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Review

Cardiac therapies for Duchenne muscular dystrophy

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***Abstract*:** Duchenne muscular dystrophy (DMD) is a devastating disease that results in life-limiting complications such as loss of skeletal muscle function as well as respiratory and cardiac complications. Advanced therapeutics in pulmonary care have significantly reduced respiratory complication–related mortality, making cardiomyopathy the main determinant factor of survival. While there are multiple therapies such as the use of anti-inflammatory drugs, physical therapy, and ventilatory assistance targeted toward delaying the disease progression in DMD, a cure remains elusive. In the last decade, several therapeutic approaches have been developed to improve patient survival. These include small molecule–based therapy, micro-dystrophin gene delivery, CRISPR-mediated gene editing, nonsense readthrough, exon skipping, and cardiosphere-derived cell therapy. Associated with the specific benefits of each of these approaches are their individual risks and limitations. The variability in the genetic aberrations leading to DMD also limits the widespread use of these therapies. While numerous approaches have been explored to treat DMD pathophysiology, only a handful have successfully advanced through the preclinical stages. In this review, we summarize the currently approved as well as the most promising therapeutics undergoing clinical trials aimed toward treating DMD with a focus on its cardiac manifestations.

Keywords: ACE inhibitor, cardiac, cardiomyopathy, CRISPR, DMD, Duchenne muscular dystrophy, exon skipping, gene editing, gene therapy

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Introduction

Duchenne muscular dystrophy (DMD) is an X-linked genetic disorder affecting approximately one in every 5000 male births worldwide. The cause of this disease lies in the absence of the protein dystrophin – a crucial protein that supports the structural integrity of muscle cells by anchoring the inner membranes of their sarcolemma to the actin filaments in the cytoskeleton. The clinical symptoms typically start to manifest in patients around the age of 3 to 5 years.[1] Progressive body-wide muscle weakness is the first sign of DMD that leads to eventual loss of ambulation by the age of 12.[2] The pathological condition gradually progresses toward death due to cardiorespiratory failure.

Dystrophin is located on the cytoplasmic side of the plasma membrane and functions as part of a large glycoprotein complex called the dystrophin-associated protein complex (DPC).[3] The primary function of dystrophin is to provide mechanical reinforcement to the sarcolemma as well as to stabilize the DPC. Without dystrophin, the integrity of the sarcolemma becomes severely compromised. This absence, in turn, gives rise to a multitude of cellular defects that include loss of membrane proteins, increased inflammation, and impaired calcium homeostasis. These complications culminate in characteristic DMD pathologies such as muscle degeneration, cardiac fibrosis, and loss of motor function.

Dystrophin is encoded by the *DMD* gene, the largest known human gene at 2.4 Mb, which contains 79 exons. This large size of the gene increases its likelihood of having mutations, resulting in erroneous gene products. Deletions are the most

common type of mutation in the *DMD* gene accounting for 60–70% of all mutations.[4,5] Deletion mutations often alter the reading frame of the protein and introduce a premature stop codon. Insertions and point mutations are the next most common forms of mutation, accounting for about 20%, followed by duplication mutations that happen in about 5–15% of patients.[4] In most cases, the mutations result in premature stop codons and subsequent termination of protein synthesis. Conversely, mutations that do not produce stop codons and allow for truncated dystrophin production often result in a milder form of muscular dystrophy called Becker muscular dystrophy (BMD). BMD is much rarer than DMD, affecting less than eight per 100,000 male newborns.[6] In BMD, the reading frame is usually preserved resulting in the production of partially functional dystrophin. The clinical manifestations of BMD can range from severe DMD-like symptoms to very mild muscle weakness. This phenotypic severity depends on which regions of the dystrophin protein are lost due to the mutation.[7] Studies on this genotype–phenotype correlation have aided us in understanding the roles of different domains of dystrophin in maintaining cellular integrity and have been instrumental to the development of numerous genetic therapies like gene correction and replacement that can potentially treat DMD.

In patients with DMD, cardiorespiratory complications are usually the main determinant factor for survival. With the advancement of palliative therapies like ventilation support and assisted airway clearance, however, respiratory complications are becoming more manageable. A study in France showed that patients can now live up to their 40s with optimal care and support.[8] As respiratory complications become more manageable, cardiac conditions are now considered the leading cause of death among DMD patients. Even though there are recommended guidelines specifically designed for treating patients preemptively for delaying cardiovascular symptoms, a cure is still out of reach.

To treat DMD, new genetic and molecular therapies are being developed that work either by restoring the function of dystrophin or by compensating for the loss of dystrophin. Even though cardiorespiratory complications have become primary determinants of patient survival, most clinical trials still mainly use improvement in skeletal muscle function to assess the therapeutic efficacy of DMD therapies. As such, most of the treatments show limited to no efficacy in the dystrophic heart. The pathophysiology of DMD cardiomyopathy is complex. Numerous factors contribute to disease progression, including myocyte membrane instability, dysregulation of calcium channels, limited mitochondrial energy production, reactive oxygen species and nitric oxide dysregulation, and fibrosis.[9] Considering the importance of cardiac pathology in patient survival, in this article, we will review emerging therapeutic advancements targeted toward DMD and how well these therapies are at reducing or preventing cardiac dysfunctions. Of note, the focus of this article is on noninvasive pharmacological and biological therapies that are already in use or under investigation. Invasive modalities of treatments such as ventricular assist devices, heart transplantation, and internal cardiac defibrillators are beyond the scope of this article.

Small molecules for cardiac treatments

The lack of curative treatments for DMD has led to the use of small molecule–based therapies aimed at mitigating cardiac symptoms and disease progression. Three major classes of small molecules are currently being used to manage cardiac symptoms in DMD patients.

