Chapter 15

Advanced Treatments and Emerging Therapies for Dystrophin-Deficient Cardiomyopathies

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Abstract

Dystrophinopathies are characterized by skeletal and cardiac muscle complications because of a lack or shortened DYSTROPHIN protein. Ventilation assistance and corticosteroid treatment have positively affected life outcome but lead to an increased incidence of cardiomyopathy. Cardiomyopathy is now the leading cause of death in patients with dystrophinopathy. Thus, coherent guidelines for cardiac care have become essential and need to be communicated well. Progression of cardiac complications in patients with dystrophinopathy diverges from standard dilated cardiomyopathy development and monitoring and medical care for dystrophinopathy. This chapter summarizes current guidelines and recommendations for monitoring and clinical treatment of cardiac complications in patients with dystrophinopathy and provides a thorough survey of emerging therapies focusing on cardiac outcomes.

Keywords: dystrophin, Duchenne and Becker muscular dystrophy, dilated cardiomyopathy, symptomatic treatment, exon skipping, gene and cell therapy

1. Introduction

Dystrophinopathies are a group of diseases comprising Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD) and X-linked dilated cardiomyopathy (XLDCM), characterized by a shortened or absent *DYSTROPHIN* gene. DMD is the most prominent, with an incidence around 1:5000 live male births [1]. BMD occurs about three times less than DMD [2], while XLDCM is extremely rare [3]. *DYSTROPHIN*—part of the dystrophin-glycoprotein complex (Appendix 1)—is located on the X chromosome, and therefore, only males are affected.

The main clinical feature of patients with dystrophinopathy is an early loss of ambulation depending on the availability of DYSTROPHIN. Patients with DMD completely lack DYSTROPHIN—the pathological mechanisms are discussed in Appendix 2—and are presented with the most severe disease progression. This is indicated by a loss of ambulation around 10 years of age and consequent ventilation assistance, followed by death during the second or third decade [4]. Conversely, patients with BMD still express a truncated form of DYSTROPHIN. Although only partially functional, this still has an effect on disease progression and life expectancy, which are drastically prolonged [5]. Patients with XLDCM show the absence of DYSTROPHIN in the heart, while expression in skeletal muscles is conserved. Several hypotheses have been proposed for this peculiar genotype [3]. In patients with dystrophinopathy, the routine use of corticosteroids and nocturnal ventilation support have dramatically improved the life expectancy and its quality. Unfortunately, this brings up other complications, as the incidence of cardiomyopathy is raising and is nearly ubiquitous in older patients with DMD. In this perspective, monitoring and treatment of cardiac complications becomes more and more important.

This chapter contains an overview of the current monitoring and treatment guidelines and state-of-the-art therapies for dystrophin-deficient cardiomyopathies. The clinical features of dystrophinopathies with an emphasis on cardiac disease progression will be discussed briefly, followed by a description of the diagnostic process and current management strategies. Conclusions from recent clinical trials on current symptomatic treatments will be summarized and emerging therapies will be discussed in detail, from promising preclinical research to ongoing clinical trials.

2. Clinical features

2.1. Duchenne and Becker muscular dystrophy

Neonate boys with DMD are rarely symptomatic, and the disease will not be recognized until the second or third year of life. The patient may show evidence of mild muscle weakness before the 12th month of life (i.e., poor head control after the 6th week, mild inability to sit unsupported at the 6th month). It is possible that he achieves the motor milestones of walking (12th month) and running (2nd year) during the toddler period, but he will eventually be brought to medical attention due to being less active than expected, as well as being prone to falling [6].

One of the earliest clinical signs of DMD is pseudohypertrophy of the gastrocnemii in the calves caused by hypertrophy of muscle fibers combined with fat infiltration and proliferation of collagen. The muscles have a firm and rubbery consistency, as well as being hypotonic compared with unaffected muscles. By the age of 3–5 years, the clinical picture of DMD gradually appears and "the patient straddles as he stands and waddles as he walks". In order to rise from the ground, the typical Gowers' sign (i.e., the child first extends the arms and legs assuming a four-point position and then works each hand alternately up the corresponding thigh) is present and it is fully expressed by the age of 5 or 6 years. While standing and walking,

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the patient places the feet wide apart to increase the base of support, and as a result of gluteus medius weakness, there is waddling when walking [7].

The typical posture of a patient with DMD is lumbar lordosis and scoliosis (due to weakness of abdominal muscles and paravertebral muscles) with hip flexion and abduction, knee flexion and plantar flexion, as well as winging of the scapulae. Other common presentations in toddlers include falling, troubles in running or using the stairs and developmental delays [7, 8]. As the muscular wasting progresses, the weakness will spread to the muscles of the legs and forearms and patients may end up being confined to a wheelchair by 7 years of age, while other patients may continue walking with increasing difficulty until 10 years of age. Death usually occurs around the second decade of life, caused by pulmonary infections, respiratory failure, aspiration, airway obstruction or heart failure [6].

