis, neck flexor muscles are relatively spared compared to DMD boys (2). Gastro-intestinal manifestation is seldom (2). Cardiac involvement as seen in DMD also apply to BMD patients with a mean diagnosis of cardiomyopathy at the age of 14 years (2). BMD boys have a high risk of developing cardiomyopathy regardless of the skeletal muscle phenotype (1). Cognition is generally spared (5) and in the few cases it does occur it manifests less severe (2). Generally BMD boys live beyond the age of 30 years and death occurs between the age of 30-60 years (2).

1.1.3. IMD

Intermediate muscular dystrophy (IMD), a third frequently used dystrophinopathy, is considered an intermediate phenotype between DMD and BMD (mild DMD or severe BMD). Cut-offs for clinical diagnosis are patients who are ambulatory after the age of 12 years but lose their ability to walk before the age of 15 years. Whereas patients who still walk after the age of 15 years are classified as BMD (5) and boys who become wheelchair bound before the age of 12 years are classified as DMD (2).

1.1.4. Heterozygote female carriers

Most heterozygote female carriers of DMD mutation(s) are asymptomatic. Although some, approximately 2-8%, develop similar symptoms ranging from mild muscle weakness to a rapidly progressive DMD-like muscular dystrophy (2, 8). Cardiac involvement should always be considered in female carriers. Most commonly it manifests as subclinical ECG and echocardiographic abnormalities. 8% of female DMD-carriers develop dilated cardiomyopathy in comparison to 0% of female BMD-carriers. This emphasizes the importance of evaluating female carriers even though they are frequently asymptomatic (2).

1.2. The dystrophin protein

The functional unit of a skeletal muscle, the myofiber, is a multinucleate syncytium formed by the fusion of myoblasts. A myofiber consists out of multiple myofibrils which are subsequently formed out of sarcomeres alongside structural and regulatory proteins. Sarcomeres are formed by actin and myosin filaments. Muscle contraction results from the interaction between actin and myosin filaments that generates their movement relative to one another (9). Myofibers are enclosed by a fragile cell-membrane called the sarcolemma (10).

The 427 kDa muscle dystrophin (3,10) consists out of 4 major structural domains (Figure 3) (11). The N-terminal domain is encoded by exons 1 to 8. It consist out of 2 calponin-homology domains (CH1-2) that function as 2 f-actin binding sites (11). The central rod domain consists out of 24 spectrin like repeats (R1-24) divided by 4, and potentially a 5th, hinge region (H1-4) encoded by exons 8 to 61 (12). Specifically, R1-3 and R4-19 connect with membrane phospholipids, R11-17 holds a 3th f-actin binding site and R16-17 contains a nNOS binding site (12). Certain studies also describe the interaction between the central rod domain and microtubules and microfilaments (10, 11). The Cys(tein)-rich domain is encoded by exons 62 to 69. It consists out of the WW domain, ZZ domain and 2 EF hands and is involved in binding of β-dystroglycan (11). The C-terminal domain is encoded by exon 69 to 79 and originates in 2 coiled coil domains that connect with multiple proteins (11).

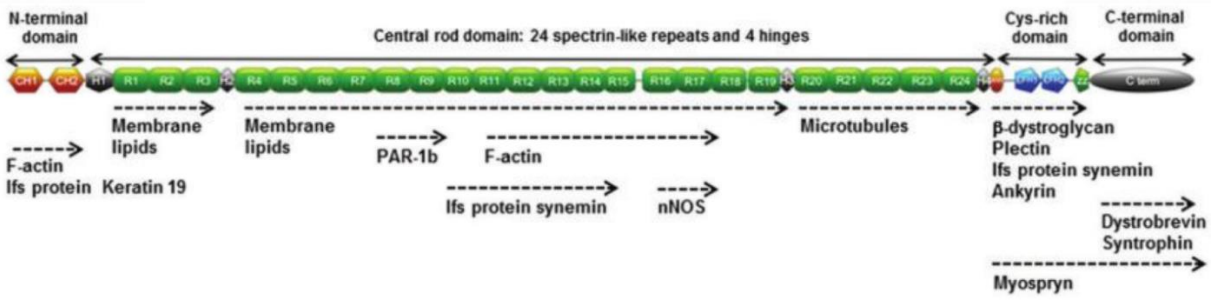

Figure 3: Dystrophin domains and partners. The dystrophin protein consists ot of an N-terminal domain (that holds 2 f-actin binding domains), the central rod domain (that binds phospholipids and nNOS, holds a 3th f-actin binding domain and possibly interacts with microtubules), a Cys-rich domain (that binds β-dystroglycan) and a C-terminal domain (that connects with multiple proteins) (11).

Dystrophin is an intracellular sarcolemmal bound protein that is highly represented in costameres in the shape of dystrophin-associated protein complexes (DAPC) (Figure 4). Costameres are sub-sarcolemmal protein complexes that prevent sarcolemma rupture during muscle-contraction with a threefold strategy 1) protection of the sarcolemma from contraction-induced damage, 2) alignment of the sarcolemma with nearby contractile structures, and 3) lateral transmission of forces from one myofiber to another (13). The C-terminal domain binds intracellular proteins (α1- and β1-syntrophin and α-dystrobrevin which subsequently bind nNOS) but also interacts with multiple other transmembrane (β-dystroglycan, α-, β-, γ-, and δ-sarcoglycan, and sarcospan) and extracellular proteins (α-dystroglycan and laminin-2) to form DAPCs (10). The Cys-rich domain binds transmembrane β-dystroglycan which in turn binds α-dystroglycan and other extracellular matrix proteins. The N-terminal domain binds intracellular f-actin. These connections result in attachment of the intracellular actin-cytoskeleton to the basal lamina of the extracellular matrix and, in such a way, provide stability as they form a bridge over the sarcolemma (3). Loss of dystrophin produces defects in the sarcolemma during muscle contraction and consequently results in a permanent state of muscle damage leading to muscle loss and chronic inflammation (12). Sarcolemmal lesions permit influx of calcium which activate calcium-dependent proteases that deteriorate the process (3). Though regeneration of muscle cells out of satellite (stem) cells occurs, periodic degeneration and regeneration will ultimately lead to depletion of the satellite pool contributing to muscle wasting (10). Diseased muscle tissue is gradually replaced by fibrous and fatty tissue causing pseudohypertrophy (10).

Studies demonstrated that general muscle weakness due to muscle wasting is distinct from exercise-induced fatigue. Intracellular components of the DAPC including α1- and β1-syntrophin, α-dystrobrevin and nNOS also function as regulators of the signal transduction at the sarcolemma. Complete loss of dystrophin will disrupt the nNOS link with the sarcolemma that is required for fast vasodilatation of muscle arteries during exercise. This will lead to insufficient supplies of oxygen not meeting up with the increased energy demand during exercise (10).

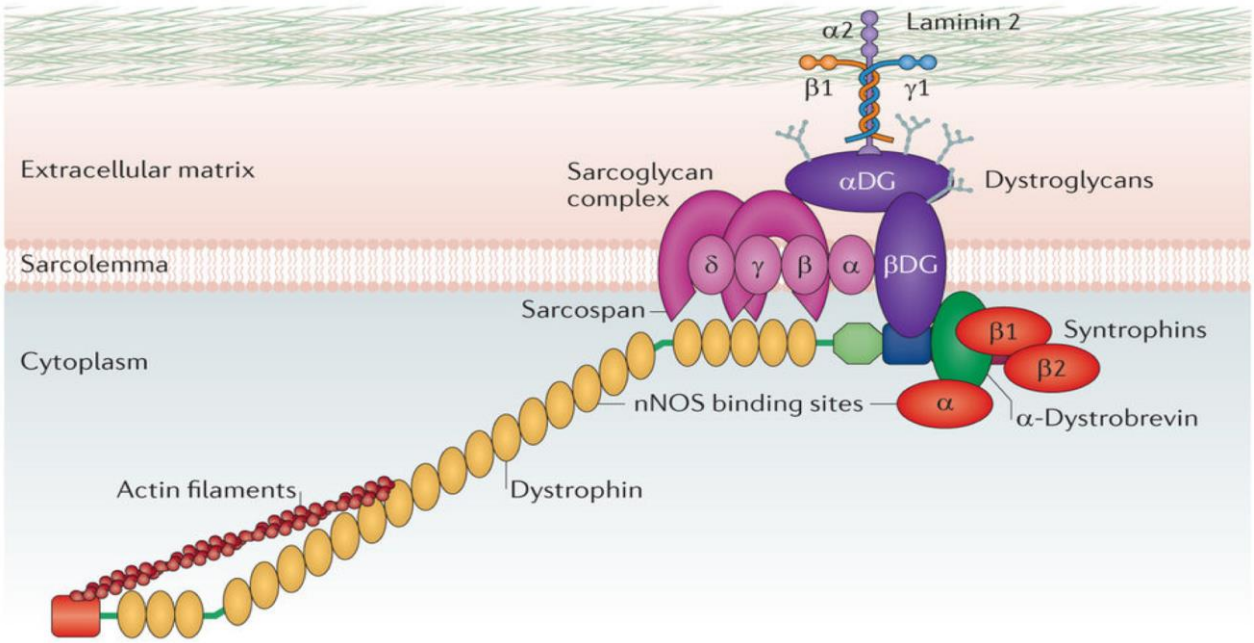

Figure 4: The dystrophin-associated protein complex (DAPC). Dystrophin associates with various extra- and intra-cellular proteins to form the DAPC. The DAPC is thought to play a structural role in linking the actin cytoskeleton to the extracellular matrix, stabilizing the sarcolemma during exercise (14).

1.3. Genotype

As previously mentioned dystrophinopathies are caused by mutations in the dystrophin gene. This massive gene is located on the X-chromosome and spans 79 exons (2). Because of its large size the gene is prone to mutations (3). Both DMD and BMD are inherited in a maternal recessive pattern (1). Approximately 20-30% of dystrophinopathies is caused by *de novo* mutations due to germline mosaicism (1, 2, 4). If this germline mutation occurs in males they risk having a female child that is a carrier. Whereas if this germline mutation occurs in females they are at substantial risk of having a DMD or BMD affected male child. Taking in account the potential risk of germline mosaicism, a non-carrier mother of a BMD or DMD affected boy risks having another affected son in at least 7–10 % (2). These sporadic cases cannot be foreseen with (prenatal) genetic counseling in a first pregnancy.

The mutational spectrum of the dystrophin gene is complex. According to Aartsma-Rus et. al (2016) the majority of DMD/BMD mutations are deletions (~68%) or duplications (~11%) but also small mutations or point mutations (~20%) and other types of mutations are found (<1%) (1).

Flanigan et al. (2009) reported the same results for duplications but a significantly higher prevalence of 'small' mutations or point mutations (46%) and significantly lower prevalence of deletions (43%). Mutation hotspots lie between exons 45–55 and exons 2–10 for deletions and duplications, respectively (1). Point mutations are distributed evenly across the gene and have no hot spots (15). The frequency of type of mutation varies within the phenotypic subgroups (4).

Deletions and duplications can cause 1) in-frame mutations (the number of lost or additional bases is dividable by 3), preserving the open reading frame (ORF), and 2) out-of-frame mutations (the number of lost or additional bases is not dividable by 3), disrupting the ORF. Small mutations involve point mutations and small deletions or insertions within an exon (15). According to their size, location and alteration in DNA sequence they may result in 1) nonsense mutations, converting an amino acid codon into a premature termination codon (PTC), 2) splice site mutations, disrupting the acceptor or donor splice site codon unrecognizable for the splicing machinery resulting in an exon deletion, and 3) in-frame or out-of-frame mutations (1). Other types of mutations are rare and involve deep intronic mutations, cryptic exons, missense mutations and translocations (1).

1.4. Genotype- phenotype correlation

Gene mutations that result in dystrophin with some retained protein function are consistent with BMD. Such mutations are usually in-frame mutations. Although the produced dystrophin proteins are altered in size and/or reduced in amount, they are still partially functional as they contain the N-terminal and C-terminal domain crucial for connecting the actin cytoskeleton to the extracellular matrix. Mutations that result in minimal (< 5%) (2) or complete absence of functional dystrophin are consistent with DMD (1). Such mutations are usually out-of-frame. Often out-of-frame mutations result in stop codons leading to premature termination of the protein thus inhibiting the production of dystrophin that contains the crucial N-terminal and/or C-terminal domain (1).

Although correlations between genotype and phenotype apply to abovementioned reading-frame rule in 96% of all DMD patients and in 93% of all BMD patients according to Tuffery-Giraud et al. (2009) (4) and 92% for both DMD and BMD according to Koenig et al. (1989) there are known exceptions to this rule (16). In accordance to the reading frame rule size or location of deletion or duplication leading to an out-of-frame-mutation should not affect the clinical phenotype as they

will all manifest as DMD. Nevertheless, three exceptions to this hypothesis were well documented. 1) Out-of-frame mutations before exon 8 can occur as a BMD phenotype, 2) patients with nonsense mutations in in-frame exons can present as a BMD phenotype, and 3) out-of-frame mutations flanking exon 44 show a milder DMD phenotype (1). Similarly, size and location of deletions or duplications that result in in-frame mutations, can only predict the clinical phenotype to a certain extent. 1) If an in-frame mutation affects all three N-terminal domains (exons 2-10 and 32-45) or the C-terminal domain (exons 64-70) a DMD phenotype will develop, 2) preservation of the third N-terminal domain while the other two are lost will manifest as a severe BMD phenotype and, 3) mutations between exons 10 and 40 cause milder and even asymptomatic phenotypes (1).

Female carriers are generally asymptomatic as a result of the protective effect of having two X-chromosomes. Random inactivation of X-chromosomes leaves 50% of the non-mutated X-chromosomes functional, making it possible to produce enough dystrophin to preserve good muscle function. Non-random inactivation of X-chromosomes, on the other hand, switches of >50% of the non-mutated X-chromosomes, resulting in <50% dystrophin positive muscle fibers. In these cases female carriers will be symptomatic (1, 2). Non-random inactivation occurs in twin pregnancies, Turner syndrome (XO) or Turner mosaic syndrome (XO/XX or XO/XX/XXX) (2) and in translocation mutations of the dystrophin gene where the unaffected gene will always be inactivated (causing a DMD phenotype) (1, 2).

Mutations in exon 1, the muscle promotor, and mutations that alter dystrophin domains of specific importance for cardiac muscle all result in X-linked dilated cardiomyopathy (2). Patients who suffer from this condition have cardiac muscle cells that lack normal dystrophin production. Skeletal muscle cells can successfully produce enough dystrophin by two alternative promoters that are normally only active in the brain (2). Consequently, X-linked cardiomyopathy patients produce solely cardiac symptoms in the absence of skeletal muscle pathology (1).

The presumption that genetic modifiers play a critical role in the development of a specific phenotype is based upon the observations that similar mutations can result in both DMD and BMD and that severity of presentation can vary for the same mutation even between relatives (1).