Angiotensin inhibitors

Cardiac fibrosis is one of the main contributors to cardiac dysfunction, as it affects cardiac muscle stiffness as well as diastolic and systolic function.[10,11] The angiotensin-converting enzyme (ACE) plays a crucial role in the development of fibrosis. This enzyme is responsible for converting the inactive angiotensin 1 (Ang 1) to its active form angiotensin 2 (Ang 2) that stimulates the secretion of aldosterone. Aldosterone can cause a fibrogenic response by directly acting on fibroblasts or indirectly stimulating immune cells to release pro-inflammatory cytokines.[12] Several animal studies have demonstrated the antifibrotic role of ACE inhibitors (ACEIs), delineating their efficacy at reducing interstitial collagen deposition and improving left ventricular (LV) function.[13] In a 10-year follow-up study, Duboc *et al.*[14] documented the benefits of perindopril on patient survival. Among 28 patients treated with perindopril, the survival rate was 92.9% after 10 years compared with the 65.5% survival rate in

the placebo treatment group of 29 patients – a clear indication of the beneficial effects of ACEIs in DMD therapeutics. Even though the causes of mortality reported in this study were not limited to cardiac failure, a previous study from this group has shown the benefits of perindopril on LV function in which only one patient had an LV ejection fraction (EF) $< 45\%$ compared with the eight patients in the placebo group.[15] These promising outcomes from clinical investigations coupled with the data from preclinical studies have made ACEIs the first line of therapy for treating DMD. Another class of widely used drugs is angiotensin II type I receptor antagonist or angiotensin receptor blocker (ARB) that also acts on the renin-angiotensin-aldosterone system to improve vasorelaxation. These two classes of drugs, ACEI and ARBs, are currently used to reduce the effects of angiotensin in the heart of DMD patients. ACEIs are recommended in patients approaching 10 years of age as a pre-emptive treatment for cardio-protection.[15] ARBs are recommended as a secondary option in cases of poor ACEI tolerance.[16]

Beta-adrenergic receptor blockers

The benefits of beta-adrenergic receptor blockers or beta-blockers (BBs) in the treatment of heart failure have been well established. Tachycardia, a condition in which the heart rate is significantly increased, is one of the common characteristics of DMD. The heart expresses β-adrenergic receptors (β-AR) in which catecholamines bind to increase heart rate and myocardial contractility. Loss of dystrophin results in a decreased capacity of the cardiac cells to respond to stress and cause myocyte damage. As such, treatment with BBs causes the heart to beat slower and with less force. BBs are now regularly prescribed in combination with ACEIs to increase survival rates. In the mdx mouse model, the most well-characterized and most commonly used animal model of DMD, combined treatment of ACEI and BB has shown to normalize stroke volume and cardiac output with improved diastolic function.[17] In contrast, a 2012 report on DMD patients found no significant difference in EF between groups treated with ACEI and BB *versus* ACEI alone.[18] As BBs are used as a second line of therapy, usually prescribed when tachycardia becomes detectable in patients and always in combination with ACEIs, there is no conclusive evidence about the efficacy of BBs alone on DMD-related cardiomyopathy and it is unlikely to see such a study in the future.[19] Hence, efforts should be made to evaluate the effectiveness of BBs in patients receiving treatments with and without BBs to determine the benefits that are specific to BB usage.

Corticosteroids

Corticosteroids are the most widely prescribed medications for DMD. These are a class of steroid hormones that are secreted by the adrenal cortex. Glucocorticoids, one of the major classes of corticosteroids, are involved in a wide range of physiological processes such as regulating the immune response/inflammation, metabolism, and behavior.[20] They carry out these functions by diffusing through the cell membrane and binding to the glucocorticoid receptor (GR). This receptor–ligand complex then gets translocated into the nucleus and suppresses the activity of pro-inflammatory nuclear factor kappa B (NF-κB). As a result, glucocorticoids can exert a potent anti-inflammatory effect and thus are one of the most prescribed drugs to treat autoimmune and inflammatory diseases.[21] In DMD, elevated NF-κB activity is recognized as one of the key molecular features responsible for increased inflammation, and glucocorticoids such as prednisone/prednisolone and deflazacort are considered to be the gold standards of care (SoC) for treatment.[22] GR, however, can also bind to negative glucocorticoid response element (nGRE) sites on other genes and can induce adverse effects that include reduced bone density, excessive weight gain, cataracts, and delayed growth.[23] Despite these side effects, large studies have found glucocorticoids to be beneficial for DMD patients.[24,25] The use of glucocorticoids was associated with significant improvements in motor functions.[26] A recent study on patients with DMD found no effects of corticosteroids on delaying LV function; however, the patients had a significantly later onset of respiratory complications after continued corticosteroid use.[27] In another study, Guglieri *et al.*[28] conducted a trial on 196 boys with DMD to compare the three most common corticosteroid regimens – daily prednisone (0.75 mg/kg), daily deflazacort (0.90 mg/kg), and intermittent prednisone (0.75 mg/kg). Their primary outcome comprised changes in motor function (rise from the floor velocity), respiratory function (forced vital capacity), and satisfaction with treatment, and both daily prednisone and daily deflazacort treatments provided better outcomes

compared with intermittent prednisone treatment. Their report showed no significant difference between the two daily treatment groups. In terms of safety, influenza was, however, more frequently reported in the daily prednisone group compared with the other two, whereas cataracts were more frequent in the daily deflazacort group. Both prednisone treatment group participants gained more weight than the deflazacort group. Taken together, deflazacort appears to be the safer option; however, patient health conditions and genetic background need to be carefully evaluated before prescribing these corticosteroids. The study did not include any cardiac assessments.