In BMD, the weakness and hypertrophy appear in the same muscles as DMD, but the onset is later (5–45 years; mean age: 12 years) with most patients losing ambulation in the third or fourth decade. However, some patients may have a milder phenotype with the onset of muscular weakness in late adulthood [6].

In both DMD and BMD groups, the median age of diagnosis of cardiac involvement is 14 years, with all patients having cardiac problems after 18 years of age [8]. While in BMD, the cardiac involvement can be the presenting symptom at diagnosis [9], and in patients with DMD, the presentation at diagnosis is usually subclinical and asymptomatic. Patients often have unspecific symptoms including fatigue, weight loss, nausea, sleep disturbance, cough, palpitations, sweating, chest and abdominal discomfort, decreased urinary output, irritability and concentration difficulties [8].

The physical examination may present some problems in patients with advanced muscular dystrophy (MD) due to scoliosis, immobility or glucocorticoid-related obesity. Tachycardia will be present, unless treated with β-blockers. Hypotension may be present—as a result of DYSTROPHIN loss in both vascular smooth muscles and low oral fluid intake—causing altered cardiac pacemaker activity, altered myocardial contractility and altered vasomotor tone. Edema is not commonly present, even in the presence of advanced right and left cardiac failure [9].

At auscultation the cardiac apex may be displaced as a result of scoliosis, with S3 gallop and S4 gallop commonly heard as a result of acute congestive heart failure and left ventricular (LV) dysfunction, respectively [9]. Moderate mitral regurgitation (due to posterior wall fibrosis and LV thinning) and moderate tricuspid regurgitation may be present [10]. Neck venous engorgement may be seen as a result of abdominal compression caused by scoliosis [8].

As dystrophy progresses, the LV function worsens, leading to the clinical picture of dilated cardiomyopathy (DCM), which can be complicated by arrhythmias. DCM is an enlargement of one or both of the ventricles combined with systolic dysfunction, usually preceding signs and symptoms of congestive heart failure. The hallmarks of DCM are decreased LV function (decreased ejection fraction), LV dilation and mitral regurgitation. The latter manifests as palpitations, vertigo, dizziness, syncope or sudden cardiac death. Moreover, in DMD, the

arrhythmia occurs even in the absence of myocardial fibrosis [11]. **Table 1** provides a summary with the most important clinical characteristics for DMD, BMD and XLDCM.

	DMD	BMD	XLDCM
Dystrophin	Absent	Partially functional	Absent (heart only)
Incidence	1 : 3500–6000 male births	1 : 18,000–19,000 male births	Very rare
Myopathy onset	3–5 years	12 years	Variable
Loss of ambulation	~12 years	~27 years	No loss
Life expectancy	Mid to late 20s	40s	Mid to late 10s
Cardiomyopathy onset	16–18 years	Variable, can precede skeletal muscle symptoms	Variable, from mild to severe cases

Table 1. Characteristics of Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD) and X-linked dilated cardiomyopathy (XLDCM).

2.2. X-linked dilated cardiomyopathy

XLDCM is a cardio-specific dystrophinopathy presenting with congestive heart failure (CHF) due to DCM in 10- to 20-year-old patients, without the dystrophinopathy-related involvement of skeletal muscles (**Table 1**). Patients show a brisk and progressive heart failure and ventricular arrhythmias, with untreated patients that may die of congestive heart failure not long after diagnosis [3].

3. Diagnosis and monitoring

Dystrophinopathy is generally underdiagnosed and definitive diagnosis can even take up to 2, 5 years from the onset of symptoms [12]. The first diagnostic test performed, when dystrophinopathy is expected, is a serum creatine kinase (CK) measurement. In most cases, CK levels are elevated, around 50–100 times in DMD, while in BMD levels are lower but still higher compared to healthy patients [13]. A second level diagnostic test is a mutation analysis, which reveals the specific genetic alteration and is useful to discriminate between DMD and BMD [14]. Differences between DMD and BMD are clarified by the reading frame concept (Appendix 3) [15]. For 5% of the cases, mutation analysis is not able to diagnose for dystrophinopathy [16]. In this circumstance, a muscle biopsy is taken and the reduction or the absence of DYSTROPHIN is analyzed by tissue staining (immunohistochemistry and immunofluorescence) or immunoblot (Western blot).