1.5. Diagnosis

Given the importance of early diagnosis and management of DMD/BMD, all medical workers who come in contact with children and family practitioners in particular should be attentive to early symptoms or signs of dystrophinopathies. Suspicion, family history not taken in consideration, is most commonly triggered when muscular problems develop in a male child (Gower's sign is typical especially when accompanied by a waddling gate) or when creatine kinase or transaminases (produced by both muscle and liver) are elevated in a regular blood check-up. In these cases, a **screening test measuring the creatine kinase** plasma concentration should be conducted (if creatine kinase is not readily determined). Normal creatine kinase concentrations rule out DMD/BMD diagnosis thus advocating for another diagnosis. Markedly increased concentrations should elicit further investigations by a neuromuscular specialist who will ultimately confirm or exclude the diagnosis of DMD/BMD. Importantly, creatine kinase cannot differentiate between DMD or milder forms of dystrophinopathies. In boys with a positive family history a creatine kinase screening test in the absence of suspicious signs may be useful (taken in account the age related differences in creatine kinase levels) and any small abnormality of muscle function should elicit high suspicion (17).

Diagnosis can be confirmed by genetic testing on a blood sample or muscle biopsy. Although it is encouraged to do genetic testing first as this can prevent the unnecessary invasive procedure of a biopsy, a muscle biopsy can be done depending on the availability of genetic testing in a clinical setting. **Muscle biopsies** are examined for histopathological features and protein expression by using techniques such as immunohistochemistry and Western blot analysis. Both immunohistochemistry and western blot make use of commercially available antibodies targeted against dystrophin protein domains. Immunohistochemistry detects the presence of dystrophin in the biopsied tissue (Figure 5.A.). In DMD boys, staining is absent, although in more than 50% of all patients small groups of so called 'revertant' fibers (arising from somatic mutations that correct the reading frame) can account for minimal staining. BMD affected boys show normal or partial immunostaining. Western blot determines the amount and the molecular size of dystrophin in biopsied tissue (Figure 5.B.). Next to its ability to distinguish DMD from milder phenotypes, this technique predicts disease severity. In patients diagnosed by genetic testing a muscle biopsy remains optional. In rare cases where creatine kinase is elevated, DMD-specific muscular problems are present but genetic testing turns out negative, muscle biopsy is the designated test to confirm or exclude the diagnosis. The same goes for boys that show suspicious signs, have a positive family history, but have no known family-specific mutation.

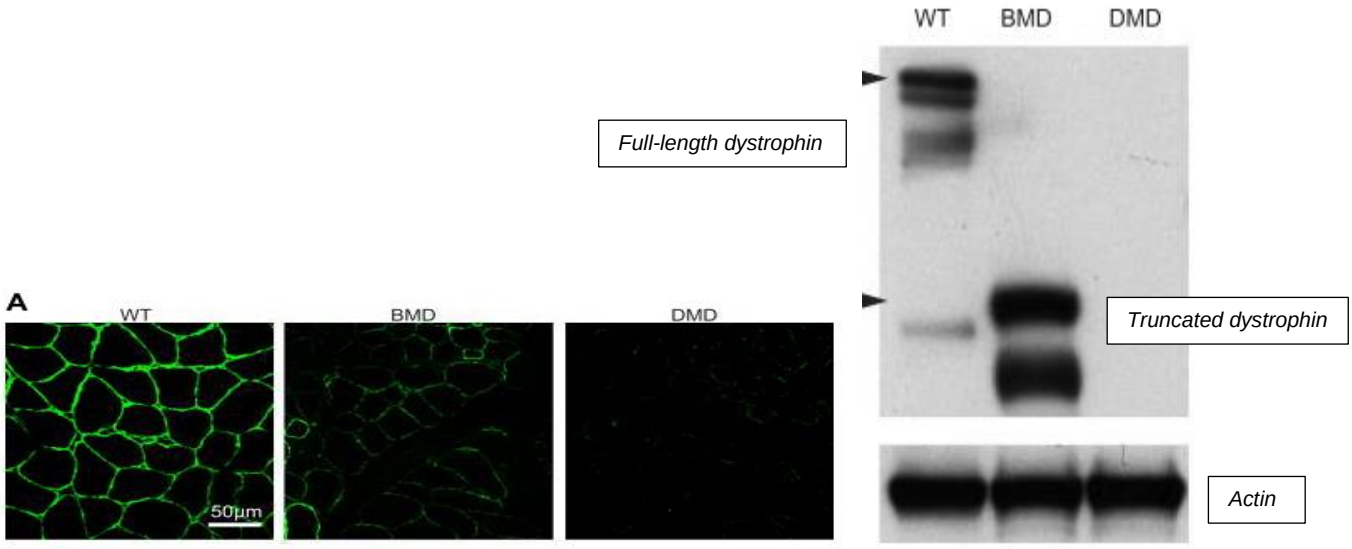

Figure 5: Diagnostic testing for dystrophinopathies. (A) Immunofluorescent analysis of muscle biopsy sections. Dystrophin (C-terminal antibody (dys1)) is detected at the sarcolemmal membrane of all muscle fibers in control tissue (WT). In BMD, the intensity of staining and the number of dystrophin positive fibers are diminished. In DMD, only rare dystrophin-positive fibers are detected. (B) An example of a Western blot (C-terminal antibody (dys1)). In control tissue (WT), full-length dystrophin is detected. In BMD tissue, shortened protein is present. In DMD, no dystrophin is present. Actin is used here as a loading control (18).

Genetic testing is mandatory even after positive muscle biopsy as it holds implications for phenotypical presentation and thus prognosis, genetic counseling, prenatal diagnosis and patients' compatibility for mutation-specific therapeutics. Detection of a deletion/duplication by PCR or the relatively rapid and inexpensive multiplex ligation-dependent probe amplification (MLPA) and multiplex amplifiable probe hybridization (MAPH) provides the first step of genetic diagnosis. When a boy is affected by a mutation other than a deletion/duplication, these tests will turn out negative making DNA sequencing to detect subexonic rearrangements or point mutations obligatory (2, 17).

After final diagnosis further investigations are elicited. As such, neuromuscular function and implications of the disease on everyday living are analyzed. Tests on muscle strength, range of motion, motor function, timed function and ADL are conducted. Pulmonary function, cardiac function, a standard medical- and family history and psychosocial wellbeing of patient and family are evaluated (17).

Genetic counselling, that uses the detected family-specific mutation as a screening marker to determine carrier status, is recommended for any at risk female family member. Similarly, confirmed family-specific mutations are used for prenatal diagnosis on amniocentesis or chorionic villus biopsy (2).

2. Objective

The purpose of this thesis is to analyze and synthesize the literature on experimental studies of cell and gene therapy in Duchenne muscular dystrophy. Hereby producing an integral summary of the progress made in the search for a curative treatment of Duchenne muscular dystrophy and Becker muscular dystrophy.

3. Methodology

Sources used to find relevant information were Pubmed, Embase and the library of the university of Ghent. In *Pubmed* a specific search was performed: Title: Duchenne, and: all fields: Gene therapy *((duchenne[Title]) AND gene therapy)*. This search yielded 564 publications. All publications were exported to Endnote and were subjected to strict exclusion criteria. Exclusion criteria were: publication older than 2000, publication type conference proceedings, editorials and letters to the editor, publication language other than English, female carriers of the Duchenne gene and no free full text available. Subsequently studies were screened on title and abstract. 35 references were retrieved for detailed evaluation. These involved 26 studies and 9 reviews concerning gene therapy. Ultimately, some of these studies were rejected because they were at the margin of relevance or were thought to have bad scientific quality.

The same search strategy was performed in **Embase**. This search came up with 406 publications. Studies were excluded in the same manner as mentioned above. After screening based on title and abstract we retrieved 47 references for detailed evaluation. These involved 35 studies and 12 reviews concerning gene therapy. Similarly, some of these studies were rejected because they were at the margin of relevance or were thought to have bad scientific quality.

Online e-books were retrieved from the library of the university of Ghent (URL: www.kcgg.ugent.be) by the implementation of the search question ‘muscular dystrophies’. Three online books and one book in paperback were withheld and provided a great deal of valuable information.

Reviews were used as a source of information for the introduction of this paper, functioned as a guideline for the results topic and served as a helpful reference source. Furthermore, articles were withheld through the automatically **‘similar articles’** suggestions at the side bar in Pubmed.

4. Results

4.1. Hurdles in DMD gene therapy

Gene therapy for DMD poses specific challenges. Firstly, the dystrophin gene is the largest gene thus far known. Consequentially, conventional vectors cannot encapsulate the full dystrophin cDNA because of its large size and the substantial gene brings about a complex transcriptional regulation. Secondly, muscle tissue is distributed bodywide and presents as most abundant tissue in the human body which makes target delivery extremely challenging. Hypothetical, it is not hard to imagine that drugs injected directly into muscle fibers do not have the potential to cure DMD as this approach would not be applicable to clinical practice. That being said, systemic delivery poses a desirable approach although this method shows specific challenges too. Thirdly, insertional mutagenesis is seen in many gene therapy approaches and is a point of concern because of the risk of tumorigenesis. Lastly, auto-immune reactions against all 'new' incorporated products are seen in most gene therapy strategies and are not to be taken lightly as they can be very severe (19).

4.2. Supportive therapy

At present, most DMD treatments are palliative at best, aimed at managing problems with ambulation, respiration, and cardiac health. Up till recently, corticosteroids (Prednisone and Deflazacort) were the only available drugs that delay the decline in muscle strength and functioning in DMD. Still, as only Ataluren (Stop codon read-through therapeutic, see chapter 4.3.2.) and Eteplirsen (exon skipping therapeutic, see chapter 4.3.1) have just recently been approved in the European Union (EU) and United States (US) respectively, corticosteroids remain standard of care in dystrophinopathies and should be considered in all affected patients until definite treatment is available. Corticosteroids reduce the risk of developing scoliosis consequently resulting in delayed deterioration of pulmonary function. Prolonged ambulation was seen after corticosteroid treatment. Furthermore, corticosteroids are suggested to have a positive influence on cardiac function (although no hard outcomes were measured in studies) (17). Significant intellectual improvements in DMD patients that received corticosteroid therapy were also demonstrated (20). Unfortunately, all these improvements are temporary and the numerous potential serious side effects that come along with chronic corticosteroid therapy limit their use. If adjustments in dosing or changing to an alternative regimen cannot manage side effects,

corticosteroid therapy should be discontinued. Typical side effects include: small stature, weight gain, osteoporosis, increased risk of cataracts, delayed puberty, and a tendency toward behavioral changes (17).

When initiation of corticosteroid therapy should proceed is an individually based choice as no fixed moment is described. Most commonly, treatment is initiated when a DMD boy enters the plateau phase usually between the age of 4-8 years. Treatment is preferentially started before the decline phase and is highly recommended in this stage of disease. Corticosteroid therapy after loss of ambulation is not studied well and is most commonly discontinued although some studies suggest positive effects (5, 17).

Prednisone and Deflazacort have equal efficacy. The recommended starting dose for Prednisone and Deflazacort is 0.75 mg/kg/d and 0.9 mg/kg/d, respectively. The dose is commonly increased as the child grows to a maximum of 40 kg (17).

4.3. Gene correction therapy

4.3.1. Exon skipping

Exon skipping therapy is based on the use of antisense oligonucleotides (AO). AOs are chemically modified nucleic acid sequences that are employed to bind complementary to target regions of pre-mRNA and regulate the splicing machinery, thereby making it possible to induce (multi-)exon skipping of the final transcript. AOs aim to restore the ORF by turning an out-of-frame mutation in an in-frame mutation, allowing the production of a smaller but (partially) functional protein. The DMD phenotype will consequently alter to that of the milder BMD (21).

One of the first AOs that held potential for DMD treatment was **PRO-051** or **Drisapersen,** developed by BioMarin Pharmaceutical, and targets DMD exon 51. Drisapersen is a 2'O-MePS type of AO and is negatively charged in physiologic pH resulting in the need of high doses for its potential effect (21). Although it proceeded into stage III of clinical trials, it was rejected by the FDA[1] in 2016 due to long-term (kidney) toxicity side-effects (caused by the high doses) and insufficient evidence of clinical utility (21, 22). Currently the focus is shifted to Eteplirsen.

[1] The Food and Drug Administration (FDA) is a federal agency of the United States Department of Health and Human Services responsible for protecting the public health by ensuring the safety, efficacy, and security of human, veterinary drugs, biological products, and medical devices *(source: https://www.fda.gov).*

AVI-4658 or **Eteplirsen** (brand name **Exondys 51**), developed by Sarepta therapeutics and conditionally approved by the FDA on September 2016, is the first and hereto only available drug on the US market that treats DMD on a molecular level (21). Eteplirsen is a 30-nucleotide phosphorodiamidate morpholino oligomer (PMO) type of AO. Like Drisapersen, Eteplirsen targets the pre-mRNA of DMD exon 51 (Figure 6). Covering ~14% of DMD affected boys who have a confirmed mutation of the DMD gene that is amenable to exon 51 skipping, it addresses the largest subgroup that can potentially benefit from a single-exon therapeutic (21). Because of its different backbone, Eteplirsen is uncharged in physiological pH, making it stable and resistant to biological degradation compared to the unstable Drisapersen. Eteplirsen can be administered intramuscular (IM) but is most commonly administered intravenously (IV) as systemic delivery is desirable (21).

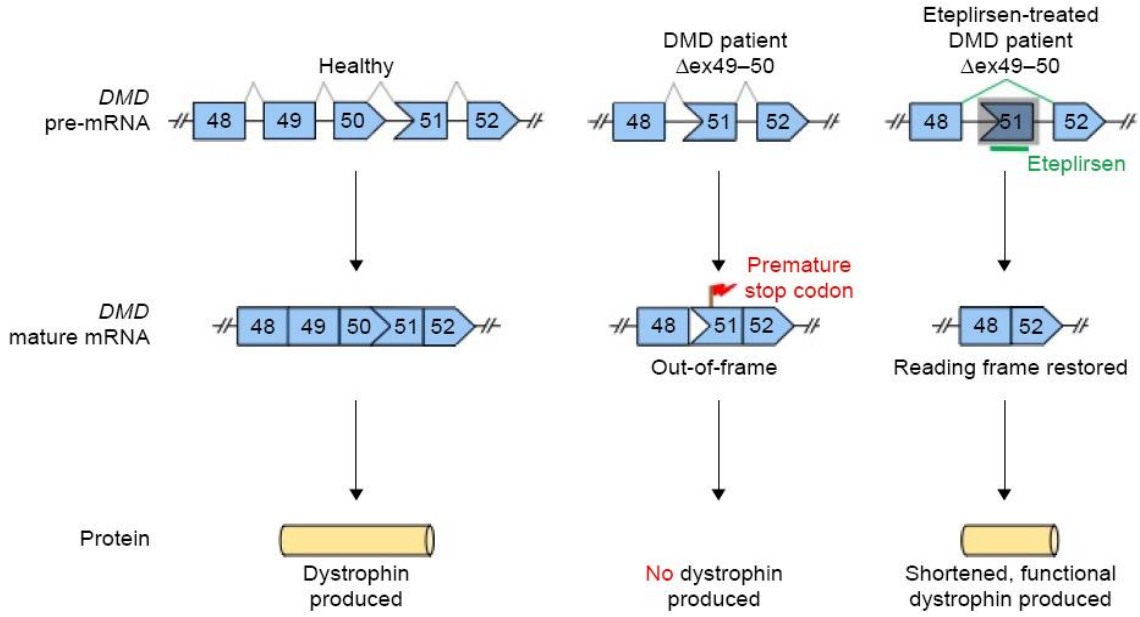

Figure 6: working mechanism of exon skipping therapeutic Eteplirsen. A exons 49-50 deletion results in an out-of-frame mutation which results in a premature stop codon. In this circumstance no dystrophin is produced consistent with a DMD phenotype. Eteplirsen targets the splicing machinery with the aim of skipping exon 51 in the pre-mRNa transcript to restore the open reading frame. Shortened but (partially) functional dystrophin is produced consistent with a BMD phenotype (21).

