Review

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Review

Mitochondria in Duchenne Muscular Dystrophy-Induced Cardiomyopathy: A Prospective Therapeutic Target to Improve Treatment Response

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Abstract: Duchenne muscular dystrophy (DMD) is a progressive neuromuscular disease caused by mutations to the dystrophin gene - resulting in deficiency of dystrophin protein, loss of myofiber integrity in skeletal and cardiac muscle, and eventual cell death and replacement with fibrotic tissue. Pathologic cardiac manifestations occur in nearly every DMD patient, with development of cardiomyopathy - the leading cause of death - inevitable by adulthood. As early cardiac abnormalities are difficult to detect, timely diagnosis and appropriate treatment modalities remain a challenge. There is no cure for DMD – treatment is aimed at delaying disorder progression and alleviating symptoms. A comprehensive understanding of the pathophysiological mechanisms is crucial to development of targeted treatments. While established hypotheses of underlying mechanisms include sarcolemmal weakening, upregulation of pro-inflammatory cytokines, and perturbed ion homeostasis, mitochondrial stress has recently come into focus as a potential key contributor. Several experimental compounds targeting the skeletal muscle pathology of DMD are in development, but effects of such agents on cardiac function remain unclear. Synergistic integration of small molecule- and gene-target-based drugs with metabolic, immune, or ion balance-enhancing compounds into a combinatorial therapy offers potential for treating dystrophin deficiency-induced cardiomyopathy, making it crucial to understand the underlying mechanisms driving the disorder.

Keywords: duchenne muscular dystrophy; cardiomyopathy; mitochondria; elamipretide; therapy; inflammation; ion dysregulation; sarcolemmal tearing; metabolism; calcium balance; skeletal muscle; gene therapy; antioxidants; bioenergetics; reactive oxygen species

1. Introduction

1.1. Overview

Duchenne muscular dystrophy (DMD) is a devastating progressive neuromuscular disorder caused by X-linked recessive mutations to the dystrophin gene (*DMD*), located on chromosome Xp21 (Nigro et al., 1983; Jacobs et al., 1981). The mutation results in deficiency of functional dystrophin protein and subsequent loss of myofiber integrity in skeletal and cardiac muscle, thus leaving muscle fibers susceptible to contraction-induced damage (Petrof et al., 1993). As DMD progresses, muscle

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turnover and repair cannot keep pace with the constitutive cellular damage that arises, resulting in cell death and replacement with fibrotic and/or fatty tissue (reviewed in Wallace and McNally, 2009).

Phenotypically, dystrophin deficiency is characterized by progressive locomotor-skeletal, respiratory, and cardiac muscle weakness, ultimately resulting in loss of ambulation as well as respiratory and cardiac failure (McNally et al., 2015; Bach et al., 1987; Zambon et al., 2022; reviewed in Schultz et al., 2022). Pathologic cardiac manifestations occur in nearly every DMD patient, with development of cardiomyopathy inevitable by adulthood (Nigro et al., 1990; de Kermadec et al., 1994), and ultimately progressing to heart failure and mortality in 44-57% of patients (Szabo et al., 2021). Due to early pharmacological intervention and advancements in respiratory care for DMD patients, cardiomyopathy is now the leading cause of death in this patient population (Bushby and Connor, 2011; Passamano et al., 2012). While timely detection and management are essential to slow progression of dystrophin deficiency-induced cardiomyopathy, uncovering appropriate diagnostic and treatment modalities still remains a challenge due to the early cardiac abnormalities that are often difficult to detect and limitations in cardiac imaging techniques. Additionally, functional measurements in dystrophin deficiency-induced cardiomyopathy are confounded by skeletal muscle weakness given that typical heart failure symptoms, such as exercise limitations and dyspnea, are masked in this population (reviewed in McNally et al., 2015).