Recognizing cardiac complications in patients with dystrophinopathy is challenging, especially because of physical inactivity and respiratory complications [8]. Hence, cardiomyopathies are underdiagnosed in these patients [17]. Clinical guidelines were created in 2010, recommending an echocardiogram every 2 years from the moment of diagnosis of dystro-

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phinopathy or from the age of 6 years. From 10 years of age, a yearly screening to asses LV function is suggested [14]. Recently, it has been documented that cardiac MRI is more sensitive and can distinguish cardiac complications in an earlier stage for patients with dystrophinopathy [18]. Therefore, it is recommended to perform a cardiac MRI instead of echocardiography, also because patients with dystrophinopathy can suffer from scoliosis, which makes diagnosis with echocardiography more complicated. However, cardiac MRI can also be challenging for pediatric patients because of the need for sedation, cost and lack of accessibility. Sinus tachycardia is also known to precede any cardiac complications in patients with DMD [19].

As mentioned before, early diagnosis of cardiomyopathy onset is essential in patients with dystrophinopathy. Hence, clinical trials are still undertaken to study whether electrocardiogram (ECG), echocardiography, cardiac MRI and sera biomarkers can improve early detection of myocardial involvement and clinical outcome (NCT02020954).

4. Clinical management

4.1. Pharmacological treatment

Early diagnosis of dystrophinopathies—before the onset of cardiac complications—gives the opportunity to treat patients in a presymptomatic stage (**Figure 1**). However, for dystrophinopathy there is no general agreement on the treatment of cardiomyopathy [20]. There are some guidelines published to guide the decision-making process; however, still a huge variability in treatments exists between centers and clinicians [11].

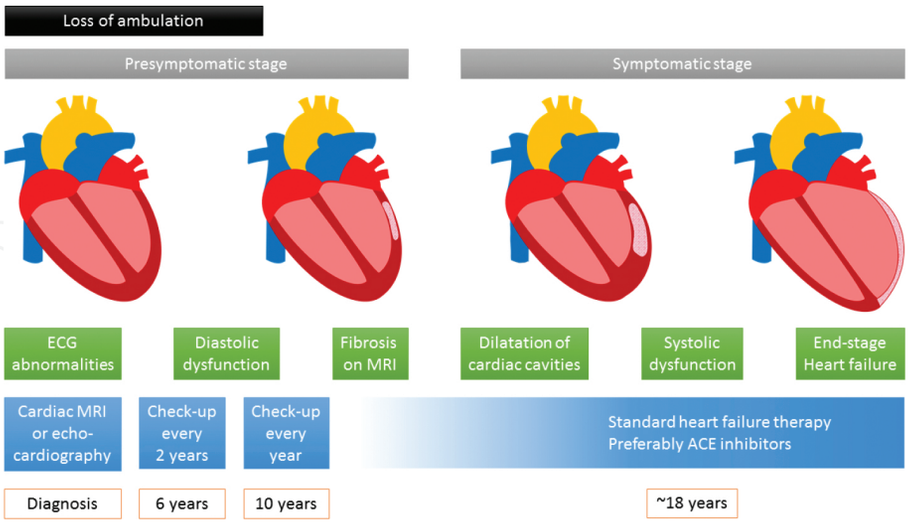

Figure 1. Progression of dystrophin-deficient cardiomyopathy and guidelines for monitoring and treatment. ACE, angiotensin-II-converting enzyme.

Corticosteroids improve muscle performance and delay loss of ambulation and also have beneficial effects on ventilation and scoliosis. However, this is accompanied by many side

effects such as weight gain, delay of puberty, decrease of vertebral bone mass, increase of vertebral fragility, cataract formation and growth-failure [11]. Evidence exists that corticosteroids also have advantageous effects on the heart of patients with DMD, several clinical trials suggest a delayed onset of cardiomyopathy in patients with dystrophy [21]. However, these results have to be interpreted with caution; all studies were retrospective without the objective to treat for cardiomyopathic complications. In addition, corticosteroids treatment in X-linked muscular dystrophy (*mdx*) mice—an animal model for DMD with a stop codon in exon 23—led to cases of heart failure, myocardial fibrosis and sarcolemmal damage [22]. Prospective clinical trials are necessary to address the effect of corticosteroids on heart functionality. Preclinical results in dystrophic mice and hamsters are concerning, especially given the broad use of corticosteroids for treating dystrophinopathies, and reveal the gap in our understanding about effects of corticosteroids on the heart [11]. In addition, the most suitable duration of corticosteroid therapy still has to be determined.

The effect of angiotensin-converting enzyme inhibitors (ACEIs) is more clear-cut and proved to postpone cardiomyopathy onset in both preclinical animal models and patients. A three-year treatment did not show any effect among a group of DMD patients treated with perindopril and another placebo-treated group. However, after 2 additional years of perindopril treatment – in which both of these groups now received this drug - a significant reduction in LV ejection fraction was observed between the 5 year-treated and 2 year-treated group [23]. A 10-year follow-up study, observed a significantly higher survival rate in a group of DMD patients that received a presymptomatic treatment with perindopril for 3 years [24]. Current guidelines propose a start of ACEIs for DMD patients only after development of LV dysfunction [14]. However, because aforementioned results demonstrated a clear beneficial effect of presymptomatic treatment, it is now recommended to initiate treatment with ACEIs before the onset of LV dysfunction in patients with DMD [25] (**Figure 1**). In case of observed intolerance against ACEIs, angiotensin-II receptor blockers (ARBs) could be used instead, since they have been shown to be as effective as ACEIs [26].