A first open label, dose-ranging study by Cirak et al. (2010) enrolled nineteen patients in 6 cohorts. Cohorts were given 0, 5, 1, 2, 4, 10 or 20mg/kg/week IV Eteplirsen therapy for twelve weeks. Seven patients showed improved dystrophin levels post-treatment. Six of them had received 20mg/kg/week and one had received 2mg/kg/week (21). More extensive studies were performed by Mendell et al. (2013,2016). A double blind, randomized, placebo-controlled trial consisting of 3 cohorts of four patients each, received 30mg/kg/week, 50mg/kg/week or placebo for 24 and 48 weeks. The 30mg/kg/week cohort showed a significant mean increase of 22,9% and 51,7% in dystrophin-positive fibers, after 24 weeks and 48 weeks of therapy, respectively. Likewise, the group of 50mg/kg/week showed an increase of dystrophin positive fibers, be it that the mean increase at 48 weeks was lower (42,9%) than that of the 30mg/kg/week group. Clinical outcomes showed a significant difference in 6 minute walking test[2] (6MWT) in both the 50mg/kg/week group and in the combined dose group 3 years 48 weeks post-treatment. Delay in pulmonal deterioration was seen in the combined dose cohort. All positive effects of the study drug arise from a delay in disease progression rather than an increase in clinical performance (21). After a subsequent controversial debate concerning the efficacy of the drug because of FDA doubts on the reliability of the Mendell et al. (2013,2016) study results, a conditional approval was granted by the FDA in 2016. In continuation of this conditional approval, Sarepta therapeutics proceeded with a, currently still ongoing, phase III trial (NCT02255552). In this trial 2 cohorts of both eighty patients received 30mg/kg/week IV Eteplirsen therapy or placebo for 96 weeks. Preliminary data of 13 patients 48 weeks post-treatment showed a statistically significant increase of dystrophin from 0,22%-0,32% of normal values. For a DMD patient to experience clinical benefit, dystrophin values should be at least 10% of healthy dystrophin amounts. Due to discrepancy between abovementioned study results and this critical clinical threshold, it is still controversial if Eteplirsen therapy is of any clinical meaning (21).

According to Lim et al. (2017) abovementioned studies lack efficacy and good clinical practice. Lack of efficacy could be due to several factors. One of them is the issue of fast renal clearance of Eteplirsen due to its neutral nature and water solubility. This makes systemic delivery and target tissue uptake challenging. A higher dose and administration frequency could potentially ameliorate uptake but were not examined or could not be clearly established in these studies. Administration with hexoses[3] and conjugation with cell-penetrating peptides could benefit cell uptake and show

[2] The six minute walking test (6MWT) measures the distance walked over a span of 6 minutes. A change in 6 minute walking test is considered of clinical importance and is a predictor of disease progression in ambulatory DMD patients. The 6MWT is commonly used as a primary clinical endpoint (endpoint that functions as the main outcome of a study) in ambulatory DMD trials (23).

[3] Hexose solutions include 5% glucose/fructose or 5% fructose solutions (21).

promising results in animal models. Additionally an assumption was made that the lack of efficacy could be due to the fact that the produced truncated dystrophin post-treatment was not as functional as was initially hoped. Lastly, it was questioned if the Eteplirsen sequence is the most optimal target sequence. Lack of good clinical practice include absence of good controls, sample heterogeneity (as enrolled participants were often of different age and had different types of mutations), limited outcome measures (as the 6MWT is restricted to ambulatory patients and is motivation dependent), low sample size and problems with reliability and reproducibility of results (21).

It could be concluded that Eteplirsen is a highly stable therapeutic which makes unwanted insertional mutagenesis very unlikely. Considering the fact that Eteplirsen does not bind Toll-like receptors[4] it cannot initiate an innate immune response and no anti-dystrophin antibodies were found post-treatment. Eteplirsen was well tolerated in all trials resulting in an excellent long-term safety and tolerability profile. Notwithstanding this fact, 50% of the study population in the Mendell et al. (2013,2016) trials showed general side-effects like vomiting, headaches, balance disorders and proteinuria (21).

Eteplirsen shows immense promise for future curative treatment as it directly addresses the molecular etiology of DMD and has a favorable safety and tolerability profile. Although Eteplirsen is now accessible to patients in the US, there are still efficacy uncertainties. So far the treatment has not proven its curative character for DMD patients. Final approval of the FDA is based upon further investigations of the efficacy of the drug. Eteplirsen has the drawback of only addressing patients affected by an exon 51 out-of-frame mutation covering only ~ 14% of DMD affected boys and leaving other patients in the dark. Another disadvantage is the obligatory (IV) readministration (21).

On a related note, AO-based exon-skipping therapeutics targeting other exons or targeting multi-exons are being produced by Sarepta therapeutics and other biopharmaceutical companies. **SRP-4045** and **SRP-4053** target exon 45 and 53, respectively, and are currently in a phase III trial (NCT02500381). **NS-065/NCNP-01** targeting exon 53 is currently recruiting for a phase II clinical trial (NCT02740972) (21). Production of these therapeutic drugs is greatly encouraged as they are applicable to a broader spectrum of mutations. In theory 83% of all DMD patients could benefit from (multi-) exon skipping strategies (25).

[4] Toll-like receptors (TLRs) are transmembrane proteins expressed by cells of the innate immune system, which recognize invading pathogenic microbial infections and induce immune and inflammatory responses (24).

4.3.2. Stop codon readthrough

Mutations that result in premature stop codons, also called premature termination codons (PTC) or nonsense mutations, cover ~ 13% of the DMD population (26). PTC-generating mutations alter DNA base pair(s) so that a sense codon is recoded in a stop codon (UAA, UAG, UGA) that is premature to the normal stop codon at the 3' end. This results in premature translation termination, consequently producing a truncated, usually non-functional and/or unstable protein product. Stop codon readthrough at PTCs is a process that occurs when recognition of the default termination codon by the release factor complex is replaced by recognition of the termination codon by a near-cognate aminoacyl-tRNA (tRNA that forms weakly pairs with multiple mRNA codons). In this manner stop codon readthrough therapeutics help near-cognate aminoacyl-tRNAs to compete with the release factor complex. Instead of premature protein termination, the tRNA will implement an amino acid of own choice whereafter normal sequence translation can continue (Figure 7). Stop codon readthrough is expected to create a (partially) functional dystrophin protein unless the overridden PTC sequence used to code for a protein-crucial amino acid (27). Certain factors make PTCs more susceptible to stop codon readthrough therapy than normal stop codons. If this was not the case, readthrough of normal stop codons would potentially increase protein elongation and the amount of misfolded proteins (with consequences on cellular responses) (26).

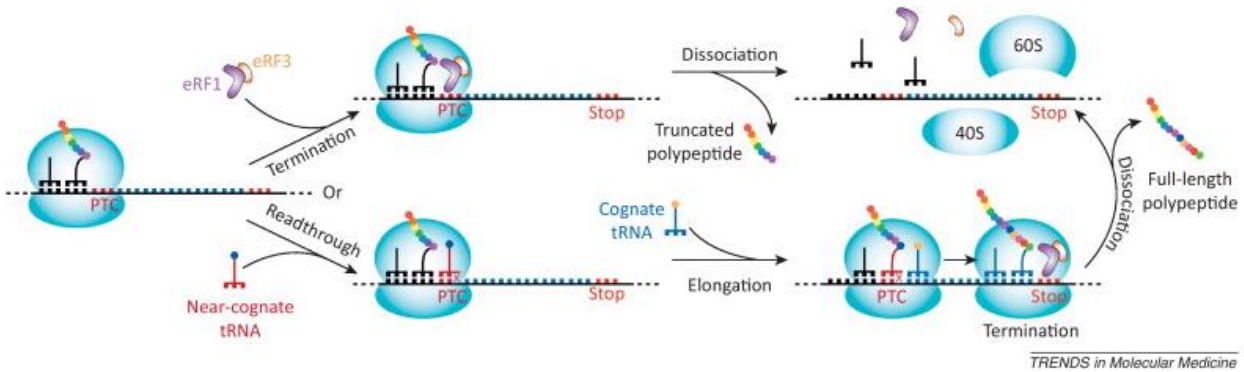

Figure 7: Working mechanism of stop codon readthrough therapeutics. Release factors bind the A site of the ribosome at a stop codon and facilitate cleavage of the polypeptide chain. Consequently, a PTC-generating mutation in the dystrophin gene manifests in premature translation termination. This results in a truncated, usually non-functional dystrophin. Stop codon readthrough therapeutics help near-cognate aminoacyl-tRNAs to compete with the release factor complex and aim to stimulate the production of a (partially) functional dystrophin protein (28).

The first developed stop codon readthrough drug**, Gentamicin**, is a small-molecule drug that can be administered IV (primary administration route in most studies) or IM. An extensive study by Malik et al. (2010) provided the evidence that Gentamicin works selectively on PTC mutations. A second study group consisted of 14 boys that received 7,5mg/kg weekly or twice weekly for 6 months. A significant increase in dystrophin levels was seen in six boys. Moreover, dystrophin levels increased markedly in three boys. The first boy (administered 7,5mg/kg once weekly) showed a 3,54 fold increase in dystrophin levels and the two other boys (administered 7,5mg/kg twice weekly) showed a 5,06 and 3,44 fold increase in dystrophin levels. All three boys reached dystrophin levels between 13% and 15% of normal dystrophin amounts bypassing the 10% threshold of functional benefit. Serum creatine kinase reduced significantly under treatment. Although no significant clinical benefit could be stated and average muscle score and time function tests (TFT)[5] showed no improvements what so ever, forced vital capacity increased slightly and maximal voluntary isometric contraction showed little improvements. An increase in dystrophin levels after Gentamicin treatment was predictable in patients that had measurable dystrophin levels at baseline but was not seen in patients who lacked any baseline production. This suggests that Gentamicin therapy is more prone to so called 'leaky' mutations[6] (29).

In this study no adverse events were seen in patients who received the correct dose of Gentamicin. Immune responses were seen in patients that lacked all baseline production pretreatment but produced 'foreign' dystrophin post treatment (29).

Gentamicin brings about obstacles with its use. That is to say, its working mechanism depends strongly on the neighboring sequence of the target codon. Furthermore the narrow therapeutic window and IV administration of the drug results in intensive treatment and monitoring. Long-term side-effects, specifically renal toxicity and ototoxicity, are also feared (26, 27).

Although the working mechanism of **Ataluren** or **PTC124** is similar to Gentamicin, it binds another part of the ribosome. In comparison to Gentamicin, Ataluren benefits from the fact that it has an excellent safety profile and is administered orally. In a study by Bönnemann et al. (2007) Ataluren was administered in 38 DMD/BMD boys for a period of 28 days. Three cohorts received 16, 40 or 80mg/kg/day in 3 daily doses. Results showed an increase in muscle dystrophin levels and a

[5] Time function tests (TFT) are a set of tests that measure functional capability in ambulatory patients. It includes timing of a 10-meter walk, standing from supine, and 4-stair climb and descent. It is considered as complementary to the 6MWT (23).

[6] The natural process of nonsense mutation-mediated decay contributes to the decay of aberrant mRNA. Some nonsense mutations however provide the ability to escape this important mRNA control. So-called 'leaky' mutations allow low levels of (partially) functional protein consequently resulting in the BMD phenotype (27).

reduction in creatine kinase concentrations in most patients post-treatment. *In-vivo*, no clear dose dependent response and no correlation to mutation type or mutation site could be observed (26).

A phase IIb, double-blind, placebo-controlled trial by Bushby et al. (2014) investigated Ataluren treatment in DMD boys with confirmed nonsense mutations. Three cohorts were administered 40mg/kg/day (N=57), 80mg/kg/day (N=60) Ataluren or placebo (N=57) in 3 daily doses for 48 weeks. Although results were not significant, the 6MWT showed a mean difference of 31,3 meters (m) for the 40mg/kg/day cohort compared to the placebo group (Figure 8). As a minimal difference of 28,5 to 31,7m in the 6MWT is expected to improve clinical outcome, Ataluren offers promise as a treatment for DMD (30).

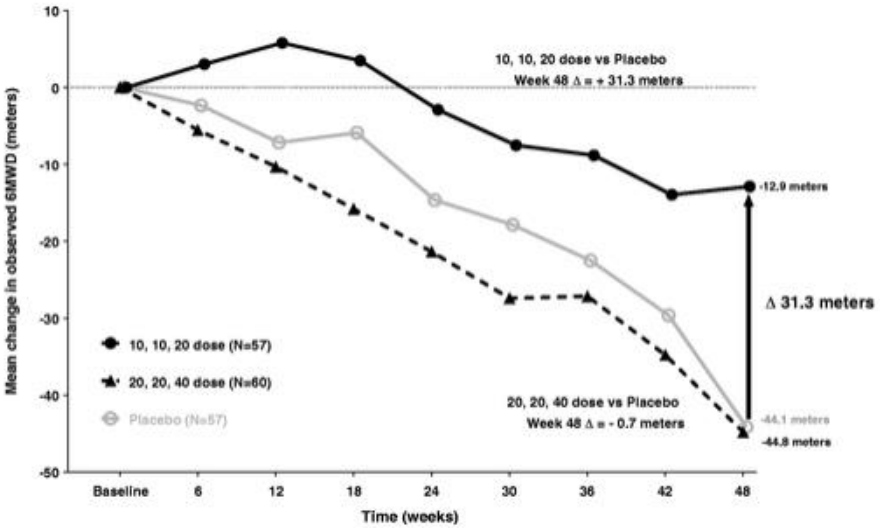

Figure 8: Mean change in 6MWT after Ataluren treatment with 40mg/kg/day, 80mg/kg/day or placebo for 48 weeks (31).

According to the expectation that effect of Ataluren treatment would be more pronounced in patients that have entered the decline phase, this subpopulation[7] was looked at more distinctly. As predicted, the mean difference in 6MWT increased to almost 50 m in this subpopulation compared to the placebo cohort (Figure 9) (30).