No cure exists for DMD—therefore, treatment is aimed at delaying disorder progression and alleviating symptoms. A comprehensive appreciation of the pathophysiological mechanisms that underlie cardiomyopathy is of the utmost importance to develop targeted treatments. It is worth noting that a single mechanism cannot explain the multifactorial pathogenesis of dystrophin deficiency-induced cardiomyopathy; rather, it is a result of several, interrelated mechanisms. While prevailing hypotheses of underlying mechanisms include sarcolemmal weakening, upregulation of pro-inflammatory cytokines, metabolic disruptions, and perturbed calcium homeostasis, mitochondrial dysfunction has come into focus as a potential key contributor (reviewed in Bellissimo et al., 2022; Schultz et al., 2022). Several experimental compounds targeted to the skeletal muscle pathology of dystrophin deficiency are in development, but the effect of agents on cardiac function remain unclear. Combinatory therapies are most likely the best approach for treating dystrophin deficiency-induced cardiomyopathy, again making it crucial to understand the underlying pathologic mechanisms driving the disorder.

1.2. Epidemiology of Duchenne muscular dystrophy (DMD)

DMD is the most common inherited muscular dystrophy diagnosed in childhood, with an incidence of approximately 1 in 5,000 newborn males (Crisafulli et al., 2020), although variations have been reported elsewhere (Ryder et al., 2017; Mah et al., 2014; Bladen et al., 2013). Since DMD is X-linked, it primarily affects males. Female carriers are typically asymptomatic, however, females with mild to severe symptoms have been reported (Florian et al., 2016). Approximately 25% and 59% of DMD patients develop cardiomyopathy by 6 and 10 years of age, respectively, with electrocardiogram (ECG) abnormalities and sinus tachycardia (ST) representing the primary early clinical manifestations (Nigro et al., 1990; Finsterer and Cripe, 2014; McNally et al., 2015; van Westering et al., 2015). By 18 years of age, cardiomyopathy occurs in 98% of patients and progresses to heart failure in 40% of patients (Nigro et al., 1983; Judge et al., 2011; Verhaart et al., 2011). Although limited, some studies have shown that female carriers present with indices of cardiac abnormalities, including cardiomyopathy, and similar to affected males, cardiac risk increases with age (Florian et al., 2016; Birnkrant et al., 2018). A higher mortality is linked to dystrophin deficiency-induced cardiomyopathy than to other cardiomyopathies (McNally et al., 2015).

1.3. Genetics

The *DMD* gene is the largest in the human genome (Koenig et al., 1987; Hoffman et al., 2020), spanning 2.4 Mb in the Xp21 region (Kamdar and Garry, 2016). It consists of 79 exons and has several internal promoters that produce an array of dystrophin isoforms expressed in striated and smooth

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muscles, brain, retina, and kidney (Kamdar and Garry, 2016). Thousands of mutations in the *DMD* gene have been found to cause dystrophin deficiency (Bladen et al., 2013).

Deletions account for over 70% of mutations and typically result in an altered reading frame that produces a premature stop codon (Bladen et al., 2015; Flanigan, 2017). Approximately 20% of dystrophin mutations are point mutations, small deletions, or insertions, whereas duplications make up 5-15% of mutations (Aartsma-Rus et al., 2006; Magri et al., 2011). Deletions and duplications tend to cluster in hotspot regions, located at exons 45-55 and 3-9 on the *DMD* gene (Nakamura et al., 2016). *De novo* mutations are responsible for one-third of DMD cases (Bladen et al., 2015; Laing, 1993). Mutations that disrupt the reading frame or introduce a premature stop codon cause the absence of dystrophin. Mutations that maintain the reading frame allow for truncated dystrophin to be produced resulting in the phenotype of Becker muscular dystrophy (BMD) (Aartsma-Rus and den Dunnen, 2019). Mutations can potentially confer cardioprotective properties against dilated cardiomyopathy (DCM) (exons 51-52) (Jefferies et al., 2005), perpetuate cardiomyopathy (exons 12, 14-17, 31-42, 45, 48-49, and 79) (reviewed in Yamamoto et al., 2018), or be neutral. As mutations can affect different exons on the *DMD* gene, it is unlikely that a single genetic therapy will benefit patients across the spectrum of mutations. Instead, there is substantial value in targeting secondary dysfunctions, such as mitochondrial stress, which are ubiquitously exhibited in most dystrophin-deficiency models.