The use of β-blockers as a combined therapy with ACEIs is common for heart failure treatment; however, for dystrophinopathies, this is not well documented. One study described improvements in LV systolic function, when patients with DMD were treated with carvedilol [27]. Unfortunately, these findings were never reproduced in dystrophic *mdx* mice [28]. Study outcomes for combined therapy were mixed and showed additional beneficial effects for β-blockers in some cases, and no additional advantageous effects in others [29]. Future clinical trials need to evaluate whether the use of β-blockers for dystrophinopathy as mono- or combination therapy have beneficial effects. At the moment, it is recommended to initiate a therapy with β-blockers in patients with dystrophinopathy that have LV dysfunction [25]. Future studies will need to assess whether therapies combining corticosteroids with β-blockers and ACEIs could be of added benefit.

Mineralocorticoid receptor antagonists are a standard heart failure therapy due to their anti-fibrotic effect in DMD and ability to attenuate cardiomyopathy [30]. In a recent randomized double-blind clinical trial, one group of DMD patients with normal LV function were treated with only ACEIs or ARBs and the other group additionally also received eplerenone (aldos-

terone inhibitor). Results showed a lower LV circumferential strain in both treated groups compared to control, but not between treated groups [31]. This study is the only study of mineralocorticoid-receptor antagonists on dystrophin-deficient cardiomyopathy, and although it was not able to demonstrate a significant improvement, it is essential to investigate further whether aldosterone inhibitors could be of any benefit for delaying cardiomyopathy onset.

4.2. Nonpharmacological treatment

Heart transplantation is the only remedy for end-stage heart failure. Some cases have been published in which patients with DMD received a successful transplantation with no significant adverse effects [25]. Heart transplantation occurs more frequently in patients with BMD because of the higher incidence of cardiomyopathy and is only recommended for patients who have end-stage heart failure [25]. A deficit in surrogate organs complicates heart transplantation; therefore, left ventricular assist devices (LVADs) are an interesting substitution, demonstrating effectiveness in DMD and BMD patients with advanced heart failure [25]. However, possible postoperative complications, such as arrhythmias, bleeding, respiratory failure, stroke and rehabilitation, need to be further addressed [25].

4.3. Treatments with indirect effects

Treatments that have no direct effect on the heart can also have considerable benefits on cardiac function. For example, pain reduction lowers blood levels of catecholamines, which on its turn generates no further stress on the heart.

Lung and heart function are known to affect each other. When lung function needs to be assisted by noninvasive positive pressure ventilation (NIPPV)—because of breathing difficulties—it also has favoring results on cardiac function. It leads to less strain on the heart and a correspondingly reduced heart rate. Reduced lung function is also a strong negative predictor of survival when the vital capacity hits one liter [32]. In addition, assisted ventilation increased the mean survival of DMD patients with more than 10 years; however, patients who need constant ventilation will not exceed 20 years of age [32].

Although corticosteroids treatment has positively affected the incidence of scoliosis, it still occurs that thoracolumbar surgery becomes necessary to correct the spinal curvature [33]. In this case, timing is important and certain risk factors are bound to patients with DMD that have LV dysfunction [32]. Even when surgery is undertaken, it is not certain if scoliosis will not develop. This could lead into feeding and swallowing problems [32]. When surgery is able to prevent scoliosis onset, not only does it improve positioning and pulmonary function, but it also ameliorates cardiac function [33]. It is important to note that—although spinal surgery is performed in many neurological centers—it is not uniformly supported [11]. If patients with dystrophinopathy undergo surgery, an appropriate use of anesthetics is essential and needs to be assessed and monitored carefully before, during and after surgical intervention [34]. **Table 2** gives an overview of all current clinical interventions available for the treatment of dystrophin-deficient cardiomyopathy.