[7] The decline-phase subgroup encompassed patients >7 years of age, treated with corticosteroids, 6MWT ≥150 m, and <80% predicted 6MWT (30).

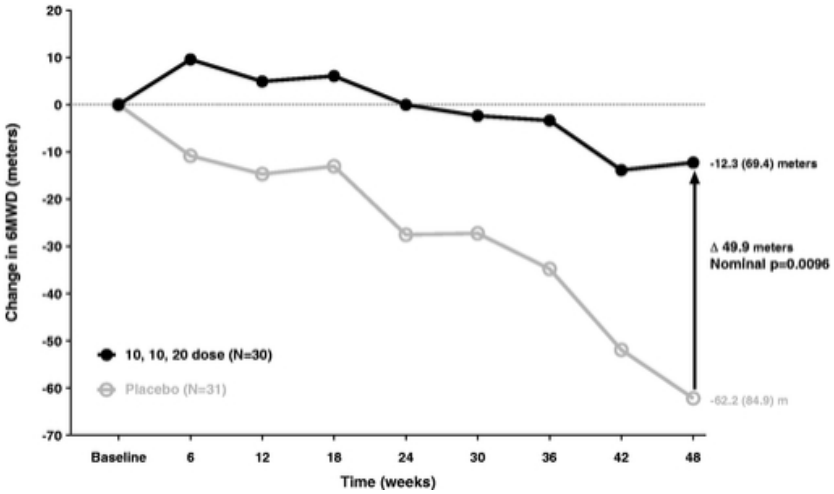

Figure 9: Mean change in 6MWT in the decline phase subgroup after receiving 40mg/kg/day of Ataluren or placebo for 48 weeks (31).

Furthermore, the 10% change in walking ability[8] showed a meaningful delay in decline in ambulation for the 40mg/kg/day cohort compared to the placebo group (Figure 10) (30).

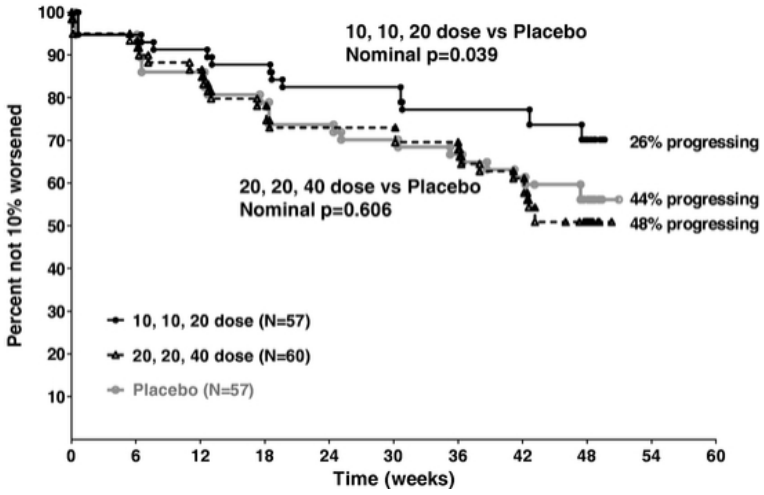

Figure 10: The proportion of patients that progress into persistent 10% worsening in 6MWT after Ataluren treatment with 40mg/kg/day, 80mg/kg/day or placebo for 48 weeks (31).

A minimal effect of less deterioration in muscle function (assessed by TFTs) and muscle strength was seen in the 40mg/kg/day cohort compared to the placebo group. Although small, these effects are considered of great clinical benefit in daily life activities of DMD patients. No effect was seen in the 80mg/kg/day cohort compared to the placebo group. This is consistent with the bell shaped

[8] A change of at least 10% in walking ability in one year is a predictive parameter for the clinical decline in DMD patients (30).

dose-response curve of Ataluren, as earlier described in *in vitro* studies on myotubes of *mdx*-mice and DMD-patients (appendix 7.1.) (30, 32).

In July 2014, the EMA[9] granted a conditional approval for Ataluren in the EU. Because the benefits of the drug outweigh the uncertainties and no other treatment is readily available, pharmaceuticals can since produce this drug under certain circumstances (like performance of further studies). Translarna, a PTC therapeutics drug, is now available in the EU for DMD boys of 5 years and older with a confirmed nonsense mutation (33). Ataluren is currently being reviewed by the FDA for approval in the US.

As a condition to the early approval, PTC therapeutics proceeded studies in the investigation of Ataluren. In September 2017 results of a phase III, double-blind, randomized, placebo-controlled, trial that investigated Ataluren treatment in DMD boys with confirmed nonsense mutations were published. Two cohorts were administered 40mg/kg/day Ataluren (N=115) or placebo (N=115) over a period of 48 days. Delay of disease progression, assessed by 6MWT, showed a least-square means (LS means) change of - 47,4m in the Ataluren group compared to - 60,7m in the placebo group post treatment. Although not significant, this results in a LS means difference of 13m.The subpopulation[10] of patients in the decline phase showed a significant LS means difference of 42,9m compared to the placebo group (34). Less decline of muscle and physical function, assessed by TFTs, was seen in the Ataluren group compared to the placebo group but was only significant for the 4-stairs descent test. Exploratory endpoints like change in physical capacity, assessed by the North Star Ambulatory Assessment resulted in 12,9% loss of physical function in the Ataluren group versus 18,8% loss in the placebo group resulting in an overall reduced risk of 31%. Pediatric Outcomes Data Collection Instrument and activities of daily living showed no significant differences between both groups. It was well proven that the decline-phase subgroup benefitted most from Ataluren treatment compared to the other subgroups (34).

It is worth nothing that this study has its limitations. First, non-stringent inclusion criteria resulted in a study population that varied greatly in baseline 6MWT. This affected study results as the decline-phase subgroup benefits most from Ataluren treatment. As such, more stringent inclusion criteria can contribute to favorable outcomes for Ataluren. Secondly, considering the slow degenerative progression of the disease, longer treatment observations should be pursued in

[9] The European Medicines Agency (EMA) is an agency of the European Union (EU) that is responsible for the scientific evaluation, supervision and safety monitoring of medicines in the EU *(source: http://www.ema.europa.eu).*
[10] The decline phase subgroup included patients that performed 300m or more, but less than 400m at a baseline 6MWT (34).

future studies. An extensive study on efficacy and safety of Ataluren that will span 72 weeks of observation and a 72-week open-label extension is currently planned (NCT03179631). Lastly, as the 6MWT is a motivation dependent test, it is favorable that we keep looking for more objective measures to evaluate disease progression in DMD patients (34).

All abovementioned studies confirmed the excellent safety and tolerability profile of Ataluren. Adverse effects were not more commonly seen compared to the placebo group. However, some studies indicated that Ataluren can result in a, dose dependent, reversible increase of liver enzymes, plasma cholesterol and triglycerides. Ataluren may also potentiate the aminoglycoside nephrotoxicity. Concomitant use of Ataluren and IV aminoglycosides is contra-indicated (32).

4.4. AAV-mediated transport

Adeno-associated virus (AAV) is a small, non-enveloped, single stranded DNA virus that belongs to the *Parvoviridae* family and is subcategorized as a *Dependovirus*. *Dependorviruses* normally require the presence of a helper virus such as adenovirus or herpesvirus to replicate (35). Although AAV infection is common in human adults, as more than 90% show anti-AAV antibodies, no human pathogenicity could be linked to the virus (36). The wild-type AAV genome is approximately 4.7 kB in size. It comprises two Inverted terminal repeat (ITR) regions at the terminal ends and two open reading frames known as the *rep* and *cas* region. The *rep* genes code for replication and packaging proteins while the *cas* genes code for three capsid proteins of the virus. ITRs are important replication originators as they contain a primer for second-strand DNA synthesis by DNA polymerase present within host cells. ITRs are the only crucial regions for vector replication and as such need to be preserved in genetically engineered recombinant AAV (rAAV) (35, 36).

In rAAV vectors, all viral genome sequences are deleted except for the indispensable ITRs, between which a transgene cassette and its promotor are inserted. In such a way, engineered viral vectors exploit the efficient transfection mechanism of native viruses while simultaneously inserting a transgene into host cells (37). For DMD treatment, the ideal approach would be to engineer a rAAV that encapsulates the entire dystrophin gene. However, as the cargo capacity of AAV vectors is limited to approximately 5 kb, a much smaller or so called '**micro-dystrophin**' gene needed to be developed, reducing the native dystrophin gene to a 3,6 kb cDNA construct (Figure 11).

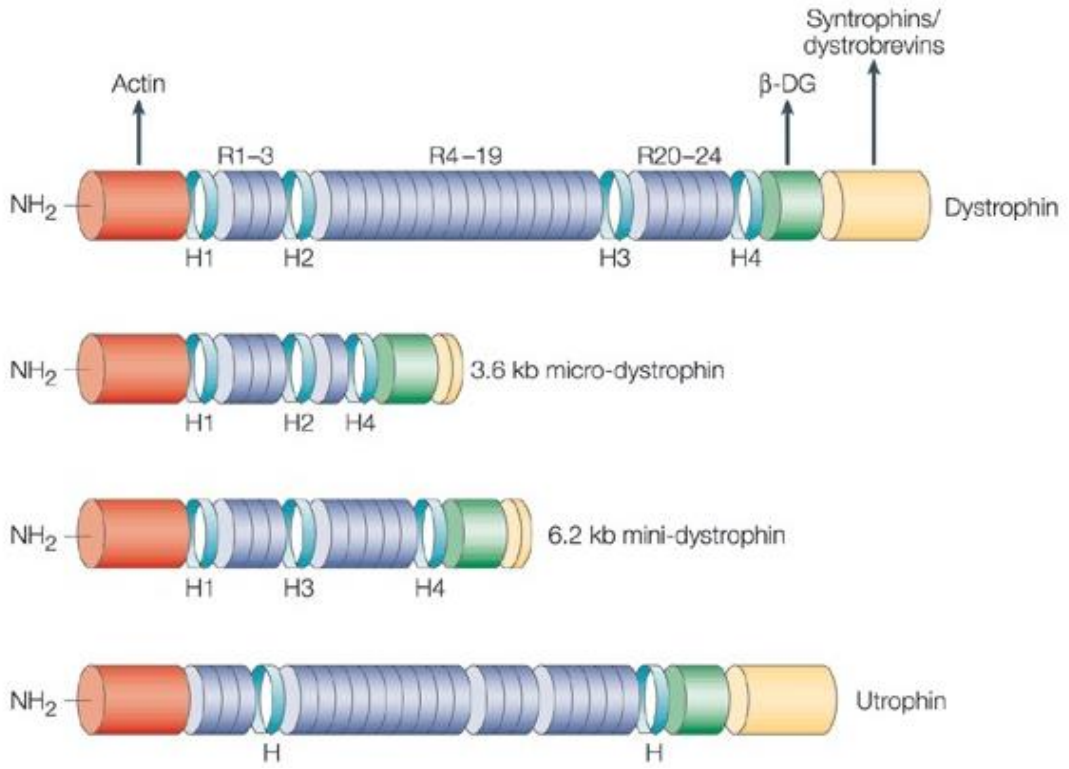

Figure 11: Presentation of the full-size dystrophin gene and the smaller micro-dystrophin and mini-dystrophin genes. Utrophin can be used as surrogate protein for dystrophin (38).

The ultimate balance between reducing the dystrophin gene to its protein-crucial domains and preserving the stability and functional activity of the truncated dystrophin product has been studied in great dept. Protein-crucial regions are the N-terminal, actin-binding domain and the Cys-rich, dystroglycan-binding domain. Dystrophin is most tolerable for removal of the C-terminal domain and secondly for the rod domains. However, 4 or more rod domains need to be preserved for high functionality of the protein construct (35).

As producing a smaller micro-dystrophin transgene is one option to overcome the limited cargo-capacity of AAV vectors, co-delivery methods using overlapping vector approaches is another option to address this problem. In this manner, multiple AAV vectors that contain parts of the split dystrophin transgene can recombine *in vivo* providing the advantage of a larger dystrophin construct called '**mini-dystrophin**' (Figure 11). Studies showed amelioration of exercise-induced muscle injury and functional ischemia in a *mdx*-mouse model after dual vector therapy. In a study by Odom et al. (2011) expression of a 'highly functional' micro-dystrophin was seen after IV administration of two rAAV6-microdystrophin vectors as a result of dual delivery (37). In contrast, triple vector strategies showed less positive results due to safety factors (39).

AAV vectors are often assumed to transduce solely as extra-chromosomal episomes[11] in the nucleus but at least a small fraction of transgenes is integrated in the host genome. The episomal transgenome provides long-term expression of transgenes mostly in non-dividing cells as this extra-chromosomal DNA gets rapidly lost after cell division. The integrated transgenes are replicated and segregated during mitosis. Consequently, it results in long-term expression in both dividing and non-dividing cells (35, 40). Skeletal and cardiac muscle tissue consist of post-mitotic, non-dividing cells (37). Although AAVs integrate vector DNA in the host genome at a much lower frequency rate than retroviruses and insertion occurs in a stable and predictive manner, it is important to give thought to the effects of possible insertional mutagenesis. One of the most feared consequences of this process is malignant transformation of host cells (41).

Many AAV serotypes have been acquired so far. Serotypes differ in capsid proteins while they encapsulate the identical genome. Different capsid proteins bind other cell receptors which results in specific tissue tropism for each serotype. A number of studies showed high transduction efficiency in striated muscle with AAV1,6,7,8 and 9. Be that as it may, serotype 2 AAV vectors are most frequently employed in studies (35).

Both localized IM and systemic IV/ intra-arterial (IA) administration routes have been proposed for rAAV vectors. As skeletal muscle tissue is distributed bodywide and is bulky in size, systemic delivery is a favorable approach. However, systemic delivery, resulting in target (muscle) as well as well as off-target (non-muscle) transduction, elicits immune responses. Luckily, immune responses seen after rAAV administration are mild compared to approaches with other recombinant viruses. Nevertheless, evading immune responses against rAAV capsid proteins, transgenes and newly produced dystrophin are of great importance because of immunocytotoxic consequences but also because of rAAV neutralization by antibodies (35). Numerous strategies that aim to bypass unwanted immune responses have been described. First, transient immune suppression during rAAV administration or delivering a dystrophin surrogate (which would not be recognized as foreign), like micro-utrophin (see chapter 4.7.1.), aim to prevent immune responses. Secondly, the use of alternative serotypes, capsid engineering and plasmaphoresis are proposed strategies to evade pre-existing antibodies. Lastly, efficient muscle targeting is not only desired for efficacy purposes, but holds a second motive of diminishing immune responses. Target delivery has been optimized by codon-optimizing and development of stronger muscle-specific promotors (39). Apart from delivery route administered dose also plays a critical role in eliciting an

[11] In episomes, transformation of the vector DNA results in circular dsDNA. The circular dsDNA generally forms concatemers (long DNA molecules that consist of copies of the entire transgenome) but can also be present as monomeric episomes (35).

immune response (Figure 12). Although the dose threshold for effective delivery of rAAV-microdystrophin to target tissue has yet to be defined in humans, animal trials suggest 10^14 vector genomes/kg in order of magnitude (35, 39).