Mineralocorticoids are another class of corticosteroids produced in the adrenal cortex that are responsible for maintaining salt and water balance. The mineralocorticoid receptors (MRs) are ligand-activated transcription factors that reside in the cytosol and are activated by mineralocorticoids like aldosterone. Once activated, the receptors dimerize and translocate to the nucleus to induce gene expression.[29] MRs are present in many cell types like endothelial cells, myeloid cells, and cardiomyocytes. MR overactivation in pathophysiological conditions has been shown to increase the expression of pro-inflammatory and fibrotic proteins that ultimately lead to cardiovascular damage and dysfunction.[30] Over the years, MR antagonists have been demonstrated to be effective in treating hypertension and cardiac patients by lowering blood pressure and fibrosis.[31] Now drugs like eplerenone and spironolactone are routinely prescribed for managing heart conditions with low EF.[32] In a randomized, double-blind, placebo-controlled trial on 42 patients, Raman *et al.*[33] reported administration of eplerenone with ACEI/ARB resulted in the preservation of LV systolic function and improvement in LV EF and systolic circumferential strain when compared with ACEI/ARB treatment alone. While this combined use of eplerenone with aldosterone inhibitors has shown improved outcomes, the effects of eplerenone alone are not clear. The patients enrolled in the study were receiving different treatments such as ACEI, ARB, or BB, further complicating a proper assessment.[34] This is understandable given the limited number of DMD patients who meet the criteria for enrolling in clinical trials. Hence, future studies with a larger cohort of patients would be necessary to properly evaluate the therapeutic efficacy of eplerenone.

Vamorolone (previously known as VBP-15) is a novel anti-inflammatory steroid analog that is currently being studied as a replacement for traditional glucocorticoid treatment for DMD.[35] This drug acts in a similar way to other glucocorticoids by binding to the GR but not to GREs. As such, the adverse effects associated with traditional glucocorticoids are expected to be significantly lower with the use of vamorolone. Moreover, vamorolone also acts as an MR antagonist, further minimizing the side effects seen with the use of traditional glucocorticoids. The effectiveness of vamorolone has already been demonstrated in preclinical studies[36] and the drug has advanced to the clinical trial stage. Cohorts from a randomized, placebo-controlled phase II trial (NCT03439670) receiving vamorolone showed improved outcomes in time to stand (TTSTAND) and 6-min walk test (6MWT) velocity, indicating that this drug is just as effective as the prednisone while having fewer side effects.[37] No data on cardiac improvement, however, have been reported. Currently, the drug has been granted Orphan Drug status in the United States and in Europe.

Other symptomatic treatments

Apart from the abovementioned major classes of small molecules, several new drugs aimed at managing the downstream effects of DMD are under clinical investigation. FG-3019 (Pamrevlumab; FibroGen Inc., USA) is a monoclonal antibody designed to interfere with the connective tissue growth factor [CTGF/(cellular communication network factor 2) CCN-2] – a key factor responsible for muscle fibrosis. Administration of FG-3019 in mdx mice has been shown to reduce the dystrophic phenotype and functional improvements.[38] A report on the FG-3019 phase II trial (NCT02606136) showed a 0.29% increase in patients' LV EF with improved lung function and upper limb performance.[39] Currently, this drug is in a phase III clinical trial in which it will be administered as an intravenous infusion in combination with systemic corticosteroids (NCT04632940). Ifetroban (Cumberland Pharmaceuticals, USA), a thromboxane receptor antagonist, is in its phase II clinical trial and aims to treat dilated cardiomyopathy in DMD patients (NCT03340675). Preclinical studies on dystrophic mouse models reported Ifetroban to be effective at reducing cardiomyopathy as well as improving heart function and survival.[40,41] Rimeporide (EspeRare Foundation, Switzerland) is a Na^+–H^+

exchanger 1 (NHE1) inhibitor that has been developed to treat advanced congestive heart failure. NHE1 is a transmembrane protein responsible for balancing the intracellular Na^+–H^+ levels. This protein also plays part in regulating intracellular calcium concentrations. Selective inhibition of this protein using Rimeporide has been shown to preserve LV EF in the Golden retriever muscular dystrophy (GRMD) dog model.[42] The drug was found to be safe and well tolerated in a phase Ib trial on DMD patients (NCT02710591) and is currently under preparation for a phase II trial.[42]

Genetic therapies

Genetic therapy is another promising approach to treat DMD in which the goal is to induce the expression of a functional gene that can restore dystrophin production. This can be achieved either by correcting the mutated gene or by delivering a new copy of the gene. Considering the length of the gene and the variety of mutations among patients, most of the current genetic approaches, however, need to be tailored for certain patient subgroups. In this section, the most promising genetic therapies will be discussed.

Nonsense readthrough

Nonsense readthrough or nonsense suppression therapy is targeted toward patients who have a nonsense mutation in their *DMD* gene. About 10% of DMD patients have this kind of mutation in which an amino acid codon gets replaced by a stop codon. This premature stop codon (or premature termination codon, PTC) results in a *DMD* mRNA that either produces truncated, dysfunctional dystrophin or no dystrophin at all. In nonsense readthrough, the goal is to interfere with ribosomal activity such that its ability to recognize PTCs is mildly disrupted. This enables the ribosome to add an amino acid in place of the PTC and continue with the rest of the translation process without having any significant effects on natural stop codons.[43] Barton-Davis *et al.*[44] first reported that administration of the aminoglycoside antibiotic, gentamycin, can restore dystrophin expression in mdx mice that have a PTC in exon 23 of the *DMD* gene. This drug, however, failed to show any dystrophin restoration in DMD or BMD patients.[45] Later, based on the same principle, Ataluren (brand name Translarna) was developed by PTC Therapeutics Inc., USA which shows higher nonsense suppression activity with less renal toxicity.[43] Currently, Ataluren has been approved in Europe [European Medicines Agency (EMA)], but not in the United States [U.S. Food and Drug Administration (FDA)]. A report from a phase III clinical trial (NCT01826487) suggests that Ataluren administration showed nonsignificant but improved motor function in the 6MWT.[46] In a more recent report from another phase III clinical trial (NCT01557400), patients treated with Ataluren in combination with SoC showed a significant 2.2-year delay in age at loss of ambulation and a 3-year delay in respiratory complications compared with patients receiving SoC alone.[47] Other clinical trials (NCT01247207, NCT03179631) are still ongoing and any significant effect of Ataluren on cardiac improvement is yet to be observed.