Method	Level of evidence	Recommendations	References
Pharmacological			
Corticosteroids	Mid	Initiation based on functional state and pre-existing risk factors for adverse side-effects	[14]
		Be aware of controversial cardiac results in animal studies and clinical trials	[83]
ACEIs and ARBs	High	First-line therapy upon development of LV dysfunction	[18]
		Initiate therapy from 10 years of age or earlier	[14]
β-Blockers	Low	Follow guidelines for adults with chronic heart failure	[84]
		Variable, normally initiation after ACEIs start on the basis of ventricular dysfunction or elevated heart rate	[18]
Mineralocorticoid -receptor antagonists	Low	Timing not adequately addressed and variation in clinical practice	[18]
Nonpharmacological			
LVAD	High	Currently bridge to heart transplantation but potential for destination therapy High-risk factor: scoliosis, respiratory muscle weakness and difficulties in recovery and rehabilitation	[85]
Heart transplantation	High	Should be considered for patient with end-stage heart failure	[25]
Indirect effects			
NIPPV	High	Main need for pulmonary care is from the onset of ambulation loss. Decisions for respiratory care must be taken by a care team including a physician and skilled therapist.	[86]
Thoracolumbar surgery	Mid	For patients not receiving glucocorticoids: surgery warranted when spinal curvature > 20°. For patients receiving glucocorticoids: surgery warranted upon further spinal curve progression.	[33]

ACEIs, angiotensin-converting enzyme inhibitor; ARBs, angiotensin II receptor blockers; LVAD, left ventricular assist device; NIPPV, noninvasive positive pressure ventilation.

Table 2. Clinical treatments of dystrophin-deficient cardiomyopathy.

5. Emerging therapies

5.1. Utrophin upregulation

In 1972, UTROPHIN—a DYSTROPHIN homolog with a shortened rod domain but many similar binding proteins—was discovered [35]. These resemblances started the speculation

that UTROPHIN could partially compensate for DYSTROPHIN loss. More clarity was brought by the creation of a *DYSTROPHIN/UTROPHIN* double knockout (*mdx/utrn*$^{-/-}$) mouse model, that showed a worsened skeletal and cardiac muscle phenotype with a drastically lower life expectancy of only 2–3 months compared to *mdx* mice [36]. Several studies have been performed testing UTROPHIN upregulation in preclinical models, the most promising one coming from a small molecule screening. SMT C1100 treatment demonstrated an increase of UTROPHIN levels in *mdx* mice and improved muscle function [37]. A phase Ia and recently also a phase Ib clinical trial were completed, concluding that no serious adverse effects were accompanied by SMT C1100 treatment in healthy controls and pediatric DMD patients, respectively [38]. It is important to mention that UTROPHIN will never be able to replace symptoms of DYSTROPHIN deficiency because of its structural differences and lack of a NO binding site (Appendix 1), presuming an incomplete surrogate effect.

5.2. Nonsense suppression

About 10–15% of patients with DMD carry a premature stop codon that abrogates translation of *DYSTROPHIN* [39]. Aminoglycosides are mainly used as antibiotic agents but are also able to cause a ribosomal read-through—meaning it is able to ignore premature stop codons while still detecting normal stop codons—and in this way generating a shortened, although functional DYSTROPHIN protein. Although promising, clinical results have been poor with inconclusive or very low expressed DYSTROPHIN levels and major concerns about side effects caused by Gentamicin and Negamicin [40]. Henceforth, PCT124 or ataluren was discovered by another small-molecule drug screening, showing promising results in preclinical animal models without skipping normal stop codons [41]. In addition, ataluren was considered safe and showed upregulation of DYSTROPHIN in certain subgroups of patients with DMD, although nothing was mentioned about the heart [42–44]. These clinical trials confirmed the need for further understanding and a division of patients with DMD in subgroups. Currently, ataluren is involved in a randomized, double-blind and placebo-controlled phase III trial, with the primary outcome being an improvement in the 6-minute walking test (6MWT) after 48 weeks; however, cardiac function will not be monitored (NCT01826487). A search for more effective read-through compounds (RTCs) ended up with two more drugs: RTC13 and RTC14. The highest efficiency was reached by RTC13. This compound restored DYSTROPHIN levels the most in the skeletal muscle and the heart compared to gentamicin, PTC124 and RTC14. Moreover, CK levels were reduced and muscle function was improved in *mdx* mice [45].

5.3. Exon skipping

The idea of exon skipping originated from BMD, due to the fact that these patients express a shorter isoform of DYSTROPHIN and show a much milder phenotype [46]. Skipping the genetic alteration of *DYSTROPHIN* so that an out-of-frame shift is turned into an in-frame shift would result in the expression of a truncated DYSTROPHIN isoform such as in BMD (Appendix 2) [47]. Antisense oligonucleotides (AONs) are short, single-stranded DNA sequences that are complementary to a target pre-mRNA splice site and able to skip exons by sterically hindering splice enhancers [48]. This procedure should be able to treat 35% of patients with

DMD by targeting one exon and even 83% when two exons are simultaneously targeted [49]. The downside is that AONs can only target one exon, for this reason research currently focuses only on the most frequently involved exons [49].