1.4. Dystrophin and membrane instability

Dystrophin is a rod-shaped protein, located beneath the muscle fiber membrane, that acts as a molecular shock absorber by transmitting forces generated by sarcomere contraction to the extracellular matrix (ECM) (Le et al., 2018). Dystrophin consists of four major functional domains: an amino-terminal actin-binding domain, a large central rod domain, a carboxyl-terminal domain, and a cysteine-rich domain that binds dystroglycan (reviewed in Kaspar et al., 2009). Dystrophin is an integral part of the dystrophin-glycoprotein complex (DGC) that connects the intracellular cytoskeleton to the ECM (Ervasti and Campbell, 1993) and provides structural support to the sarcolemma. Aside from structural support, dystrophin also serves as a scaffold for proteins involved in various signaling cascades, including sarcolemmal ion channels, to be discussed in detail later (Allen et al., 2010). In cardiomyocytes, the Dp427 isoform of dystrophin is expressed and located at the cardiac sarcolemma as well as in the cardiac T-tubules (Masubuchi et al., 2013). DMD-induced cardiomyopathy appears to be predominantly attributed to the loss of the Dp427 isoform (Masubuchi et al., 2013).

The absence of dystrophin results in sarcolemmal membrane fragility and susceptibility to contraction-induced damage leading to micro-tears in the membrane (Kaspar et al., 2009). In one study examining dystrophin deficiency-induced membrane tears, it was determined through an Evans blue dye assay that left ventricular (LV) cardiomyocyte leakage was significantly increased in C57BL/10 *mdx* mice compared to age-matched control mice (Van Erp et al., 2010). Membrane fragility and tears are hypothesized to precipitate secondary pathophysiological mechanisms, such as excessive extracellular calcium influx and altered calcium homeostasis, that lead to myocyte degradation and necrosis (Allen et al., 2016; reviewed in Meyers and Townsend, 2019), which will be discussed in detail later. Furthermore, elevated serum cardiac troponin I (cTnI) (as a result of destruction to the myocardium) reflects the increased cellular permeability of the dystrophin-deficient heart (Matsumura et al., 2007). Use of membrane sealants, such as Poloxamer 188, have demonstrated stabilization of the sarcolemma and improvement in acute cardiac function in murine and canine dystrophin-deficiency models (Yasuda et al., 2005, Townsend et al., 2010).

In skeletal muscle, dystrophin mutations also lead to disorganized microtubule organization and oxidized actin which contributes to weakness and is thought to contribute to metabolic stress and other abnormalities (Ramos et al., 2020; Prins et al., 2009; Belanto et al., 2016; Nelson et al., 2018; Belanto et al., 2014; Olthoff et al., 2018).

 doi:10.20944/preprints202401.2158.v1

1.5. Preclinical models of DMD

Animal models have been critical in delineating the pathophysiologic mechanisms of dystrophin deficiency, and for subsequently trialing therapeutic candidates. The C57BL/10 *mdx* mouse, a genetic and biochemical homolog of human DMD, is the most widely used animal model of dystrophin deficiency and has been invaluable in providing knowledge for therapeutic strategies (Coley et al., 2016). The C57BL/10 *mdx* mouse lacks full-length dystrophin due to a nonsense point mutation in exon 23 of the *DMD* gene (Coley et al., 2016). This model only exhibits a mild cardiomyopathy late in life that never fully advances into heart failure (Howard et al., 2021). Additionally, cardiac fibrosis and dysfunction present very late, if at all, in this model (Bulfield et al., 1984). Although the milder skeletal muscle phenotype and subtle nature of cardiac involvement limits translation to human disorder progression (reviewed in McNally et al., 2015), some features such as cardiomyopathy can be unmasked in C57BL/10 *mdx* mice via cardiac stressors, such as ischemia-reperfusion (IR) insults (Burelle et al., 2009; Ascah et al., 2011). Due to the ease of breeding and maintaining, with a long-life expectancy, the C57BL/10 *mdx* mouse models remain a common tool in dystrophin deficiency research (Shirokova et al., 2013). Other mouse models exist with a variety of backgrounds and mutations, but the majority result from the crossing of the C57BL/10 *mdx* mouse with different mutations to worsen the dystrophin deficiency phenotype (Swiderski and Lynch, 2021; Wong et al., 2020)). However, the natural history of cardiomyopathy in each model remains largely uncharacterized.