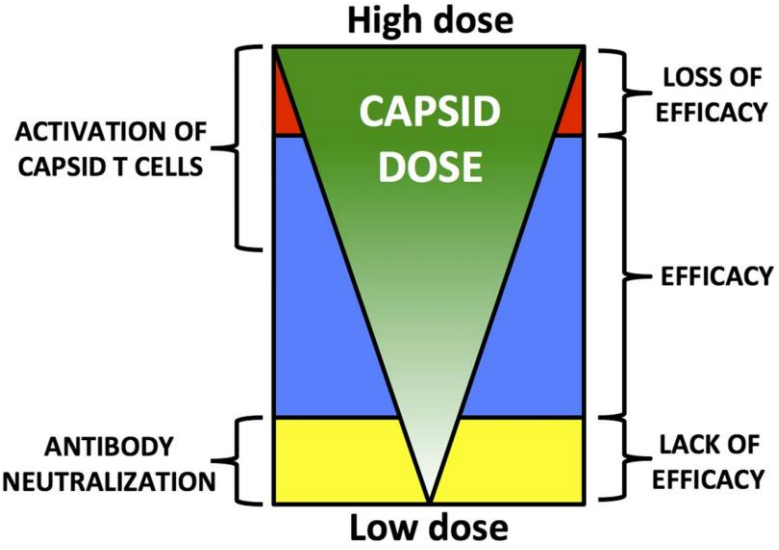

Figure 12: Model of the relationship between rAAV dose, immune response against rAAV capsid proteins and efficacy in humans. A low rAAV dose elicits anti-AAV antibody production which neutralizes AAV vectors before they are able to enter cells. This results in lack of efficacy. Increasing rAAV dose potentiates efficacy. When a certain threshold is reached, rAAV activates T-cell immunity which results in loss of efficacy by immune-mediated clearance of transduced target cells and holds implications for safety of the host (42).

Pre-clinical DMD mouse, dog and rat models have demonstrated long-term gene delivery in various tissues accompanied by correction of dystrophin production after micro-dystrophin rAAV treatment. The ongoing optimization of micro-dystrophin molecules has led to more positive outcomes. IM and IV rAAV-microdystrophin administration resulted in complete rescue of muscle mass, specific force and resistance to eccentric contraction in a *mdx*-mouse study. In dystrophic dogs, dystrophin levels ,expressed post-treatment, resulted in functional improvements and amelioration in clinical outcome (37). These studies also demonstrated that higher quantities of rAAV-microdystrophin are obligatory for functioning in larger animals which heightens the risk of developing immune responses (35). Regarding present challenges, clinical trials have only proceeded into phase I. A double-blind, dose escalating trial that investigated micro-dystrophin delivery with a rAAV2 serotype variant (brand name Biostrophin) and an accessory CMV promotor was completed in 2008 by Asklepios Biopharamaceuticals. Six DMD boys received 2x10^10 or 1x10^11 vector genomes/kg Biostrophin injections in one biceps muscle for 6 weeks and received corticosteroids during the trial. Not a single biopsy showed significant improvements of dystrophin

levels post-treatment. According to the authors this relied on elicited immune responses or other design flaws. Other phase I clinical trials, also focusing on IM rAAV-microdystrophin transduction, are still ongoing. As such, a study by Mendell et al. that evaluates the effects of IM administered rAAV-microdystrophin to the extensor digitorum brevis muscle has been initiated (NCT02376816). A more carefully designed phase I/II clinical trial investigating IV rAAV transgene delivery is currently planned (Trial ID: US-1245) (37).

AAVs are not solely used as micro- or mini-dystrophin delivery vectors but also produce a feasible delivery method for other therapeutic agents. Firstly, **CRISPR-Cas9 AAV delivery** (see chapter 4.5) has been presented as a useful approach. As *in vivo* CRISPR-Cas9 approaches have been limited by problems with Cas9 delivery the potential of combining an advantageous delivery system (AAV) with an advanced genome modification technique (CRISPR-Cas9) poses very appealing (37). Secondly, **AON delivery by AAV vectors** (see chapter 4.3.1) improves efficiency of muscle targeting and could lead to long-term transgene expression in comparison to naked AON molecules. However, the requested high dose for this approach could diminish its applicability. Lastly, **utrophin AAV delivery** (see chapter 4.7.1) approaches can be applied (43).

Apart from the proven efficacy of rAAV-microdystrophin approaches in animal models, many other factors contribute to the excellent AAV vector profile. Mild immune responses (compared to other viral vectors) and low frequency of integration into the host genome providing a good safety profile, the ability to infect both dividing and non-dividing cells, the long-term transgene expression in non-dividing cells, the wide range of infectivity and the simplicity in producing high amounts of rAAV all provide favorable contributions. As this strategy is applicable to all DMD patients, it presents as an attractive treatment approach (35, 39).

Downsides to AAV-mediated transfer approaches are the necessity to ensure a high enough dose for efficient delivery without consequently eliciting an immune response. The limited cargo-capacity of AAV vectors poses another hurdle as the dystrophin gene is of enormous size. Further, as this approach predominantly results in non-integrated episomes in the cell nucleus, both dividing cells (by losing transgenes after mitosis), and non-dividing cells (by normal cell turnover after years) result in low frequent readministration (35, 39).

4.5. CRISPR-CAS9

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas9 (CRISPR associated 9 endonuclease) is a genome engineering technique providing the potential of a one-time treatment that would make a permanent correction in the DMD mutation by implementation of 4 approaches: exon skipping, (multi-)exon deletions, frame shifting and exon knock-in (described in detail further along the chapter) (44).

The CRISPR-Cas9 systems were discovered as a natural protection mechanism against bacteriophages used in bacteria. CRISPR-Cas9 consists of single guide RNA (sgRNA), which is a fusion of transactivating RNA (tracrRNa) and CRISPR RNA (crRNA), and the endonuclease Cas9. SgRNA provides target site specificity by a programmable 20 base pairs sequence also known as the protospacer. In such a way, sgRNA directs Cas9 to the desired target site. Cas9 will not successfully bind to or cleave the target DNA sequence if the protospacer is not immediately followed by a so called protospacer Adjacent Motif (PAM) consisting of 2-6 base pairs of DNA. Once the target site is reached, Cas9 cleaves the DNA double-strand. To repair DNA double-strand breaks, cells depend on two main mechanisms: non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ is the predominant pathway in higher eukaryotes as it works more efficiently (presumably because of a higher ligation speed) than HR. NHEJ has the tendency to introduce mutations at the break sites such as small insertions and deletions (indels). These indels form the basis for restoration of the ORF. Although less efficient, HR contributes to the repair mechanism but requires a DNA repair-template. According to studies, NHEJ accounts for 92% of the edited genomes after CRISPR-Cas9 application (44).

Among the CRISPR-Cas systems, type II, comprising different types of Cas9, is the most simple in use. The most commonly used SpCas9, derived from *streptococcus pyogenes,* is associated with a PAM sequence (NGG) that is common in the human genome which provides flexibility in engineering sgRNAs. SaCas9, derived from *staphylococcus aureus*, is associated with a PAM sequence (NGGRRT) that is not often seen in human DNA and therefore lacks this high flexibility. Other type II CRISPR-Cas9 or type V CRISPR-Cpf1 systems have been adapted for use in animal cells and recognize their own specific PAM. Recently, new programmable nucleases have been discovered that do not require a PAM for functioning. These nucleases could function as a powerful tool in the genome editing field as they provide a potential therapy for DMD boys affected by multiple mutations (44).

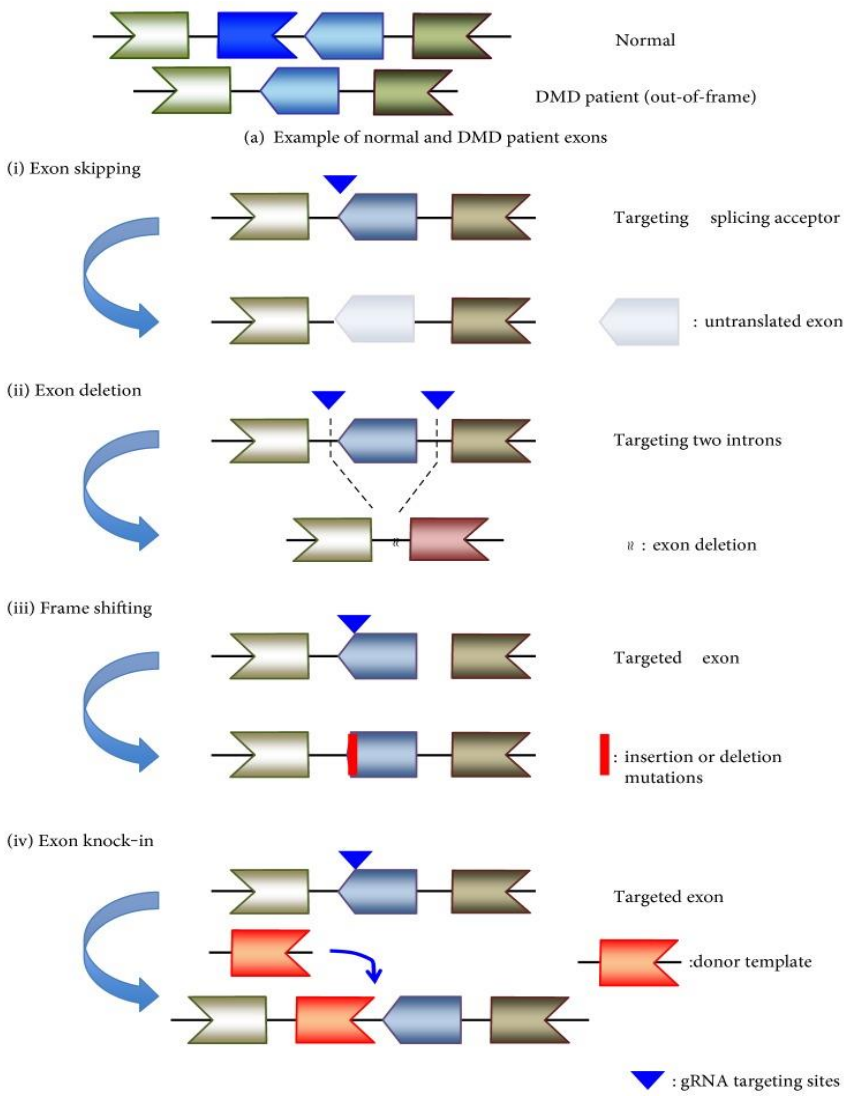

Figure 13: DMD-mutation targeting strategies by CRISPR-Cas9. By cutting DNA double-strands at a specific site and the subsequent help of DNA repair mechanisms, CRISPR-Cas9 rescues dystrophin synthesis in a 4-way manner. Exon skipping can be obtained by targeting splice site acceptors, (multi-)exon deletion can be obtained by targeting two introns that flank a target exon and frame shifting can be obtained by targeting nonsense mutations. These three latter approaches restore the open reading frame. The 4th approach, exon knock-in, is based on the insertion of a healthy exon into the host DNA (44).

The desired result of the CRISPR-Cas9 approach is derived by four mechanisms (Figure 13). The first one is **exon skipping.** By targeting splice site acceptors in front of the target exon, CRISPR-Cas9 can correct out-of-frame mutations. This approach resembles the working mechanism of AON therapeutics described previously. Alternatively, CRISPR-Cas9 systems have the possibility to induce **(multi-)exon deletions** by targeting the flanking introns of the target exon(s). This

approach can be administered for out-of-frame mutations and was also found useful in patients with nonsense mutations. Importantly, the exon deletion approach can only occur when Cas9 is administered in the presence of 2 SgRNAs as Cas9 needs to be directed to the 2 flanking introns of the target exon. (Multi-)exon deletion mechanisms, which aim to restore the production of (truncated) protein, are predicted to cause adverse events. Despite this disadvantage, it is a favorable technique from a cost-effective point of view as up to 60% of DMD patients could potentially benefit from this approach. **Frame shifting** is a third approach of CRISPR-Cas9 genome editing. By targeting premature stop codons, indels produced by NHEJ can restore the ORF. Although frame shifting only occurs in 1 out of 3 times after application of CRISPR-Cas9, studies have proven the effectiveness of the approach in DMD boys. The last and most ideal approach, **exon knock-in,** poses the potential to restore the production of full-length dystrophin. A genetic engineered exon of choice, administered in the presence of CRISPR-Cas9, provides as a DNA template for repair by HR. Consequently, the desirable DNA is inserted in the host genome. Although Li et al. (2015) recommend this as the approach of choice based on the proven precision and efficacy (45), Gee et al. (2017) emphasized multiple disadvantages that come along with it. Pleading against this approach are the low frequency of the HR pathway, less efficient *in vivo* functioning and limitations of template DNA length depending on the used delivery vector (44).

CRISPR-Cas9 was applied in 229 induced pluripotent stem cell (iPSC) clones that lacked exon 44 (of the dystrophin gene) in a study by Li et al. (2015). Forty clones showed indels at the target site of which 6 resulted in exon-skipping (skipping of exon 45 producing a 43-46 ORF) and 12 resulted in frame-shifting. Both approaches successfully restored (truncated) dystrophin production after iPSC differentiation into myoblasts. Li et al. (2015) also succeeded in knocking-in exon 44 and this resulted, as only approach, in the restoration of full-length dystrophin (45). In myoblast cells with a deletion of exons 45-50 (of the dystrophin gene), Ousterout et al. (2015) demonstrated frameshift restoration after CRISPR-Cas9 administration. After differentiation of the myoblasts, restoration of dystrophin production was seen. Using the same cell-line, an exon 51 deletion was seen after administration of two sgRNAs and Cas9. Similarly, restored (truncated) dystrophin production was seen after myoblast differentiation. Aiming to produce a single method that could potentially benefit up to 60% of all DMD boys, Ousterout et al. (2015) investigated the potential of the CRISPR-Cas9 multi-exon deletion approach. Although the efficiency of producing such large deletions is less than that of a single-exon deletion technique, restored (truncated) dystrophin expression was still observed after deletion of the exons 45-55 (46). A similar multi-exon deletion of exons 45-55 was demonstrated by Young et al. (2016), using two sgRNAs and Cas9. Results of this trial showed positive dystrophin fibers *in vitro* and *in-vivo* in *mdx*-mice (47).

As stem cell therapy shows promising results (see chapter 4.6), a rational approach for DMD treatment would be to restore DMD mutations with CRISPR-Cas9 in autologous or HLA-matched allogenic iPSCs, subsequently, after differentiation into myoblast progenitor cells, transplanting them back into human muscle tissue (Figure 14). A study showed that genetically corrected iPSCs, transplanted back into *mdx*-mice by systemic delivery or engraftment led to dystrophin production in skeletal muscle cells, expression of satellite cells and improved muscle strength (44). Although Ousterout et al. (2015) observed a rather low frequency of dystrophin production after IM injection of genetically corrected iPSCs, they also validated consistent *in vivo* expression of dystrophin in a fraction of implanted cells in *mdx*-mice (46).