Exon skipping

Exon skipping is one of the most promising therapeutic techniques for DMD that aims to restore the disrupted *DMD* mRNA reading frame by skipping specific exons. The open reading frame (ORF) is crucial for protein translation, and a shift in the *DMD* ORF, usually caused by deletion mutations, results in the formation of a PTC downstream, thereby interfering with the translation of the full dystrophin protein. The idea behind exon skipping is to skip one or more exons responsible for the frameshift and restore the ORF. Skipping specific exons to restore the reading frame of the mRNA induces the production of truncated but functional dystrophin, converting the severe DMD phenotype to the milder BMD-like phenotype. This can be achieved by using antisense oligonucleotides (AOs) that are usually designed to bind and obscure one or more splice acceptor or enhancer sites of the mutated exon. As a result, the spliceosomes move on to the next splicing site omitting the exon from the final mRNA product (Figure 1). The *DMD* gene contains various hotspots in which deletions of certain exons are more prevalent in the population. This means that groups of DMD patients can potentially be treated by the same exon-skipping strategy. For instance, skipping exons 51, 53, 45, and 44 would be applicable for approximately 13%, 8%, 6%, and 6% of DMD patients, respectively.[48]

The first phosphorodiamidate morpholino oligomer (PMO)-based exon-skipping drug to get FDA approval for treating DMD was eteplirsen

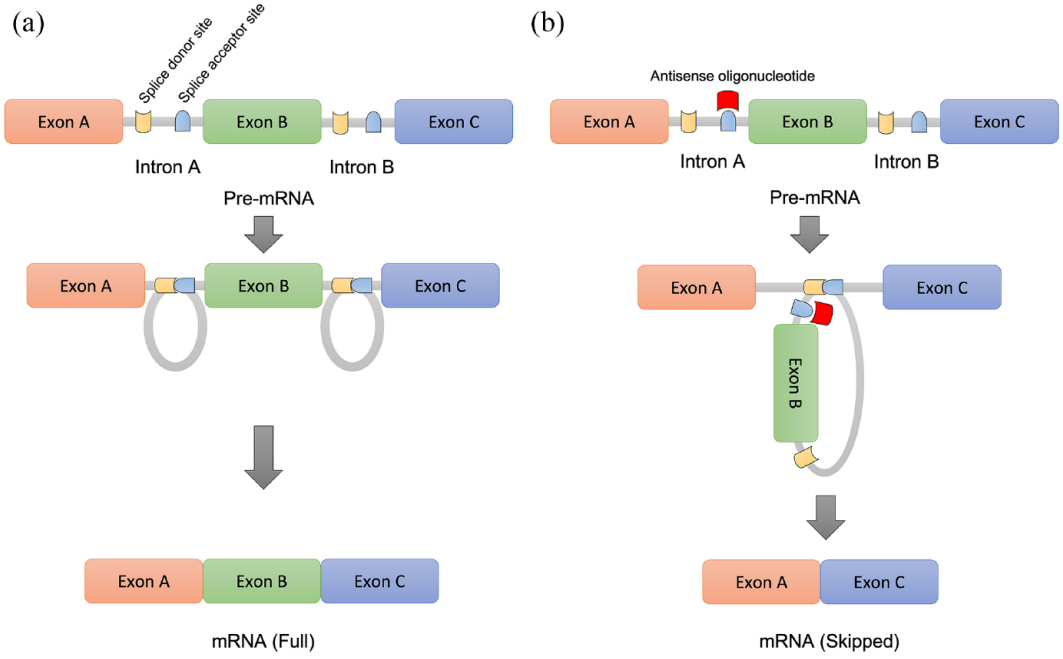

Figure 1. Schematic diagram of exon skipping. (a) Regular splicing. (b) Antisense oligonucleotide–mediated exon skipping.
Antisense oligonucleotide binds to and restricts the splice acceptor site causing the spliceosomes and the donor site to move onto the next splice acceptor site. As a result, exon B gets skipped and a shortened mRNA is produced.

(EXONDYS 51®; Sarepta Therapeutics Inc, USA) in 2016. PMOs are short, single-stranded DNA analogs, built upon a backbone of morpholine rings connected by phosphorodiamidate linkages. These charge-neutral nucleic acid analogs bind to the target mRNA *via* sequence complementarity and induce exon skipping. The resistance of PMOs to degradation by a variety of enzymes present in biological fluids makes them highly suitable for *in vivo* applications. This PMO-based drug, eteplirsen, showed limited dystrophin restoration in skeletal muscles by skipping exon 51.[49] A 4-year study on 12 patients has reported that patients receiving eteplirsen performed better in the 6MWT and had an attenuated ambulatory decline compared with the placebo group.[50] The difference, however, was not statistically significant. There has been no evidence of significant eteplirsen uptake or activity in the heart.[51] Following the approval of eteplirsen, three more PMO-based drugs have been approved by the FDA –golodirsen (exon 53), viltolarsen (exon 53), and casimersen (exon 45). Golodirsen (SRP-4053, Vyondys 53™; Sarepta Therapeutics Inc, USA) got the FDA approval in 2019. Reports from phase I/II study (NCT02310906) of this exon 53 skipping PMO demonstrated increased dystrophin restoration (baseline 0.095%, treated 1.019%) in skeletal muscle biopsies.[52] The 6MWT report shows the treated patients were able to cover more distance than the control group; however, an external control natural history cohort served as the control group in this case. Viltolarsen (NS-065/NCNP-01, Viltepso®; NS Pharma Inc., USA), an exon 53 skipping PMO, was the next PMO that got FDA approval in 2020. A phase II clinical trial (NCT02740972) reported that viltolarsen significantly improved patient muscle functions, including TTSTAND from supine, 10 m run/walk velocity (viltolarsen: 0.23 m/s; control: −0.04 m/s), and 6MWT (viltolarsen: 28.9 m; control: −65.3 m).[53] In this case, as well, an external group served as control. Casimersen (SRP-4045, Amondys 45™; Sarepta Therapeutics Inc, USA), approved by FDA in 2021, is applicable to DMD patients who are amenable to exon 45 skipping. According to an interim report from an ongoing phase III trial (NCT02500381), casimersen was able to increase the dystrophin production in patients by about 0.59%.[54] The data from clinical studies on these drugs mainly focus on the improvement of

skeletal muscle functions for the most part, while no significant improvements in cardiac function have been reported so far.[3]