The DBA/2J-*mdx* (D2.*mdx*) and utrophin-dystrophin double knockout mice have drawn attention as representing better models of DMD-induced cardiomyopathy, including earlier onset cardiac deficits (Coley et al., 2016). In the D2.*mdx* mouse, cardiac fibrosis is noted at 18 weeks of age (Hayes et al., 2022), but the degree to which cardiac dysfunction occurs at later ages is not understood. Several mouse models for dystrophin deficiency have been developed, but none of the models completely recapitulates the phenotype and disorder progression seen in humans. However, more severe models have a reduced lifespan, are more difficult to breed, and carry an additional mutation not affected in humans, which causes difficulty in reliably extrapolating data to the human phenotype (Shirokova et al., 2013; Coley et al., 2016).

To bridge the gap between small rodent (mouse) DMD models and the human phenotype, several different rat DMD models have been investigated. To date, the majority of rat DMD models have been developed to genetically target the genomic region that spans exons 3-26 (Taglietti et al., 2022; Iyer et al., 2020; Larcher et al., 2014; Nakamura et al., 2014). This is a major limitation in the model given that a major mutation hotspot for the classical, progressive DMD phenotype is between exons 45-55 (Taglietti et al., 2022). For this reason, a new rat DMD model was generated with a deletion mutation in exon 52 on the rat *DMD* gene, known as the R-DMDdel52 rat (Taglietti et al., 2022). In comparison to the *mdx* mouse model, the R-DMDdel52 rat exhibits remodelling of the entire striated musculature, including both the heart and diaphragm, which culminates leading to premature lethality between 10-14 months of age (Taglietti et al., 2022).

The golden retriever muscular dystrophy (GRMD) model offers remarkable resemblance to the progressive locomotor, respiratory, and cardiac muscular phenotype observed in human DMD patients (Howell et al., 1997). Similar to humans, these canines are characterized by highly variable cardiomyopathy progression, making assessment of therapies challenging (Le Guiner et al., 2017; Felsburg, 2002). Unfortunately, high costs, limited supply, and difficulty in maintaining a colony preclude widespread use of GRMD models (Shirokova et al., 2013).

In order to more closely recapitulate the human DMD phenotype (in comparison to small rodent models), without limitations imposed by the financial burden of large mammals (e.g., GRMD), a DMD rabbit model was developed by cytoplasmic microinjections of Cas9 mRNA and single guide RNA (sgRNA) (Sui et al., 2018). The rabbit DMD model was developed by targeting exon 51 (commonly mutated in human DMD patients) to disrupt the open reading frame of *DMD* in rabbits, thereby generating *DMD* KO (knock-out) rabbits (Sui et al., 2018). This model exhibits many hallmark pathologies of DMD, including elevated serum creatine kinase, impaired mobility/ambulation, muscle necrosis and regeneration, and importantly, cardiomyopathic manifestations (increased

 doi:10.20944/preprints202401.2158.v1

cardiac fibrosis, impaired function, etc.) (Sui et al., 2018). Although further research is still required to comprehensively validate and characterize this model, the clear evidence of cardiomyopathy at 4 months of age (demonstrated by reduced left ventricular ejection fraction and fractioning shortening) paired with the close resemblance to its human DMD counterpart, makes this an attractive model for preclinical drug screening studies (Sui et al., 2018).

Induced pluripotent stem cells (iPSCs), another preclinical model for dystrophin deficiency, are reprogrammed from patient-specific somatic cells and carry the same genetic defects as DMD patients (Lin et al., 2015). These cells make it possible to obtain functional cardiomyocytes from DMD patients and offer an important complement to animal models in studying dystrophin deficiency (reviewed in Law et al., 2020). However, iPSCs do not manifest a fully mature phenotype, which may limit their utility in pre-clinical research (Svobodova et al., 2021).