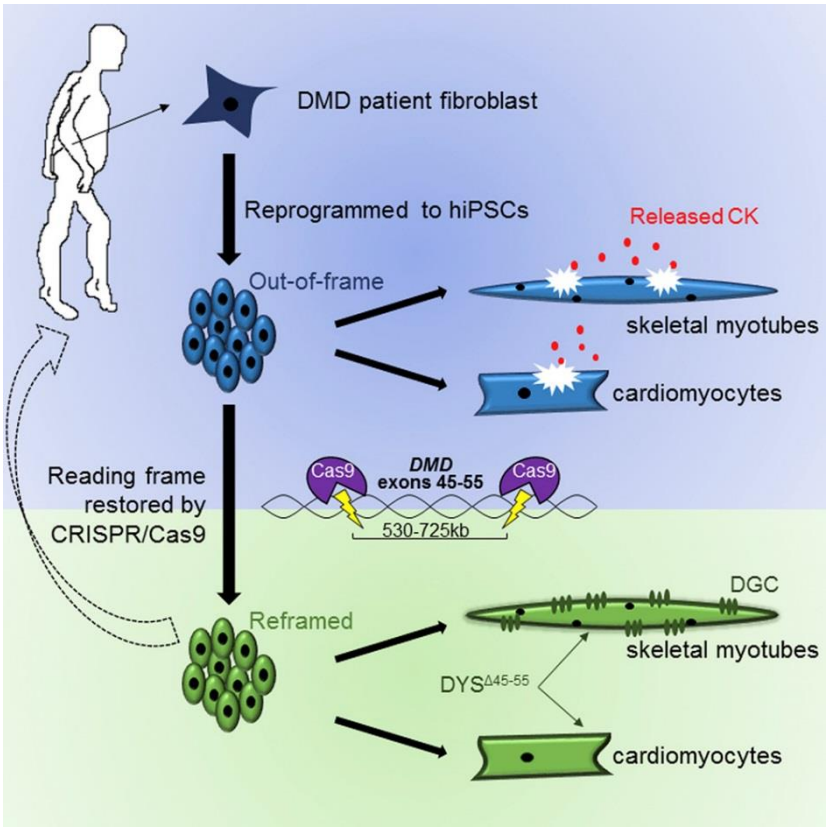

Figure 14: ex vivo gene therapy with CRISPR-Cas9 in autologous stem cells. Fibroblasts are derived from a DMD patient affected by an out-of-frame mutation. Subsequently, fibroblasts are reprogrammed into iPSCs. CRISPR-Cas9 corrects the ORF of iPSCs by using an multi-exon deletion approach. Corrected iPSCs are differentiated into muscle stem cells and transplanted back into the patient (47).

Alternatively, rAAV vectors have been used as a delivery technique for CRISPR-Cas9. Injected in *mdx*-mice, partial restoration of dystrophin expression was shown. However, challenges regarding AAV mediated transport came along with this delivery method (see chapter 4.4). First, host immune responses are feared in AAV mediated gene therapy. Secondly, AAV vector delivery is associated with long-term expression of transgenes, making off target cleavage by CRISPR-Cas9

more likely. Lastly, AAV size limitations result in divided packaging of Cas9 and sgRNA in 2 different AAV vectors. Consequently, efficiency is reduced and production cost is raised (44).

Although up till now, no off-target mutations have been reported in *in vitro* or *in vivo* iPSC studies with CRISPR-Cas9, it remains a point of concern. Moreover, it is seen as one of the most important obstacles for the therapeutic use of this technique (44, 45). The risk of off-target mutagenesis by programmable nucleases is associated with the specificity to its target sequence. As such, before CRISPR-Cas9 systems can be implemented in clinical practice, target specificity must be improved (45). Given the severity of DMD, the lack of apparent cytotoxicity in human cells and the absence of adverse events in ongoing trials that investigate other endonuclease genome editing approaches, this lack in specificity may be tolerable (46). In addition, we lack good screening tools to detect off-target mutations. A second point of concern are elicited immune responses against Cas9 endonuclease and the corrected dystrophin protein (44). Furthermore, advancements in delivery techniques and efficiency will enhance the utility of CRISPR-Cas9 (46).

Targeted genomic cleavage has been possible for some time with other nucleases like meganucleases, zinc-finger nucleases and TALENs (transcription activator-like effector nucleases) but they are expensive and difficult to generate. In contrast, CRISPR-Cas9 enables us to edit DNA in an inexpensive, practical and quick manner (44). Additionally, greater flexibility in selecting target regions is described for both TALEN and CRISPR in comparison to other programmable endonuclease techniques (45). As a result, the powerful CRISPR-Cas9, that addresses a variety of dystrophin mutations, providing applicability to more than 60% of all DMD patients, has received much attention and has become the technique of choice in the genome editing field in recent years (44).

On a related note, a long patent war over CRISPR-Cas9 between the Broad Institute in Cambridge Massachusetts, and the University of California over intellectual-property rights recently concluded in favor of the Broad institute (48).

4.6. Stem cell therapy and ex vivo gene therapy

DMD patients in advanced stages of disease benefit less to nothing from gene therapy for there are not enough remaining muscle cells to target and/or therapeutics cannot reach target cells as connective tissue withholds small molecules. Because stem cell therapy aims at promoting muscle growth apart from dystrophin restoration it does not only slow disease progression but moreover improves normal muscle function. Stem cell approaches with or without *ex vivo* gene correction theoretically hold great potential for all DMD patients (6).

Many types of cells hold intrinsic potential to differentiate into myogenic progenitor cells also called myogenic stem cells or myoblasts. The most apparent ones are **satellite cells (SCs)** as they are key players in the natural repair mechanism of muscle tissue. SCs are mesodermal from origin. Localized between the basil lamina and muscle membrane in adult muscle tissue they account for 2,5-6% of all muscle nuclei. In healthy circumstances these cells remain dormant whereas they are activated when muscle cells are damaged (49). Stimulated SCs differentiate into myogenic progenitors who at their turn differentiate into end stage myocytes that fuse with other regenerating myofibers. As a result from the ongoing muscle damage / muscle regeneration cycle In DMD patients SC reserves eventually deplete. Consequently, DMD is hallmarked by impaired regenerative capacity of muscle tissue.

Pioneer studies in stem cell therapy administered HLA-matched allogeneic myoblasts, derived from healthy donors, in *mdx*-mice via IM delivery route. Unfortunately, these approaches failed due to poor cell survival, bad migration to target tissue, immune rejection and *in vitro* expansion issues (6, 49). Furthermore, as SCs are incapable of crossing vessel walls, systemic delivery, considered as most beneficial administration route to target muscle tissue, is not applicable (6).

In the search for alternative cells that could distribute to a wider range of target tissue by using systemic delivery, myogenic progenitor cells, originating out of tissues other than skeletal muscle, were established.

Hematopoietic stem cells (HSCs) derived from bone marrow, peripheral blood or placenta cord, were shown to have myogenic progenitor capacity. A big advance in using HSCs is that the recruitment of progenitors (after bone marrow transplantation or systemic administration) can be achieved systemically. However, none of the HSC subpopulations showed myogenic progenitor capacity at a high frequency (49).

Mesoangioblasts (MABS), mesodermal of origin, are derived from the vessel wall. Because they have the ability to efficiently cross vessel walls, creating options for systemic delivery, they pose intriguing candidates for stem cell strategies. Additionally, MABS are easily transduced with viral vectors facilitating possible *ex vivo* gene therapy. In a study by Sampaolesi et al. (2006), IA administration of allogeneic HLA-matched MABS (derived from healthy dogs) into dystrophic golden retrievers, resulted in recovery of dystrophin expression, better muscle morphology and amelioration of functional presentation (50). Another study resulted in dystrophin-positive muscle fibers after allogeneic MABS (derived from healthy humans) were administered in *mdx*-mice (51). Note that in both studies MABS contributed to muscle regeneration with a lower efficiency than did satellite cells. After intensive preclinical research clinical trials initiated. A dose-escalating phase I/II clinical trial administered allogeneic HLA-matched MABS in 5 DMD boys. MABS were administered via the limb arteries and all study subjects were under immunosuppressive treatment. This study resulted in safe outcomes apart from one patient that developed a thalamic stroke with no clinical consequences. Efficacy, on the other hand, was low. Presumably caused by low engraftment of donor cells (52). Hereafter, research pursued *ex vivo* gene therapy in MABS. This procedure basically involves the use of patient-specific stem cells (autologous stem cells) for culture expansion, correction of the DMD mutation with *ex vivo* gene therapy and transplant them back into the patient as differentiated myogenic progenitor cells (Figure 14). Immune responses are restrained as this approach makes use of autologous cells. Notwithstanding the great potential of these techniques, only small remaining amounts of MABS, insufficient for cell transplantation, were recently seen in DMD boys (6).

Human multipotent adipose derived stem cells (hMADS), mesodermal of origin**,** show great expansion capacity and elicit low immune responses next to their multipotent differentiation potential (49). Moreover, adipose tissue is easily available**.** In addition to other mesodermal cells **(Mesenchymal stem cells, multipotent adult progenitor cells, MDSCs, CD133+ cells** and **endothelial progenitor cells)**, **neural stem cells** derived from ectodermal tissue also show potential regenerative muscle capacity (49).

Pluripotent stem cells (PSCs) have the beneficial potential of differentiating into any cell type. Amongst PSCs **Embryogenic stem cells (ESC)** are well studied. Due to ethical controversy and the fact that ESCs can only result in allogeneic cell lines, their role in DMD therapy will not be discussed in this thesis. Another type of pluripotent stem cell, first generated in 2006 by the team of Yamanaka et al. (2006), are **induced pluripotent stem cells (iPSC)** (53). IPSCs are

manufactured out of somatic cells like blood or skin cells. Although they resemble certain ESC potencies, iPSCs bypass ethical issues as they are derived from mature human tissue and they can provide desirable autologous cell lines for *ex vivo* gene therapy purposes. Furthermore, since iPSCs are self-renewing and pluripotent, they represent as an unlimited source of autologous cell lines and pose as a theoretically excellent stem cell therapy agent. *Ex vivo* gene therapies in iPSCs basically consist of reprogramming autologous somatic cells into iPSCs (most commonly by viral vector gene transfer), correction of the DMD mutation with *ex vivo* gene therapy, culture expansion, reprogramming these cells into myogenic progenitors (by transferring regulatory genes that serve as important transcription factors in muscle cells (MyoD, Pax7, Pax3)) and transplanting the myogenic cells back into the patient (Figure 14). As MABS can be administered systemically, while iPSCs cannot, production of MABs out of iPSCs is an alternative approach to overcome the quantity issues of MABs seen in DMD patients, while obtaining a systemically applicable therapeutic agent (6). A *mdx*-mouse study in which autologous fibroblasts were reprogrammed into iPSCs, genetically corrected with *ex vivo* transposon therapy (see chapter 4.6.2.) and transplanted back into the *mdx*-mice as myogenic progenitors resulted in dystrophin production, establishment of satellite cells and improved muscle strength (44). IPSC cultures are readily being used to study disease mechanisms of genetic disorders and to obtain information on novel therapies. Up till today, clinical studies have not commenced yet as certain safety and efficacy issues need to be looked at. One of the biggest concerns comes from the observations that insertional mutagenesis occurs during iPSC reprogramming (49).

To summarize, stem cell therapy comprises two main approaches. Firstly, allogeneic HLA-matched cells derived from healthy subjects can be used as agents for *in vivo* stem cell therapy. Secondly, autologous cells derived from DMD affected subjects can be genetically corrected with *ex vivo* gene therapy and pose as favorable stem cell agents as they elicit low immune responses (Figure 15). *Ex vivo* gene therapy can be applied in a classical manner, using viral vector transduction, but other vectors such as human artificial chromosomes (see chapter 4.6.1.) or transposons (see chapter 4.6.2.) are also applied as cDNA transduction vehicles. More recently, genome editing approaches using CRISPR-Cas9 (see chapter 4.5) have been an evolving approach in *ex vivo* gene therapy (44). Many different body cells have myogenic capacity but MABS derived out of iPSCs hold the greatest potential as they showed survival, engraftment and therefore efficacy in preclinical trials, safety in clinical trials and can be administered systemically.

4.6.1. Human artificial chromosomes

Human artificial chromosomes (HAC) inserted in cells by microcell-mediated chromosome transfer technology (MMCT), are artificially produced microchromosomes that act as a new chromosome in human cells. That is, instead of containing 46 chromosomes, HAC induced cells contain 47 chromosomes. HACs provide a large cargo capacity that can contain the entire dystrophin gene, hereby overcoming the limitations in size seen in conventional viral vector techniques. Furthermore, as DNA stays episomal (non-integrated in host genome) HAC corrected cells do not bear consequences of transgene silencing, interference with endogenous gene expression and most importantly, insertional mutagenesis (19, 54). Replication and segregation of HACs takes place independently from the host genome, which makes them mitotically stable agents. All advantages taken together, human artificial chromosomes that contain full-length dystrophin including the regulatory elements (DYS-HAC) hold the exceptional potential for complete genetic correction applicable to all DMD patients in a safe manner.

The first DYS-HAC was successfully generated by Hoshiya et al. (2009) (55). A proof of principle study by Kazuki et al. (2010) showed complete correction of iPSCs using a DYS-HAC approach in both *mdx*-mouse cells and cells of a DMD patient. Corrected iPSCs resulted in dystrophin expression *ex vivo*. I*n vivo* transplantation of these cells in *mdx*-mice resulted in DYS-HAC presentation in all biopsied tissues with production of tissue-specific dystrophin (56). In parallel with these results, Tedesco et al. (2011) fully corrected MABS, derived from *mdx*-mice, with DYS-HAC and transplanted them back into immune suppressed *mdx*-mice. Morphological and functional amelioration was seen next to dystrophin expression in muscle fibers of the recipient mice (57). The Tedesco group is currently focusing on DYS-HAC correction in human stem cells (19). As earlier proposed, a suitable therapeutic strategy for all DMD patients would be *ex vivo* gene correction in autologous stem cells with DYS-HAC followed by transplantation. Additionally, Kazuki et al. (2010) recommended HACs as feasible and safe tools to generate iPSCs, for they are non-integrating vectors (56). HACs cannot be used for *in vivo* gene therapy due to certain safety limitations (19). These limitations will not be further discussed in this thesis for it would lead us to far from the subject.

The efficiency of transferring large constructs like HACs into target cells is lower than that of conventional viral vector transfer. Along with complex engineering, this low transduction efficiency makes the approach challenging, time consuming and costly. Due to these limitations clinical trials with DYS-HAC have not commenced yet (19).