There are currently two different types of AONs undergoing clinical trials: 2′O methyl phosphorothioate (2′OMePS)—also called drisapersen [50]—and phosphorodiamidate morpholino oligomers (PMO)—also known as eteplirsen [51]. Both were unable to improve DYSTROPHIN expression in the heart [52, 53]. Recently, a high degree of DYSTROPHIN rescue was achieved in the respiratory and cardiac muscles with tricyclo DNA oligomers in *mdx* mice [54]. However, these results were accomplished by multiple injections and high doses of administration (200 mg/kg per week).

Previous clinical trials picked up the inefficiency of systemic delivery of naked AONs [50, 51, 55]. For this reason, attention quickly converted toward the development of delivery methods for AONs. Systems like cell-penetrating peptides (CPPs) or encapsulation techniques such as liposomes or nanoparticles appeared. CPPs are small peptides that are conjugated to AONs. They facilitate the penetration through the plasma membrane and can be divided into three groups: arginine rich, Pip (PNA/PMO internalization peptide) or phage and chimeric peptides. Arginine-rich CPPs associated with a PMO showed the first robust cardiac DYSTROPHIN expression and an improved cardiac function [56, 57]. More recently, Pip6 conjugated with a PMO also showed cardiac Dystrophin expression and functional improvement at low doses [55] and prevented exercise-induced cardiomyopathy after long-term treatment [58]. Phage peptides were less successful with the exception of a 7-mer phage conjugated to 2′OMePS that resulted in an enhanced uptake and exon skipping in the cardiac muscle [59].

Instead of a molecular approach with AONs conjugated to CPPs, it is also possible to deliver small nuclear RNAs (snRNAs) or nucleases to the cardiac muscle. These effectors need to be incorporated into the genome; recombinant adenoviral-associated viruses (rAAVs) are the preferred choice because of their long persistence in myonuclei. However, the disadvantage is their relatively small cloning capacity (5 kb). In addition, to safely apply AAV therapy, all viral genes have to be removed except for the components necessary for replication [60].

Delivery of U7snRNA by rAAV6 has shown to restore cardiac DYSTROPHIN expression, even one year after injection higher DYSTROPHIN levels were still found [61]. The safety profile and optimal concentrations of rAAV8 U7snRNA delivery in the forelimb of a large cohort of GRMD dogs have been monitored carefully and concluded that this treatment is safe, since no adverse immunologic responses were observed [62]. Recently, an observational study was initiated, monitoring clinical and radiological changes of patients eligible for exon 53 skipping, while testing their immunization against viral serotypes (NCT01385917).

Exon skipping with nucleases such as "clustered regularly interspaced short palindromic repeats" (CRISPR) together with a Cas9 nuclease brought new insights and possibilities for gene-editing treatments. CRISPR/Cas9 is an immune protection system originating from bacteria that is able to edit the genome by introducing a double-strand break. Afterwards genomic damage is repaired by one of the two possible repair mechanisms: nonhomologous end-joining (NHEJ) or homology-directed repair (HDR). NHEJ was used as a technique for

DYSTROPHIN exon skipping in *mdx* mice, a CRISPR/Cas9 was designed targeting exon 23, leading into a removal of this exon by double-strand breaks where after these two ends are linked back together again by NHEJ [63]. Gene-editing components were delivered by rAAV9 through different administration routes and showed in all cases improved Dystrophin expression in the cardiac and skeletal muscle [63].

5.4. Gene therapy

Differently from exon skipping, it is also possible to incorporate *DYSTROPHIN* into the genome instead of designing techniques to modulate native *DYSTROPHIN*. The advantage of this therapy is that it is not patient specific, all patients can benefit from the same technique. However, due to the enormous size of *DYSTROPHIN*, it is impossible to compact it entirely into viral capsids. Because of the observation that short isoforms of only 45% of full-length DYSTROPHIN can lead to a very mild phenotype, mini- and microdystrophin were created, leaving behind redundant parts [64, 65].

Some different rAAV types have shown huge promise in delivering mini- or microdystrophin to the heart. Microdystrophin delivered by rAAV6 was able to incorporate itself in the skeletal, cardiac and respiratory muscles of *mdx* mice; however, heart function did not recover, while DYSTROPHIN was clearly expressed [66]. A subtype that has shown to be particularly efficient in transfecting the heart is rAAV9 [67]. Systemic injection of minidystrophin encapsulated in rAAV9 has shown widespread expression, also in the heart of the GRMD dogs [68].

While only one phase I clinical trial for minidystrophin has been executed [69] and another one for microdystrophin is ongoing (NCT02376816), it is clear that this field is still at its starting point. Many difficulties that need to addressed in the future are immunological responses and the necessity of high viral titers [70]. However, novel techniques like fetal transduction [71], chimeric vectors [72] and plasmapheresis [73] have already shown a drastic decrease in immunologic responses in preclinical animal models. In addition, viral gene therapy struggles with compaction size and eventually delivers smaller *DYSTROPHIN* constructs in comparison to skipped *DYSTROPHIN*. Nevertheless, full-length *DYSTROPHIN* was recently successfully incorporated into the skeletal muscle of a *mdx* mouse through the usage of three vectors carrying "intandem" sequential exonic parts of *DYSTROPHIN* [74].