The applicability of single exon skipping is limited as they can only be used for a certain number of patients. Individual treatment of single exon-skipping antisense oligonucleotide (AONs) can provide population coverage of up to 13%. As such, skipping multiple exons instead of one is also an active area of investigation. For instance, exon 45–55 is one of the major mutation hotspots in *DMD* and an in-frame deletion of this region has shown to be associated with a remarkably mild phenotype compared with smaller in-frame deletions within the region.[55] And skipping *DMD* exons 45–55 region is estimated to treat up to 63% of the patients with deletion mutations.[55] The feasibility of this multi-exon-skipping strategy has already been demonstrated in DMD mouse models.[56,57] More studies on safety and efficacy, however, are needed before this therapeutic approach can be moved forward for further preclinical and clinical trials. Another major challenge of using PMOs in cardiac treatment is their increased likelihood of endosomal entrapment in cardiomyocytes[58] (Figure 2). To address this issue, different delivery methods have been reported. Conjugating PMOs with peptides (producing peptide-conjugated PMOs or PPMOs) to enhance cell permeability is a promising solution that has shown a considerable amount of dystrophin restoration in the heart of dystrophic mice and dogs[56,59–62] In one study, Lim *et al.*[56] reported a remarkable 7% dystrophin restoration in the cardiac muscles of humanized dystrophic mice after injecting PMO conjugated to a peptide called DG9. But these studies were primarily conducted on animal models and the safety of using these drugs remains to be an ongoing concern. For example, unexpected toxicity can result from an immunogenic response like complement system activation.[63] Another study found PPMOs to be associated with lethargy and weight loss in rats.[64] These toxic effects, however, are dependent on the species, the nature of the peptide sequence, and the structure. Well-designed long-term preclinical studies with better dosage optimization will be necessary to come up with a safer and well-tolerated peptide suited for treating human patients.

At present, in addition to the ongoing trials on the FDA-approved PMOs, several new PMOs targeting different exons are undergoing clinical trials. These include SRP-5051 (Vesleteplirsen, exon 51; Sarepta Therapeutics, USA), PGN-EDO51 (exon 51; PepGen Inc., USA), and NS-089/NCNP-02

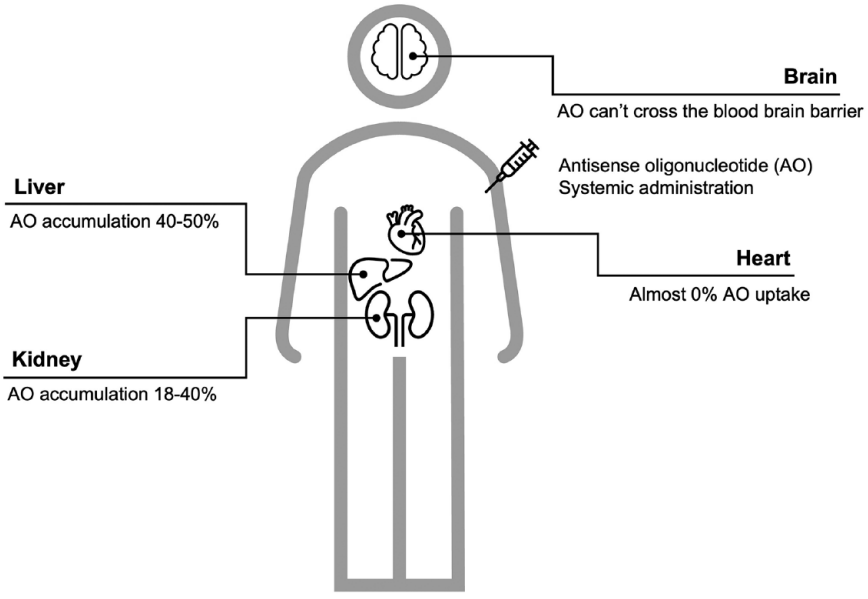

Figure 2. Challenges with antisense oligonucleotide delivery.
Figure adapted from Godfrey *et al.*[65]

(exon 44; NCNP/Nippon Shinyaku, Japan).[66,67] Among them, SRP-5051 and PGN-EDO51 are the only two PMOs undergoing clinical trials for DMD that are conjugated to peptides. SRP-5051 is the modified version of eteplirsen in which it has been conjugated to a peptide for better delivery. A report from a phase II study (NCT04004065) showed increased dystrophin restoration in patients, but the treatment also had adverse effects.[68] More than half of the patients exhibited hypomagnesemia causing the trial to be put on hold. In September 2022, this hold, however, was lifted after Sarepta extended their protocol to include urine biomarkers.[69] PGN-EDO51 is a PPMO from PepGen that is using a proprietary peptide called enhanced delivery oligonucleotide (EDO). This PPMO was able to induce 24% exon skipping in the left ventricle of nonhuman primates.[70] In its phase I Healthy Normal Volunteer (HNV) trial (undisclosed identifier), the drug showed 2% exon 51 skipping in biceps after a single dose of 15 mg/kg and is expected to move onto phase II trial in 2023.[71] A preliminary report on NS-089/NCNP-02 trial on six DMD patients has shown to be quite promising with 10–15% dystrophin restoration in skeletal muscles, but no report on cardiac assessment has been made public.[67,72]