4.6.2. Transposons

Transposons are gene elements that 'jump' from one DNA site to another by a simple cut and paste manner executed by its self-encoded transposase. A simple transposon consists of a gene, encoding the obligatory transposase, which is flanked by two inverted terminal repeats (ITR). Transposase functions as molecular scissors that cut the DNA at the ITR located binding sites. Subsequently it reintegrates the cut DNA strand in a sequence specific manner according to the type of transposon that is used. By editing a favorable transgene in between the ITR regions and supplementing it with a transposase-plasmid, virtually any DNA sequence can be incorporated in the host genome. Transposon vectors provide genome wide targeting in various cell types. The 2 most commonly used transposon systems, Sleeping beauty transposon and Piggybac transposon will be discussed (58).

Sleeping beauty transposons (SB) were initially derived from salmonid fish genomes. As SB DNA elements were brought back to life from a non-functional evolutionary sleep, they were given the name sleeping beauty. In their natural state, SB transposons encode a 1,6 Kb long transposase which is flanked by 230 base pairs of ITRs. It preferentially targets a TA-dinucleotide genome sequence and does not have the tendency to integrate in active genes (thus causing low insertional mutagenesis) (58). In a proof of principle study by Muses et al. (2011), *mdx*-mouse cells were administered SB transposons carrying a micro-dystrophin transgene and transposase. Microdystrophin expression increased up to seven fold and stable integration was confirmed at a molecular level. Although, in a *mdx*-mouse model, engraftment efficiency was lower than expected, micro-dystrophin was effectively produced (59). In parallel with these results, an *ex-vivo* gene therapy trial applied the same steps as used by the Muses group but alternatively used micro-utrophin as transgene. The *Mdx*-mice showed many micro-utrophin positive muscle fibers, biochemically corrected DGCs at the sarcolemma and improved muscle strength post-transplantation. Next to their differentiation into myocytes, myogenic progenitors also embedded as new satellite cells (60).

Piggybac (PB) transposons, were discovered in Cabbage Looper moths. In their natural state, PB transposons encode a 2,4 Kb transposase gene which is flanked by 13 base pairs of ITRs and an asymmetric 19 base pairs ITR. PB transposons preferentially target an AATT-tetranucleotide, neighbored by particular sequences. PB transposons show slight tendency to integrate into active genes and their regulatory sites (thus showing higher insertional mutagenesis) (58).

In a study by Loperofido et al. (2016), 2 different sized micro-dystrophins and full-length dystrophin genes were transduced into myoblasts by normal size PBs and large size PBs (17,7Kb), respectively. Approximately 65-66% and 78% of the corrected cell lines showed expression of the micro-dystrophins and full-length dystrophin, respectively. Subsequently, mesoangioblasts derived from a dystrophic dog were corrected with large size PBs, resulting in approximately 50% of all cells expressing full-length dystrophin (61). Efficient *ex vivo* gene transfer in mesoangioblasts was also seen after genomic correction with large size PBs in a study by Ley et al. (2014), albeit with a lesser efficiency compared to normal size transposons. After transplantation, these cells resulted in sustained transgene expression*. In vivo* therapy conducted with IM injections of PB transposons in the Tibialis muscle of *mdx*-mice, on the other hand, showed no transgene expression despite molecular evidence of PB transposition (62).

As a group, transposons show long-term transgene expression, are less prone to gene silencing, have a favorable immune and bio-safety profile compared to viral vectors and are simple and inexpensive to manufacture. Furthermore, the use of transposons showed enhanced efficiency in generating iPSCs. As specifically PB transposons hold the unique potential to get removed out of the cell without altering genome sequences, they provide a favorable approach compared to alternatively produced iPSCs with poor safety profile due to insertional mutagenesis (63). Nevertheless, insertional mutagenesis is a consequence feared in all DNA incorporating techniques. Transposons cannot cross cell membranes independently and therefore require the help of transfer devices. Consequently, *in vivo* therapy with transposons is challenging (63).

PB, specifically, holds the most promising potential for gene therapeutic purposes within the transposon group. In addition to the beneficial profile of transposons as a group, PB is highly effective, contains big transport capacity allowing up to approximately 9.1–14.3 kb of cargo, enables safe iPSC production and its transposase can be modified without substantially losing activity. This flexibility in genome engineering allows us to produce site-directed therapeutics which lowers the risk of insertional mutagenesis. PB displays overproduction inhibition (a phenomenon where increasing the concentration of transposase results in a reduction in the level of transposition) but to a much lesser extent than does SB. Nevertheless, as severe cytotoxicity was linked to overproduction inhibition in SB studies, it could account for toxic events in PB as well. For other specific concerns regarding PB use, the review article by Meir et al. (2011) is strongly recommend (63).

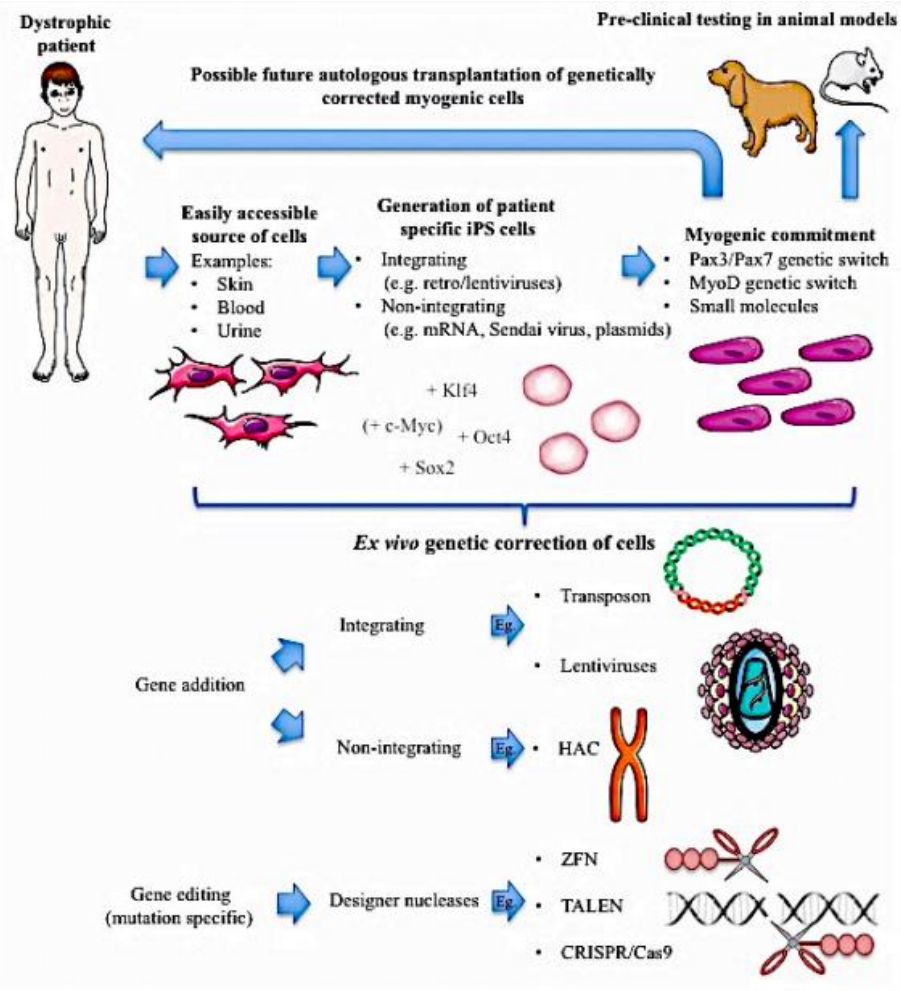

Figure 15: Potential autologous iPSC-based ex vivo gene therapies for muscle disorders

Easily accessible cells are derived from Duchenne boys and are reprogrammed into iPSCs. After culture expansion and ex vivo gene correction by different approaches the resulting iPSCs are reprogrammed into myogenic progenitor cells and transplanted back into the patient (58).

4.7. Therapies that compensate for the lack of Dystrophin

4.7.1. Utrophin

Utrophin has 80% sequence homology to dystrophin and is proven to show a similar protective function in muscle tissue. In fetal development utrophin is located at the sarcolemma while it is replaced by dystrophin postpartum. Utrophin is located in a wide range of tissues in adulthood. In normal adult muscle tissue, utrophin is located at the neuromuscular synapse and myotendinous junction but is not found at the sarcolemma of dystrophin positive muscle fibers (64). However, in dystrophin-negative muscle fibers or during muscle injury, increased utrophin levels are produced as a part of the regeneration process (65).

Similar protective function seen in utrophin derives from its ability to construct utrophin-associated protein complexes (UAPC), which resemble DAPCs to a great extent. Recruitment of the nNOS

to the sarcolemma is not achieved by UAPCs in comparison to DAPCs. This caused concern for some, while others suggested this of minimal clinical meaning as BMD patients that lack nNOS binding sites remained mildly affected (65). A possible treatment strategy for all DMD patients would be to increase the expression of utrophin rather than delivering the corrected dystrophin cDNA. This approach, called **protein upregulation**, can be achieved by transcriptional upregulation of utrophin mRNA (accomplished by activating the utrophin promoter), post-transcriptional modulation and direct protein replacement by utrophin. **SMTC100** or **Ezutromib**, produced by Summit therapeutics, is an orally available small molecule that modulates the utrophin promotor. It already passed phase I clinical trials concluding that it was well tolerated in humans and showed a 50% increase in utrophin concentrations in cells (66). A phase II clinical trial is currently set in place by Summit Therapeutics (NCT02858362). Potential immune responses associated with 'foreign' dystrophin expression can be bypassed using this surrogate protein approach (65).

However, in double knock-out (dystrophin and utrophin deficient mdx:utrn(-/-)) mammals the functional benefit of abovementioned approach is completely annulled as the utrophin gene is defective. A favorable approach in this case would be the incorporation of **(micro-)utrophin cNDA** into the host genome. A study by Tinsley et al. (1998) demonstrated that expression of full-length utrophin in *mdx*-mice after vector administration prevented the development of muscular dystrophy (67). Another study concluded that, in *mdx*-mice genetically corrected for utrophin, functional test parameters amounted to 80% of recovery (68). In parallel with these results, dystrophic golden retrievers, administered with AAVs that held mini-utrophin, efficiently expressed utrophin and showed increased expression of dystrophin-associated proteins (indicating that utrophin compensated for the lack of dystrophin) and reduced muscle fibrosis (69). Another study that conducted AAV-mediated transfer of micro-utrophin in mdx:utrn(-/-) mice, showed functional improvements compared to the untreated controls (70).

4.7.2. Downstream targets

Additionally, consequences of DMD provide many downstream targets for treatment. Pharmacological approaches that aim to reduce muscle ischemia (by altering vascular vasodilatation), increase muscle mass (by myostatin inhibitors), contribute to muscle regeneration (by histone deacetylase inhibitors), diminish inflammatory responses (by anti-inflammatory agents), reduce oxidative stress (by antioxidants), rescue the calcium homeostasis (by blocking calcium channels) and improve mitochondrial function have been tested in preclinical models or are being studied in clinical trials (71).

5. Conclusion

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are X-linked, recessive transmitted muscle disorders that affect 1/4000 boys worldwide. Primarily boys are affected, while girls that carry the mutated gene are generally asymptomatic. These severe diseases stem from a lack of (functional) dystrophin, which is a protein that is responsible for the protection of muscle fibers during exercise. Dystrophin is mainly located in skeletal muscle but other isoforms of the protein can be found in cardiac muscle tissue, smooth muscle tissue and in the brain. DMD boys are generally affected by out-of-frame mutations which result in no dystrophin production. Boys who suffer from BMD are usually affected by in-frame mutations that result in low amounts of (partially) functional dystrophin. DMD affected boys typically start developing skeletal muscle weakness at the age of 2-5 years. The disease runs a predictable course with progressive muscle deterioration, specific gait impairment and posture changes. Progressive cognitive impairment and gastro-intestinal pseudo-obstruction can also manifest. Generally, DMD boys become wheelchair bound at the age of 12 years. Respiratory failure and cardiomyopathy are the two main premature death causes seen in DMD boys, that usually don't live beyond the age of 30 years. BMD patients suffer from the same health problems, be it that they show a milder presentation and manifest symptoms later in life. Hitherto, no treatment with a curative character exists for DMD and BMD. **Corticosteroids** are the only drugs that are standard care in DMD boys. This palliative treatment aims at delaying disease progression by addressing symptoms and complications. Adverse effects of long-term corticosteroid treatment are a point of concern. In the search of a cure for this devastating disease at a genetic level, many therapeutic strategies have been investigated in the latest decennia.

Exon skipping strategies make use of AONs that regulate the splicing machinery at pre-mRNA level. In such a way, exon skipping restores the ORF. A truncated but (partially) functional dystrophin can thus be produced, altering the DMD phenotype to that of the milder BMD. *Eteplirsen*, targeting exon 51, received conditional approval of the FDA in September 2016, making it the only available drug on the US market that targets DMD at a molecular level. It is produced by Sarepta therapeutics under the brand name Exondys 51. Mendell et al. (2013, 2016) showed a significant increase in dystrophin positive fibers (in the 30 and 50mg/kg/day Eteplirsen group) and a significant difference in 6MWT (in the 50mg/kg/day and combined Eteplirsen group) in 12 DMD boys post-treatment. Preliminary data of an ongoing trial by Sarepta therapeutics showed a similar increase of dystrophin amounts post-treatment. Nevertheless, it was disputed

whether these results contribute to any clinical benefit as dystrophin levels did not reach the 10% threshold for critical clinical meaning. Patients with out-of-frame mutations amenable to Exon 51 deletions cover approximately 14% of the DMD group. Even though efficacy issues are present and repetitive treatment is obligatory, Eteplirsen has beneficial characteristics like low risk of insertional mutagenesis and a good safety and tolerability profile. We want to emphasize the importance of improvements in study design for future trials on Eteplirsen as we predict that this would advocate in favor of the study drug.

DMD boys that are affected by missense mutations that result in PTCs can be benefitted by ***stop codon readthrough*** therapeutics. This approach stimulates the continuation of dystrophin-mRNA translation by helping near-cognate aminoacyl-tRNA compete with release factors that would bind the PTC and terminate protein elongation. In such a way, stop codon readthrough enables production of (partially) functional dystrophin, altering the DMD phenotype to that of the milder BMD. Stop codon readthrough approaches are applicable to approximately 13% of the DMD population.

Gentamicin treatment showed positive results in a study by Malik et al. (2010). Significant increases of dystrophin levels were seen in 6 out of 7 participants after weekly or twice weekly administration of 7,5mg/kg Gentamicin. Three patients even showed a 3,44 to 5,06 fold increase in dystrophin levels and surpassed the 10% threshold for critical clinical meaning. However, Gentamicin brings obstacles with its use. The narrow therapeutic window and IV administration, resulting in intensive treatment and monitoring, and important side effects like renal toxicity and ototoxicity limit Gentamicin utility in clinical practice.