1.6. Clinical manifestations of DMD

The earliest symptoms in DMD patients typically present between 1 to 5 years of age and includes waddling gait, Gower's maneuver, difficulty climbing stairs, and frequent falls (Gardner-Medwin, 1980; Emery, 2002; Ryder et al., 2017). Calf hypertrophy tends to develop during early childhood due to localized accumulation of fatty and fibrotic tissue (Torriani et al., 2012). Motor skills deteriorate at 6 to 8 years of age when lordosis and scoliosis advance (Florczyk-Soluch et al., 2021). Scoliosis increases the risk of respiratory failure (Archer et al., 2016). Most DMD patients are wheelchair bound at approximately 13 years of age (Mercuri et al., 2019). Cognitive impairments are observed in roughly one third of all DMD patients (Banihani et al., 2015). Secondary to progressive muscle degradation and leakage of myofiber contents, elevated creatine kinase (CK) levels are detected early, with levels being about 10 times higher in newborns with DMD and approximately 50 to 100 times higher in children with DMD as compared to healthy age-matched boys (Birkrant et al., 2018; Mendell et al., 2012).

During disorder progression, cardiac symptoms advance from diastolic dysfunction to myocardial remodeling and fibrosis, leading to contractile weakness and subsequent DCM with decreased systolic function (Finsterer and Cripe, 2014; van Westering et al., 2015). Heart failure and arrhythmias develop gradually, increasing the risk of sudden cardiac death (Schultz et al., 2022). Largely attributable to advancements in respiratory interventions, such as assistive breathing devices (ventilators), the predominant cause of death has shifted from respiratory failure towards cardiomyopathy (reviewed in Passamano et al., 2012), and resultantly, patients may survive well into their third and fourth decades (Bushby et al., 2010; Wagner et al., 2007).

2. Cardiomyopathy in DMD: Functional and Histological Manifestations

As previously noted, typical cardiac symptoms of exercise intolerance and dyspnea are masked in DMD patients due to the influence of skeletal muscle weakness on these same parameters (McNally et al., 2015). Nonetheless, detection of early changes in the structure and function of the heart is crucial to initiating timely treatment in hopes of yielding better outcomes (Lee et al., 2021). Current guidelines recommend yearly cardiac screening at diagnosis (Birkrant et al., 2018).

2.1. Echocardiography

Echocardiography is recommended until at least 6 to 7 years of age, when the child can lie still without anesthesia, at which point cardiac magnetic resonance imaging (CMR) is recommended due to its ability to detect subtle cardiac changes prior to overt cardiac dysfunction (Birkrant et al., 2018, Lee et al., 2021). Transthoracic echocardiography (TTE) is a non-invasive, readily available diagnostic tool, but measurement of left ventricular ejection fraction (LVEF) using standard TTE rarely detects abnormal cardiac function in DMD patients within the first decade of life (Soslow et al., 2016; D'Amario et al., 2017). Two-dimensional fractional shortening (FS) and 5/6 area-length LVEF were found to be the most accurate and reproducible objective measures of left ventricular (LV) function using TTE in DMD patients (Soslow et al., 2016). However, TTE has been observed to underestimate