5.5. Cell-based therapy

Cell therapy has some advantages compared to the aforementioned therapies. The idea of cell therapy is to produce healthy cells, which express full length DYSTROPHIN that are able to integrate into the tissue upon injection. In an optimal situation, these cells should also be able to repopulate the progenitor populations such as the satellite cell pool in the skeletal muscle. Many trials have been performed with adult stem cells like myoblasts, bone marrow-derived stem cells, CD133+ stem cells and mesoangioblasts (MABs). MABs are vessel-associated progenitors that are able to migrate across the vessel wall and have been shown to repopulate the skeletal muscle of GRMD dogs upon systemic injection, resulting into a variable improvement of muscle function [75]. Treatment of *mdx*/*utrn*$^{-/-}$ mice with aorta-derived MABs led into

a delay of DCM onset and promotion of angiogenesis [76]. Recently, a completed phase I clinical trial with MABs in patients with DMD showed no sign of DYSTROPHIN expression after treatment [77]. Multiple reasons could explain this observation, such as the late age of the patients and the lengthy procedure of isolation, which does not ensure that the cells are delivered in an optimal way. However, systemic treatment with MABs has shown to be safe and efforts are being made to start a phase II clinical trial.

Method	Phase	Model/patients	References
Utrophin upregulation			
Arginine butyrate	Preclinical	*Mdx* mice	[87]
SMT C1100	I	Healthy controls	[38]
Read-through therapy			
Aminoglycosides	I, completed	Patients with DMD and BMD	[40]
Ataluren (PTC124)	III, ongoing	Patients with DMD	NCT01826487
RTC13/14	Preclinical	*Mdx* mice	[45]
Exon skipping			
Drisapersen (2′OMePS)	III, recruiting	Patients with DMD	[51], NCT01803412
Eteplirsen (PMO)	III, recruiting	Patients with DMD	[50, 88], NCT02255552
Tricyclo-DNA	Preclinical	*Mdx* mice	[54]
Cell-penetrating peptide/AON	Preclinical	*Mdx* mice	[55–58]
Phage peptide/AON	Preclinical	*Mdx* mice	[59]
rAAV6/U7snRNA	Preclinical	GRMD	[61]
rAAV9/CRISPR/cas9	Preclinical	*Mdx* mice	[63]
Gene therapy			
rAAV6-microdystrophin	Preclinical	*Mdx* mice	[66]
rAAV9-minidystrophin	Preclinical	GRMD	[67, 89]
Cell-based therapy			
Mesoangioblast	Preclinical	*Mdx*/*utrn* mice	[76]
	I, completed	Patients with DMD	[77]
iPSC-derived cells	Preclinical	Sgcb-null mice	[78]

Mdx, X-linked muscular dystrophy; DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy; RTC, read-through compound; AON, antisense oligonucleotide; rAAV, recombinant adenoviral-associated viruses; CRISPR, clustered regularly interspaced short palindromic repeats; GRMD, golden retriever muscular dystrophy; utrn, Utrophin; iPSC, induced pluripotent stem cell; Sgcb, sarcoglycan beta.

Table 3. Preclinical treatments of MD-associated cardiomyopathy.

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Recently, treatments with derivatives of induced pluripotent stem cells (iPSCs) are being explored. iPSCs are basically (patient-derived) somatic cells that are reprogrammed into pluripotent stem cells that possess similar features as embryonic stem cells. These cells have been differentiated into mesodermal-like progenitors and injected directly into the skeletal and cardiac muscles of *Sgcb-null* mice, a model for limb girdle muscular dystrophy with a worsened phenotype compared to *mdx* mice. Data showed that these unique iPSC-derived myogenic progenitors are able to integrate into heart and limb muscles and functionally ameliorate both cardiac and skeletal muscles of injected dystrophic mice [78].

Another advantage of cell therapy is the possibility of correcting patient-derived cells and reinjecting them, bypassing immunological responses. Novel strategies with CRISPR/Cas9 have been developed to repair DYSTROPHIN in iPSCs, showing functional recovery upon differentiation [79]. Eventually, differentiation of iPSCs towards cardiomyocytes can also be used to set up high-throughput screenings for detecting novel patient-tailored therapeutic molecules.

Nevertheless, there are still many aspects in cell-based therapy that need to be addressed. As of yet, there is no consensus about timing of injection, which is hypothesized to be crucial. As for integration, many cell therapies suffer from extremely low integration efficiencies. Future studies should focus on tracing the injected cells, to follow their trajectory and fate during treatment [80]. **Table 3** provides an overview of all the discussed preclinical therapies.