Ataluren received conditional approval of the EMA in July 2014, making it the only available therapeutic drug on the EU market that targets DMD at a molecular level. It is produced by PTC therapeutics under the brand name Translarna. A study by Bushby et al (2014) provided a great deal of information that contributed to the proceedings of the conditional approval. Results showed a mean difference of 31,3m at the 6MWT and an 18% difference of patients progressing into persistent 10% change in walking ability compared to the placebo group after administration of 40mg/kg/day Ataluren. This can be translated in a delay in disease progression and a delay in decline of ambulation, respectively. Ataluren has a favorable safety and tolerability profile. The fact that it is an orally administered drug makes treatment more applicable to clinical practice. It was well documented that DMD boys in the decline phase of the disease benefit most from Ataluren treatment. That being said, creating more stringent inclusion criteria or creating subgroups for phase of disease presumably contributes to favorable outcomes for Ataluren.

AAV(-mediated gene therapy) is a vector that is used for gene transport (or transport of other gene therapy constructs like CRISPR-Cas9, AONs, micro-utrophin). rAAVs are genetically engineered so that the AAV encapsulates a reduced version of the full dystrophin cDNA (so-called micro- or mini-dystrophin) instead of viral genes. rAAVs efficiently transfect host cells by utilizing viral mechanisms while inserting micro- or mini-dystrophin cDNA in the cell nucleus. Preclinical models have demonstrated long-term gene delivery with correction of (partially) functional dystrophin production and an amelioration in clinical functioning post-treatment. Clinical trials have only proceeded into phase I and did not result in a significant increase in dystrophin levels. Mild immune responses (compared to other viral vectors), low risk at insertional mutagenesis (compared to other viral vectors), long-term transgene expression in non-dividing cells, wide range of infectivity and simplicity in producing high amounts of rAAVs plead in favor of this approach. Additionally, the fact that this therapy is applicable to all DMD patients has made it an exciting transduction tool. Despite these advantageous characteristics, unwanted immune responses (seen in all viral vectors approaches) and limited cargo-capacity are challenges that still remain.

CRISPR-cas9 is a genome-editing tool that consists of SgRNA and the endonuclease Cas9. SgRNA functions as a guide for the endonuclease, leading it to the target site. Cas9 cuts the DNA double strand, whereafter NHEJ or HR repairs the DNA breaks. NHEJ, the dominant DNA repair mechanism, has the tendency to induce small indels. Indels form the molecular basis for restoration of the reading frame derived through exon skipping, (multi-)exon deletion and frame-shifting. Exon-knock in, the fourth approach applied by CRISPR-Cas9, makes use of the secondary DNA repair pathway, HR, to achieve its goal. As HR requires template DNA for its functioning, the full dystrophin gene can be inserted in between two break points. This makes exon knock-in the most ideal approach in CRISPR-Cas9 techniques. IPSCs, genetically corrected by CRISPR-Cas9, led to dystrophin production, new satellite cells and improved muscle strength in *mdx*-mice after transplantation. Apart from stem cell approaches, AAV-mediated delivery has been proposed for *in vivo* CRISPR-Cas9 applications but is challenged by difficulties associated with the AAV delivery technique. CRISPR-Cas9 has become a much favored approach as it holds potential as a one-time treatment tool that results in permanent change of the DMD gene. Furthermore, it is applicable to the majority of DMD patients (as the (multi-)exon deletion approach alone already applies to approximately 60% of DMD patients), has a good overall cost-effectiveness balance and engineering efforts are low. Nevertheless, concerns about off-target genome editing and unwanted immune responses still exist and CRISPR-Cas9 *in vivo* efficacy is limited because of delivery challenges.

Stem cell therapy can be subdivided into *in vivo* stem cell approaches and *ex vivo* gene therapy in stem cells. Allogeneic HLA-matched stem cells, derived from healthy subjects, are used for *in vivo* stem cell approaches. Autologous (stem) cells, derived from DMD affected subjects, are corrected by *ex vivo* gene therapy before they get transplanted back into the patient. Low immune responses seen after autologous cell transplantations advocate in favor of *ex vivo* gene therapy. Stem cell therapies are applicable to all DMD patients and are of specific importance in patients who have entered advanced stages of disease as stem cell therapy does not only aim at slowing disease progression but also aims at promoting muscle growth.

Many different cell types hold potential to differentiate into myogenic. **SCs,** logically pushed forward as feasible candidates, showed low cell survival, bad migration to target tissue, suffered from expansion issues *in vivo and* cannot be administered systemically. **MABS** show a better stem cell profile as they can be applied systemically and they are easily transduced by viral vectors (favorable for *ex vivo* gene therapy). Although preclinical studies showed good results, quantity issues seen in DMD boys limit their use for *ex vivo* gene therapy. **IPSCs** are derived by patient-specific cells that get reprogrammed into an embryonic-like state which provides them with self-renewing and pluripotent abilities. Trials conducted in *mdx*-mice showed restored dystrophin production, new satellite cells and improved muscle strength after administration of corrected iPSCs. Concerns regarding insertional mutagenesis and other safety factors have delayed commencement into clinical trials. IPSCs cannot be administered systemically but producing MABS out of genetically corrected iPSCs can bypass this limitation.

Ex vivo gene therapy can be applied in a classical manner, using viral vector transduction, or in a more evolved manner, using HAC, transposon or CRISPR-Cas9 approaches. **HACs** are artificially produced chromosomes that are able to carry the full-length dystrophin gene. Correction of autologous cells by DYS-HAC resulted in *ex vivo* and *in vivo* expression of dystrophin and ameliorated morphological and functional presentation in animal models. HACs are advantaged by their ability to carry large constructs (bypassing the size limitations seen in viral vector strategies), and the fact that they do not integrate into the host genome (reducing the risk of insertional mutagenesis). Even so, clinical trials have not commenced yet as low transduction efficiency *in vivo* and complex engineering makes it a costly and time consuming strategy. **Transposons** are genetic elements that can 'jump' to different locations within a genome by a simple cut and paste manner attained by their self-encoded transposase enzyme. *Ex vivo* gene therapy with transposons is based on the replacement of the transposase gene by (micro-/mini-)dystrophin genes and supplementing a transposase-plasmid with it. Opposed to HACs, transposons are simple and inexpensive to manufacture. A downside to this technique is the low

transduction efficiency which makes *in vivo* functioning very challenging. Piggybac transposon delivery is more effective, the cargo-capacity is bigger and it permits more flexibility in engineering compared to sleeping beauty transposons.

Mouse models proved that ***utrophin*** can substitute for the loss of dystrophin. With 80% sequence homology to dystrophin, utrophin is responsible for creating so-called UAPCs at the sarcolemma, which highly resemble DAPCs. Potential strategies that raise utrophin levels are subdivided into protein upregulation and (micro-)utrophin cDNA transfer into host cells. Protein upregulation encompasses transcriptional upregulation, post-transcriptional modulation and direct protein replacement. CDNA transfer is an alternative approach that addresses double knock-out (mdx:utrn (-/-)) mice, which are not benefitted by protein upregulation. Apart from proven utrophin production and structural amelioration, also functional improvements were seen in animal models post-treatment. Utrophin substitution therapies can be applied to all DMD patients. Although controversy has been raised on the significance of the lack of nNOS binding at UAPCs, it poses no huge obstacle. Immune reactions against dystrophin products can be circumvented by this substitution approach.

We have created two brief, yet sufficiently informative tables (Appendix 7.2.-7.3.) that provide a quick overview of the characteristics, advantages and limitations of described gene therapy approaches.

Investigations on the cure for DMD have never been more elaborate. As an overwhelming amount of literature on the subject is available, the mistake of failing to grasp the essential aspects is quickly made. In this regard, we have tried to create a concise guide of the recent progresses that were made in gene therapy approaches for DMD. Declaring that one of these approaches comes forward as most advantageous should be done with caution, as each approach is characterized by pros and cons that plead in favor or against it. It is plausible to say that, up to this point, CRISPR-Cas9 offers the greatest hope of a cure for DMD. It is wise to look at these gene therapy approaches in a broad context. Using and investigating them in mono-therapy, but also expanding the field to complementary use of these strategies. Undoubtedly, further research is steadily paving the way to a final cure for Duchenne muscular dystrophy.

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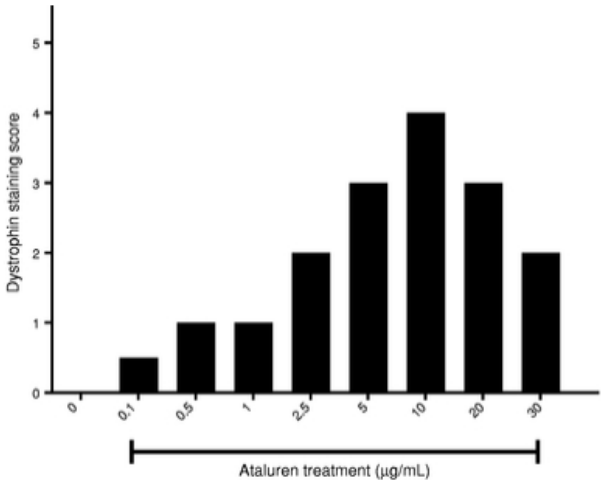
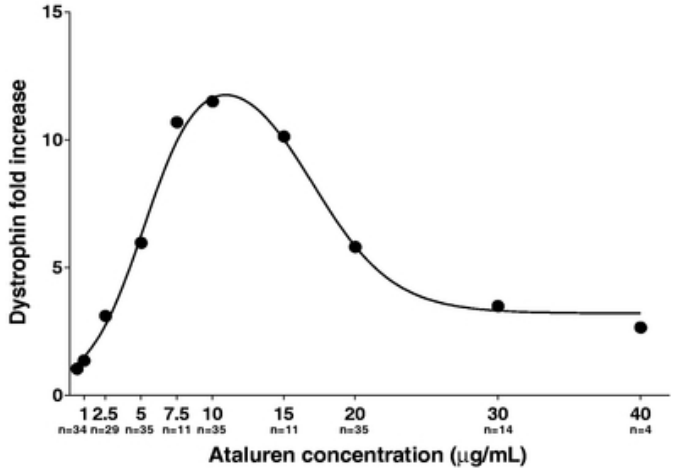
7. Appendix

7.1. Bell shaped curve of Ataluren

in vitro analysis on myotubes of *mdx*-mice presents a bell shaped dose-response curve of Ataluren. Maximal dystrophin immunohistochemistry staining (or treatment effect) was seen at 10 µg/ml (or mg/ml) and declined when higher concentrations were administered. Results are shown in the figure below (31).



In parallel, *in vitro* analysis on myotubes derived from 35 DMD patients with confirmed nonsense mutations showed similar results. Maximal dystrophin immunohistochemistry staining was seen at 10 µg/ml (or mg/ml) and staining (or treatment effect) declined when higher concentrations were administered. Results are shown in the figure below (31).



7.2. Tabel I: General characteristics of gene therapy strategies for Duchenne muscular dystrophy

	Mode of action	Final product	Route of administration	(Pre-)clinical status	Theoretic applicability in % of DMD population
Exon skipping with Eteplirsen	- Skipping of exon 51	- Truncated dystrophin	- IV - IM	- Phase III clinical status - Conditionally approved by the FDA (Sarepta therapeutics)	14%
Stop codon readthrough	- Readthrough of nonsense mutations	- Full-length dystrophin with one altered amino acid	*Gentamicin:* - IV - IM	*Gentamicin:* - Phase III clinical status	13%
			Ataluren: - PO	*Ataluren:* - Conditionally approved by the EMA (PTC therapeutics)	
AAV-mediated transport	- Micro- or mini-dystrophin cDNA transfer - Transfer of other gene therapy constructs (CRISPR-Cas9, AONs, micro-utrophin)	- Micro-dystrophin - Mini-dystrophin - Other Depends on transferred construct	- IV - IA - IM	- Phase I clinical status	100%
CRISPR-Cas9	- Exon skipping - (Multi-)exon deletion - Frame shifting - Exon knock-in	- Full-length dystrophin - Truncated dystrophin Depends on mode of action	- AAV-mediated delivery (*in vivo* gene therapy) - IPSC-mediated delivery (*ex vivo* gene therapy)	- Preclinical status	>60%

	Mode of action	Final product	Route of administration	(Pre-)clinical status	Theoretic applicability in % of DMD population
Stem cell therapies	- *In vivo transfer of allogeneic cells derived from healthy, HLA-matched donors*	Depends on mode of action	- IV - IA - IM	*In vivo transfer:* - Phase I/II status	100%
	- *Ex vivo gene therapy in autologous cells by* • AAV • CRISPR-Cas9 • HAC • Transposon			*Ex vivo gene therapy:* - Preclinical status	
Utrophin-based therapies	- *Protein upregulation* • Transcriptional upregulation • Post-transcriptional upregulation • Direct protein replacement	- (Micro-)utrophin	Depends on mode of action and delivery method	*Protein upregulation:* - Phase II status	100% (Protein upregulation is not applicable in dystrophin/utrophin knockout mammals)
	- *(Micro-)utrophin cDNA transfer*			*(Micro-)utrophin cDNA transfer:* - Preclinical status	

7.3. Tabel II: Advantages and limitations of gene therapy strategies for Duchenne muscular dystrophy.

	Advantages	Limitations
Exon skipping with Eteplirsen	- Low insertional mutagenesis risk - Good safety and tolerability profile - Low immune response risk	- High frequent (IV) readministration - Applicable to a small group (14%) of DMD patients
Stop codon readthrough	*Gentamicin:* /	*Gentamicin:* - Low safety and tolerability profile - Narrow therapeutic window - High frequent (IV) readministration - Applicable to a small group (13%) of DMD patients
	Ataluren: - Good safety and tolerability profile - PO (re)administration	*Ataluren:* - Applicable to a small group (13%) of DMD patients
AAV-mediated transport	- Low insertional mutagenesis risk - High transduction efficiency - Simplicity of production - Long-term transgene expression (low frequent readministration) - Applicable to all DMD patients	- High immune response risk - Low cargo-capacity
CRISPR-Cas9	- Permanent result (one-time administration) - Simplicity in production - Full-length dystrophin restoration - Applicable to >60% of DMD patients	- High off-target mutagenesis risk - High immune response risk - Bad *in vivo* delivery (requires delivery vectors)

	Advantages	Limitations
Stem cell therapies	*In vivo transfer of allogeneic cells*: - Simplicity in production - Low insertional mutagenesis risk - Applicable to all DMD patients	*In vivo transfer of allogeneic cells:* - High immune response risk - Limited availability of HLA-matched donors
	Ex vivo gene therapy in autologous cells: - Low immune response risk - Unlimited availability - Applicable to all DMD patients • *HAcs* - High cargo-capacity - Low insertional mutagenesis risk - Long-term gene expression • *Transposons* - Simplicity in production - Long-term gene expression	*Ex vivo gene therapy in autologous cells:* - High insertional mutagenesis risk - Labour intensive production • *HAcs* - Low transduction efficiency - Labour intensive production • *Transposons* - Low transduction efficiency
Utrophin-based therapies	- Low immune response risk - Applicable to all DMD patients	- No nNOS recruitment to the sarcolemma