Apart from PMOs, several other AOs with modified chemistry are being studied. DS-5141B (exon 45; Daiichi Sankyo Co., Japan) and WVE-N531 (exon 53; Wave Life Sciences Ltd., USA) are two such AOs that are currently under clinical trial. DS-5141B (Renadirsen) is an AO that possesses two modifications – 2′-O,4′-C-ethylene-bridged nucleic acids (ENA) and 2′-O-methyl RNA.[67] This 2′OMeRNA/ENA chimeric modification has a high nuclease resistance and an increased affinity for complementary RNA strands making it a potential candidate for exon-skipping therapeutics.[73] While the phase I/II clinical trial (NCT02667483) reports this drug to be safe, no quantifiable outcomes have been made public.[74] WVE-N531 is another drug under clinical trial that has a phosphoramidate diester (PN) backbone and can induce exon 53 skipping.[67] As of now, no clinical data have been reported.

While most exon-skipping drugs – in their respective AO forms – are directly infused in the body, Audentes Therapeutics, USA took a different approach. They used Adeno-associated viruses (AAVs) serotype 9 as their medium of delivery (discussed in the next section). The viral vector contains small nuclear RNAs (snRNAs) that target *DMD* exon 2 and is applicable to patients who have duplication in their *DMD* exon 2. In preclinical studies, this scAAV9.U7.ACCA drug has been shown to effectively restore dystrophin in multiple skeletal muscles as well as the heart.[75] Reports from the clinical trial (NCT04240314) showed improved dystrophin restoration in patients (>6% in the younger and ~1–2% in the older subject).[76] Transient nausea and vomiting were the only adverse effects exhibited by the patients. The patients showed improved functional outcomes to some extent,[77] but the phenotypic improvements in cardiac condition still need to be evaluated.

Gene replacement

A different but promising strategy to restore dystrophin production is to deliver the *DMD* gene into patient tissues. A number of ways have been studied for delivering the gene into the host cells, including direct injection, polycation scaffold-mediated transfection, encapsulation in liposomes, and viral vector–mediated delivery.[78] Among these, viral delivery is the most common technique in which viruses that have evolved to enter cells efficiently are used to transmit the DNA into the nucleus. Here, the viral gene is modified to incorporate the gene of interest – *DMD* in this case. AAVs are the most commonly used vectors for gene delivery mainly because of their decent safety profile and ease of genetic manipulation. AAV-mediated delivery, however, presents some major limitations. For one, AAV genes can integrate themselves into the host chromosome and cause undesired effects. This can be mitigated by manipulating the viral genome so that it persists as a circular episome and exists separately from the cellular chromosome. Another limitation is its gene packaging limit. While the full dystrophin protein is encoded by 11,055 DNA nucleotide bases; AAVs can only incorporate about 4700 nucleotide bases. To address this issue, new therapies are being developed in which a shortened version of the *DMD* gene is used to produce a smaller version of the dystrophin protein. The most common target for this type of deletion is the deletion in the rod domain in which most of this domain is removed and the resultant product is the miniature version of dystrophin called mini-dystrophin. These minigenes are usually ~6 kb long and require two AAV virions for delivery. A further

shortened version called micro-dystrophin is a construct that lacks all but the most crucial domains comprised of the actin-binding domain, 4–5 spectrin-like repeats, 2–3 hinge regions, and the cysteine-rich domain.[5] The ~4 kb end-product construct can be packaged into a single vector. AAV-mediated micro-dystrophin therapy has already been shown to improve diastolic function and reduce inflammation in mouse models.[79] Currently, multiple clinical trials are ongoing to evaluate the safety and efficacy of AAV-mediated gene therapy. Pfizer Inc., USA was the first to move onto the clinical trial with their AAV serotype 9–mediated mini-dystrophin gene therapy PF-06939926 (fordadistrogene movaparvovec). But after the unfortunate death of a young male participant in their nonambulatory cohort, the phase Ib trial (NCT03362502) was put on hold by the FDA in December 2021.[80] Later, the cause of death was attributed to the advanced dystrophic condition with underlying cardiac dysfunction of the patient.[80] Now they are expecting to move forward with phase III of the clinical trial (NCT04281485). At present, three different AAV-mediated micro-dystrophin therapies are on the horizon – SGT-001 (Solid Biosciences, USA), SRP-9001 (Sarepta Therapeutics, USA), and RGX-202 (REGENXBIO Inc., USA).[66] A 1.5-year interim report from a phase I/II study (NCT03368742) states that SGT-001 was able to restore 5–17.5% dystrophin levels in patients.[81] Early results from the SRP-9001 micro-dystrophin phase I/IIa trial have shown promising results in terms of safety, improved motor function, and dystrophin restoration.[82] The effects of these gene replacement therapies on cardiac improvements, however, are yet to be reported. The recruitment process for the RGX-202 phase I/II trial has already started (NCT05693142) and is expected to be completed in December 2025.