LV function compared to CMR (Soslow et al., 2016; Buddhe et al., 2016). Myocardial performance index (MPI) and doppler tissue imaging (DTI) may be capable of detecting myocardial dysfunction prior to the development of systolic dysfunction (Spurney et al., 2011). MPI, an assessment of global heart function, has been observed to correlate with EF (LaCorte et al., 2003). However, in DMD patients, calculation of the MPI showed abnormalities in 79% of patients, whereas 40% of patients had an abnormal LVEF on TTE (Bahler et al., 2005). DTI, which does not require good resolution, can detect early changes in the development of cardiomyopathy through measures of myocardial tissue velocities and strain (Spurney et al., 2011). Decreased tissue velocities have been observed in asymptomatic patients as young as 8.8 years and were able to predict poor outcomes with 85% accuracy (Giatrakos et al., 2006). Reduction in peak systolic radial strain has been observed in the posterior wall of DMD patients (Mori et al., 2007), commonly seen in the outer portion of the wall (Frankel and Rosser, 1976), and in those with normal systolic function (Ogata et al., 2007). DTI-derived strain measurement is less reliable in DMD patients due to their skeletal deformities obscuring proper Doppler beam placement and thus speckle tracking echocardiography (STE) is preferred to measure 2D strain. STE can detect subclinical LV dysfunction prior to decrease in LVEF through measurement of myocardial strain (Adorisio et al., 2020; Amedro et al., 2019) and is able to assess segmental and global myocardial function in the longitudinal, radial, and circumferential displacements (Mondillo et al., 2011). Before the appearance of overt cardiomyopathy in patients with DMD, significant reduction in global LV STE strain has been reported (Amedro et al., 2019). Myocardial strain, as measured by STE, was observed to be abnormal in approximately 50% of DMD patients, even in those with normal EF, suggesting that strain may identify early cardiac involvement (Levy et al., 2016; Patrianakos et al., 2015). Lower global longitudinal strain (GLS) values were seen in DMD patients with a decrease of 0.34% per year according to age (Amedro et al., 2019). The magnitude of difference in strain was greatest in the inferolateral and anterolateral segments (Amedro et al., 2019; Bilchick et al., 2011). Studies have reported variable findings using STE, as longitudinal peak-systolic strain was reported to be more pronounced in the apical area and mid-anterior segment in one study (Taqatqa et al., 2016), while another study revealed more prominent changes in the basal lateral segments (Bilchick et al., 2011) with decreased peak-systolic strain (Cho et al., 2018). In addition, a non-controlled study found that circumferential STE strain correlated moderately well with CMR strain and reported a trend toward reduced STE strain in patients with late gadolinium enhancement, a sign of fibrosis on CMR (Amedro et al., 2019). This could represent a good cardiac assessment option in DMD children too young to undergo CMR without anesthesia.

2.2. Limitations of echocardiography

Patients with DMD have poor acoustic windows due to increased adiposity, altered body habitus, lung hyper-inflation, and limited mobility (Romfh and McNally, 2010; Power et al., 2017). These factors degrade echocardiographic image quality and negatively impact interpretation (Buddhe et al., 2016). As a result, in several studies, echocardiography has been deemed inadequate for detecting cardiac involvement in DMD patients in the first decade of life, and sometimes beyond (Hor et al., 2013; Buddhe et al., 2016). Additionally, TTE cannot perform tissue characterization to detect early myocardial fibrosis (Prakash et al., 2022) and is unable to accurately account for the regional distribution typically apparent in DMD-induced cardiomyopathy (Lee et al., 2021). It was observed that TTE misclassified 20% of DMD patients, and 37% of the myocardial segments were unable to be visualized compared to CMR (Soslow et al., 2016; Buddhe et al., 2016). Despite these limitations, more advanced imaging may be contraindicated and TTE still remains valuable in the assessment and monitoring of DMD patients.

2.3. Cardiac magnetic resonance imaging

CMR is a non-invasive imaging tool that provides accurate volumetric measurements, assessment of wall motion abnormalities, and tissue characterization without negative effects from body habitus or other similar external factors (Soslow et al., 2016). Advantages to this technique include its excellent reproducibility and operator independency (Mavrogeni et al., 2013). CMR has

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been proven efficient in detecting myocardial fibrosis in patients with DMD (Ogata et al., 2009; Viollet et al., 2012). CMR in DMD patients has demonstrated a better diagnostic yield for detecting preclinical features of cardiomyopathy and greater sensitivity for identifying subtle changes in cardiac function, wall motion, and structural abnormalities than TTE (Prakash et al., 2022). LVEF as derived from CMR was found to poorly correlate with LVEF derived from TTE, and CMR-derived LVEF was found to moderately correlate to TTE-derived FS (Soslow et al., 2014), even with adequate imaging quality (Buddhe et al., 2018). As such, CMR is now the preferred cardiac imaging technique for patients with DMD (Birkrant et al., 2018). Even in the absence of overt cardiomyopathy, CMR has identified a pattern of fibrosis in female carriers similar to that observed in DMD patients (Mavrogeni et al., 2013). Detection of LV wall motion abnormalities on CMR has demonstrated good predictive value for the presence of regional cardiac dysfunction (Gloss et al., 2016), whereas the transmural pattern often located at the inferolateral wall independently predicts adverse cardiac event in DMD patients with normal LVEF (Florian et al., 2014b).