6. Conclusion

Since the utilization of ventilation support and corticosteroids treatment, cardiac complications in dystrophinopathies have become more prominent, being responsible for 40% of mortality in patients with DMD [11]. Cardiomyopathy development in patients with dystrophinopathy is highly variable and can be asymptomatic for a long period of time. The earlier onset of skeletal muscle symptoms and the sequential diagnosis can be used as an advantage for cardiac treatment. At the moment, it is recommended to perform a cardiac MRI at the age of 6 or right at the time of diagnosis, followed by two-year check-ups till the age of 10 where after annual check-ups are required. Momentarily, no consensus exists about initiation of treatment. It is advised to start symptomatic treatment as soon as possible because of the beneficial effects on cardiomyopathic progression. ACEIs are the preferred choice for treatment, because of their clear and advantageous effects on cardiomyopathy in patients with dystrophinopathy. Effects of corticosteroids treatment on the heart remain distrustful and cardiac deterioration should be monitored with care, while β-blockers are shown to be effective as stand-alone therapy, but additive effects together with ACEIs are not observed. In addition, transparent results about mineralocorticoid-receptor antagonist treatment on patients with dystrophinopathy are still missing. In the case of end-stage heart failure, heart transplantation or LVADs should be considered. While treatments with indirect effects on the heart like pain reduction, NIPPV and thoracolumbar surgery could also be of added benefit for cardiac health.

Many emerging therapies exist that are being investigated in preclinical models and clinical trials. These studies aim to enhance UTROPHIN expression, read through a stop codon

mutation, skip the exon—that is holding the genetic alteration in DMD to express a shorter form of DYSTROPHIN corresponding to BMD—or bring in shortened *DYSTROPHIN* variants by viral or cell-based therapy. Several of these therapies already made it into clinical trials with some undergoing phase III trials. However, as skeletal muscle complications are the most prominent, effects on the heart have not been taken up into the expected outcomes of these trials. This also because in most preclinical treatments beneficial effects on the heart were absent. Novel approaches were necessary to deliver constructs to the cardiac muscle and some preclinical studies have shown DYSTROPHIN expression in the heart together with functional improvements in small and large animal models. Emerging literature is also showing that IPSC-derived myogenic progenitors from patients with dystrophy provide an attractive model to get new insights on gene-editing treatments. This novel technology offers possibilities for autologous therapy and eventually targeting both cardiac and skeletal dystrophic muscles with a single myogenic progenitor population. It is assumed that all these preclinical therapies will make it into clinical studies and hopefully subsequently into dystrophinopathy therapies. However, many issues still exist and need to be addressed such as immunogenic reactions toward the administered cells or viruses, the need for high-dosing regimens and bodywide delivery.

Appendix

Appendix 1: The dystrophin-glycoprotein complex

DYSTROPHIN functions as a linkage rod between the actin cytoskeleton and the cell membrane, where it is connected to β-DYSTROGLYCAN, which in turn associates with the extracellular matrix via LAMININ. The SARCOGLYCAN complex is in close proximity to β-DYSTROGLYCAN and is important in signaling, which occurs through SYNTROPHIN and DYSTROBREVIN. Together these proteins are part of the dystrophin-glycoprotein complex (DGC). DYSTROPHIN also accommodates a neuronal nitric oxide synthase (nNOS)-binding site.

Appendix 2: The molecular mechanisms of dystrophin deficiency

The 24 spectrin-like repeats within DYSTROPHIN neutralizes contraction-induced stress. The absence of DYSTROPHIN leads into a loss of muscle structural integrity, which generates membrane microruptures, leading to elevated CK levels in the serum and a high intracellular Ca^{2+} concentration that subsequently activates calcium-dependent proteases [81]. Because of the lack of experimental proof, researchers have started questioning this theory. It became evident that activation of stretch-induced Ca^{2+} channels corresponds with elevated intracellular Ca^{2+} levels, although the responsible channel has still to be found [82]. Repeated contraction-induced damage leads to a continuous muscle breakdown and regeneration, with an increased build-up of fatty and fibrotic tissue, followed by an eventual loss of muscle contractility.

Appendix 3: The reading-frame concept

A reading frame consists out of codons—three nucleotide-based sequences—which translate into a specific amino acid. A shift of the reading frame basically means that the reading frame

is altered, for example by a deletion of nucleotides. If the genetic code is extended or shortened by a multitude of "3 nucleotides", the reading frame is sustained. Therefore, this is called an "in-frame shift" resulting in a partially functional protein, which corresponds to BMD. When genetic alteration occurs by a multiplication of "1–2 nucleotides", the reading-frame shifts, leading to post-translational disintegration of DYSTROPHIN; hence, this is called an "out-of-frame shift" and corresponds to DMD.

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