Surrogate gene therapy is an interesting strategy in which instead of delivering a construct as an alternative to the disrupted gene, a compensatory construct is delivered to the body. GALGT2 is one such treatment strategy currently under clinical investigation (NCT03333590). Here, the *GALGT2* gene (alternatively called *B4GALNT2*) construct is delivered using AAV.[83] In preclinical studies, *GALGT2* overexpression has been shown to inhibit muscular dystrophy,[84] contraction-induced muscle injury,[84] and prevented the loss of cardiac function in aging mice.[85] In a phase I/II study, rAAVrh74.MCK.GALGT2 delivery was well tolerated in patients, but improvements in functional outcomes were not significant.[83]

In recent years, another promising strategy to improve cardiac function has been gaining traction that focuses on restoring the abnormally elevated intracellular calcium (Ca^{2+}) concentration caused by DMD.[86] In skeletal and cardiac muscles, contraction and relaxation cycles are tightly controlled by cytoplasmic Ca^{2+} levels. Sarcoplasmic reticulum (SR)/endoplasmic reticulum (ER) is a major internal storage of Ca^{2+}, and during systole, Ca^{2+} is released into the cytoplasm *via* the ryanodine receptor in a process known as Ca^{2+} -induced Ca^{2+} release (CICR).[87] The released Ca^{2+} binds to troponin C and initiates muscle contraction. To initiate relaxation, most of the Ca^{2+} is recycled back into the SR *via* the Sarco(endo)plasmic reticulum Ca^{2+} ATPases (SERCA). The rest of the Ca^{2+} is transported out of the cells *via* sarcolemmal Ca^{2+} transport proteins with some transferring into the mitochondria *via* the mitochondrial uniporter.[86] In the case of DMD, multiple studies have reported the significantly reduced SR Ca^{2+} uptake in dystrophic muscles implying an impairment in SERCA functionality.[88,89] Indeed, a 2011 report showed that overexpression of SERCA2a *via* AAV9-mediated vector delivery was able to improve tachycardia in 12-month-old female mdx mice.[90] Later, in 2020, Wasala *et al.*[91] reported a successful restoration of skeletal and cardiac muscle functions after a single AAV9 human SERCA2a vector injection. Their treatment on mdx mice completely prevented myocardial fibrosis and restored EF to normal levels. Their treatment, however, did not improve skeletal muscle pathology. Downregulation of sarcolipin (SLN), an inhibitor of SERCA, is another potential strategy researchers have been looking into. Abnormally high levels of SLN have been reported to be present in the diaphragm and skeletal muscles of dystrophic mouse models.[88] Using AAV9-mediated RNA interference, Voit *et al.*[89] showed that reduction in SLN expression could restore SERCA function as well as ameliorate skeletal and cardiac muscle pathology. The AAV-mediated treatment in mdx:utr$^{-/-}$ mice, a severely dystrophic mouse model, also improved LV systolic function and cardiac remodeling. As a further proof of concept, the group also looked into SERCA function and intracellular Ca^{2+} handling after germline ablation of SLN expression in mdx mice in which the ablation resulted in reduced fibrosis and necrosis

along with improved cardiac function.[92] While upregulating SERCA and downregulating SLN showed promising results by restoring intracellular Ca^{2+} cycling in mouse models, Morales *et al.*[93] looked into a recently discovered positive regulator for SERCA – the Dwarf open reading frame (DWORF). They showed that overexpressing the DWORF gene using an AAV9-mediated DWORF vector in 6-week-old mdx mice could significantly enhance SERCA activity and reduce myocardial fibrosis. They also reported improvement in electrocardiography and heart hemodynamics. Even though these studies were exclusively carried out in mouse models, the results from these studies demonstrate the promise modification of Ca^{2+} regulation holds in DMD therapeutics. In a phase II trial (NCT00454818), AAV1-mediated SERCA2a therapy has already been shown to be effective in treating advanced heart failure.[94] Despite the study not being focused on DMD patients, the implications of the findings hold a promising future for gene delivery–based therapies for DMD-related cardiomyopathy.

Gene editing

With the advent of the clustered regularly interspaced short palindromic repeats/CRISPR-associated 9 (CRISPR/ Cas9) system, genome editing has become one of the major areas of investigation for DMD therapeutic development. By utilizing the components of the bacterial self-defense system, researchers have found a way to edit mammalian genes *in situ* – creating insertions and deletions at specific sites. A guide RNA (gRNA) is used to direct the Cas9 endonuclease to the target site in which it generates a double-strand break, allowing for the creation of desired gene modifications.

The tremendous potential of this tool has inspired multiple studies to explore the viability of this technique in treating DMD pathology in animal models. Using a viral vector–mediated delivery of the CRISPR/Cas9 system with a cardiac-specific promoter, several studies have reported significant cardiac dystrophin restoration as well as improved cardiac pathophysiology in dog and mouse models.[95–97] Amoasii *et al.*[96] reported a staggering 92% dystrophin restoration in the heart muscles of deltaE50-MD (*DMD* exon 50 deleted) canine model. Other notable reports include the study by Kyrychenko *et al.*[98] in which they showed the viability of genetic editing by correcting N-terminal mutations in induced pluripotent stem cell (iPSC)-derived cardiomyocytes, and by Min *et al.*[99] in which they successfully restored dystrophin production using CRISPR-Cas9-mediated gene editing in patient-derived iPSCs as well as in mouse models that had a deletion of exon 44. Recently, prime editing has been gaining attention as a way of introducing multiple changes in a small DNA segment as opposed to the traditional single-type base editors. Anzalone *et al.*[100] described a method to introduce any kind of edit, including insertions and deletions of any length using a catalytically impaired Cas9 that is fused to a reverse transcriptase. Later, Chemello *et al.*[101] showed that they were able to successfully reframe *DMD* exon 52 by inserting two bases in iPSC-derived cardiomyocytes that have exon 51 deletion mutation. Their prime-edited cardiomyocytes had a decrease in the percentage of arrhythmic calcium traces suggesting restored contractile functionality. As promising as these findings may seem, viral vector–mediated genome editing is not without its risks. Treatment using AAV carries the possible risk of immunogenicity, while CRISPR-mediated gene editing can lead to off-target effects (genotoxicity). A 1-year follow-up study on adult mdx mice has shown AAV-CRISPR-mediated immunogenicity as well as unintended genome and transcript alterations.[102] Long-term maintenance of CRISPR-mediated dystrophin restoration is another concern. Because the administration of AAV can elicit an immune response in patients, at present only a single dose can be administered. While the low turnover rate of cardiomyocytes may enable sustained dystrophin restoration in the heart, the high turnover rate of skeletal muscles presents the issue of sustained therapeutic rescue. Thus, a higher dosage might be necessary for achieving a long-term therapeutic window with an optimized gRNA that can minimize off-target mutations to avoid diseases like cancer. Before CRISPR-mediated therapies for DMD can move on to clinical trials, more preclinical studies on animal models are needed with long-term monitoring for immunogenic response and off-target effects to assess the safety and efficacy of this treatment strategy. A recent interesting study comparing the efficacy of gene editing and gene delivery in an aged CXMD dog model showed gene delivery yielded a better outcome than gene editing – a likely outcome given gene editing needs to have all the components available at the same location for a

successful editing.[103] Nonetheless, it is exciting to see the contrast between multiple techniques which may potentially create new avenues for combined treatments in the future mitigating the risks associated with any individual treatment strategy.