Late gadolinium enhancement (LGE), a sensitive marker of myocardial fat and fibrosis, appears as a result of diminished contrast washout on CMR (Prakash et al., 2022). This tool can be used to detect subclinical cardiac disease prior to LV dysfunction and may predict adverse cardiac events in DMD patients (Florian et al., 2014b; Hor et al., 2013; Silva et al., 2017). In DMD-induced cardiomyopathy, LGE is subepicardial and absent in the subendocardium (Lee et al., 2021). LGE appears to correlate with age such that patients with LGE tend to be significantly older with decreased LVEF (Hor et al., 2013; Hor et al., 2009). In fact, the presence of LGE correlated with a 2.2% decline in LVEF per year (Tandon et al., 2015). LGE in DMD patients has also been linked to greater LV dilation and dysfunction, as well as higher incidence of arrhythmias (Menon et al., 2014). LGE was reported in 30% of patients with normal LVEF and in 84% of patients with abnormal LVEF (Hor et al., 2013). One study revealed that patients with normal function on TTE had LGE on CMR and that several segments with abnormal wall motion by CMR were not detected on TTE (Soslow et al., 2016). LGE has been observed in patients under 10 years (Buddhe et al., 2016; Spurney et al., 2014), predominantly in the inferoseptal and anterolateral segments, but also typically involves the basal inferolateral free wall (Prakash et al., 2022). In addition, LGE of the subepicardium of the lateral LV wall is commonly observed, with intramural septal LGE becoming more prevalent with disorder progression (Puchalski et al., 2009; Florian et al., 2014b). It appears that the presence of LGE can predict the severity of cardiomyopathy in DMD (Tandon et al., 2015) and that it correlates to higher risk of arrhythmias in DMD (Menon et al., 2014). LGE may guide therapeutic interventions as the most recent DMD treatment guidelines recommend initiating cardiac therapy when LGE presence is first observed (Lee et al., 2021).

Strain imaging quantifies regional tissue deformation, and its measurements are potential early markers of cardiac involvement in DMD (Gotte et al., 2006). CMR strain correlates more closely with CMR LVEF compared to TTE strain (Buddhe et al., 2016). A sensitive marker used to identify cardiac pathology is peak circumferential strain (εcc) at the mid-ventricular level, which can also assess effects of therapeutic interventions on cardiac function and measure the degree of cardiac pathology (Hor et al., 2009; Siddiqui et al., 2020; Hor et al., 2015; Ashford et al., 2005; Raman et al., 2015; Bilchick et al., 2011; Batra et al., 2022). This measure was able to detect cardiac pathology as early as 5 years of age and the findings of this study revealed the heterogeneity in cardiac progression in patients with DMD (Batra et al., 2022). Additionally, circumferential uniformity ratio estimate (CURE) was identified in this study to have potential as a tool in understanding cardiac pathology in the DMD population (Batra et al., 2022). Global εcc appears to be the most sensitive strain marker for subclinical myocardial changes in DMD (Panovsky et al., 2021; Siddiqui et al., 2020) with more prominent differences in anterolateral, inferolateral, and inferior segments (Siegel et al., 2017). Reduced myocardial strain has been observed in the presence of normal LVEF (Hor et al., 2009; Ashford et al., 2005; Hagenbuch et al., 2010). Specifically, decreased global circumferential and segmental strain as well as mitral annular plane systolic excursion (MAPSE) has been observed in DMD patients with normal LV function and absence of LGE (Panovsky et al., 2021, Ashford et al., 2005), Similarly, decreased LV myocardial peak circumferential strain was observed in DMD patients under 10 years

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of age and declined with age (Hor et al., 2009). In DMD patients who develop overt LV dysfunction, CMR strain was found to be significantly worse than in those who do not develop cardiomyopathy (Siddiqui et al., 2020).