Cell-based therapy

Cardiosphere-derived cells (CDCs) are a type of progenitor cells derived from heart tissues that have been developed for regenerative therapy.[104] These cells act by secreting extracellular vesicles called exosomes that alter the pro-inflammatory phenotype of macrophages[105] and act on fibroblasts to render them antifibrotic.[106] CDCs have also been shown to reduce cardiomyocyte death and promote tissue regeneration after acute myocardial infarction.[107] These therapeutic effects have made CDCs a potential candidate for treating DMD-related cardiomyopathy. CDC treatment on mdx mouse models improved dystrophic phenotypes, partially reversing cardiac damage, and altering the expression of genes related to inflammation, oxidative stress, and muscle regeneration.[108] The impressive results from preclinical studies propelled CDCs to move on to a phase II clinical trial in DMD patients (HOPE-2; Capricor Therapeutics, USA). Results from the trial showed improved upper limb function and cardiac structure in the CAP-1002 treated group compared with placebo controls.[109] Functional improvement of the heart with an overall 4% increase in LV EF was also reported (0.1% CAP-1002 *versus* –3.9% placebo). Patients treated with CAP-1002 had a 71% delay in upper limb disease progression and a 10% delay in cardiac disease progression.[109] The study only reported hypersensitivity as an adverse effect, with no mortalities. While the results look promising, the study was limited to a small sample size ($N = 8$) over a 12-month period. Longer studies with a larger cohort are necessary to properly assess the effects of CDCs on the functional and structural restoration of cardiac tissues in DMD. Currently, phase III of the trial (HOPE-3, NCT05126758) is ongoing and is expected to be completed in 2025.

EN001 is another cell-based therapy developed by ENCell Co., Ltd., Korea that uses allogeneic umbilical cord–derived mesenchymal stem cells. Preclinical studies on mdx mice have shown EN001 to have therapeutic benefits such as muscle regeneration, and reduction in skeletal muscle apoptosis and fibrosis.[110] The effects of this cell-based therapy on cardiomyopathy still need to be investigated. EN001 has recently completed the phase I safety trial (NCT05338099); however, the findings are yet to be published.

Dietary modifications

While therapeutic interventions are important for managing this debilitating disease, the aspect of proper dietary intake often gets overlooked. Micronutrients, vitamin D, and calcium supplements are recommended for patients undergoing steroid treatment.[111] Usually, a high protein diet with low fat and carbohydrate content can be a good practice. For a healthier heart, patients are advised to replace unhealthy fats with healthier alternatives like unsalted nuts, seeds, and fish oil.[112] Processed food such as white bread, sugar, and sweetened beverages should be avoided. These recommendations are applicable to most patients with heart conditions; however, patients should always consult with their physicians as nutrient requirements can often vary depending on the health condition and the treatments they are undergoing.

Conclusion and future directions

While numerous therapeutic techniques have been explored to restore dystrophin in animal models, only a handful of them have shown promising results. Currently, exon-skipping therapies are the only treatments with FDA approval that can restore dystrophin production. Their efficacy, however, is very limited in skeletal muscles and almost nonexistent in cardiac muscles. Successful drug delivery to the target tissues, cardiomyocytes in this case, is arguably the most difficult hurdle in DMD therapeutics. While PMOs possess an excellent safety profile, their efficacy is limited due to poor cellular uptake and rapid clearance from systemic circulation. Other therapeutic strategies like gene delivery and gene editing have limited applicability owing to their construct size and the underlying safety issues with AAV-mediated delivery. As such, novel delivery strategies as well as AO sequences with modified chemistry are actively being investigated to overcome these challenges. Conjugating peptides with PMOs is one such approach that has shown quite promising results in restoring dystrophin protein levels in animal heart muscles, but their toxic effects need to be carefully evaluated. At present, PPMOs from Sarepta

(SRP-5051) and PepGen (PGN-EDO51) are being investigated in clinical trials. CDCs are another promising therapeutic avenue that can slow down the cardiac pathogenesis in DMD. Nonetheless, long-term assessments are needed to fully evaluate their therapeutic benefits.

A major concern with clinical trials is their prioritization of respiratory and skeletal muscle outcomes over cardiac improvements.[78] It is assumed that improvements in skeletal and respiratory function would translate to better patient outcomes; however, there have been studies that suggest otherwise.[113] With cardiac condition gradually becoming the main determinant of patient survival, it is important that standardized cardiac parameters like late gadolinium enhancement on cardiac magnetic resonance imaging (MRI) and changes in LV function should be introduced in clinical trials.

Finally, the current goal of genetic therapies is to turn severe DMD symptoms into less severe BMD-like symptoms. As of now, efficacy reports on most of the FDA-approved treatments are limited to those pertaining to skeletal muscles. Primary reports on ongoing trials showing higher skeletal muscle dystrophin restoration and improved cardiac conditions, however, are indicative of a better future.

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Not applicable.

Consent for publication
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Md Nur Ahad Shah: Writing – original draft; Writing – review & editing.

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