In cases where contrast is contraindicated, such as patients with vascular access issues, renal insufficiency, or contrast allergies, non-contrast CMR techniques are important (Buddhe et al., 2016). CMR-feature tracking does not use contrast and has been able to differentiate DMD patients from controls (Siegel et al., 2018; Aikawa et al., 2018). Without contrast, εcc can still detect myocardial global and regional alterations (Hor et al., 2009; Hor et al., 2015; Ashford et al., 2005).

Other CMR tools are T1 and T2 mapping. T1 mapping, which measures diffuse myocardial fibrosis and extracellular volume expansion, can identify early myocardial fibrosis even in the absence of LGE (Olivieri et al., 2016). Both pre- and post-contrast T1 mapping can detect earlier and more subtle signs of cardiac dysfunction (Magrath et al., 2018). In contrast to LGE that demonstrates focal fibrosis, T1 mapping can identify diffuse fibrosis in cardiomyopathy (Haaf et al., 2016). T1 values are increased in DMD patients and thus may serve as early markers of myocardial fibrosis (Florian et al., 2014). T1 mapping is often unable to differentiate fibrosis from inflammation or fat infiltration which may limit its use in DMD patients (Roujol et al., 2014; Kellman et al., 2015).

T2 mapping may be able to identify fat infiltration, edema, and inflammation within the affected muscle (Magrath et al., 2018). In a study using T2 mapping in DMD boys, the full-width half-max, a measure of T2 heterogeneity, correlated well with reduced LVEF and circumferential strain (Wansapura et al., 2010).

2.4. Limitations of cardiac magnetic resonance imaging

Disadvantages to CMR include expense, limited availability, patient discomfort due to position and immobility, claustrophobia, length of study, and the need for sedation in some patients (Soslow et al. 2016). The presence of hardware, such as spinal rods to treat scoliosis, may distort the image, limiting the use of CMR in DMD patients (Shih et al., 2020). With repeated exposure, gadolinium has been reported to accumulate in organs and may lead to toxicity (Perazella, 2009; Olchowy et al., 2017; Gale et al., 2017). In addition, the breath-holding segment of this procedure has been a limitation for the DMD population, but advancements have decreased breath holding times and free-breathing sequences are sometimes an option (Kellman et al., 2009; Lee at al., 2021). Despite these limitations, CMR is a powerful imaging modality for monitoring the cardiac involvement in DMD patients and circumferential strain and LGE are sensitive tools that can be utilized to detect occult cardiomyopathy prior to LVEF decline (Lee et al., 2021).

2.5. Hemodynamic biomarkers

Biomarkers indicative of DMD are suspected to be released into circulation as a result of sarcolemmal tearing, a phenomenon that that occurs in response to the mechanical stress of contraction caused by compromised membrane integrity arising from the dystrophin mutation. Cardiac troponin I (cTnI) and T (cTnT) are proteins that comprise the contractile unit of myocardial cells and are biomarkers for damage to the myocardium (Spurney et al., 2021). Serum cTnT can be elevated in the setting of neuromuscular disease and has been shown to correlate better with creatine kinase and myoglobin levels than with cTnI (Rittoo et al., 2014; Wens et al., 2016). Therefore, cTnI is more specific for myocardial injury in DMD as it is not expressed in skeletal muscle during regenerative processes (Schmid et al., 2018; Bodor et al., 1995). Cases of acutely elevated cTnI in DMD patients have been reported with abnormal ECGs, but no evidence of coronary artery disease (Hor et al., 2018; Abutaleb et al., 2018; Cinteza et al., 2017; Cucia et al., 1984; Politano et al., 2003; Schoeffler et al., 2008). Heterogeneity of cTnI levels have also been reported throughout the literature (Spurney et al., 2021). Significantly elevated levels of cTnI have been reported in DMD patients with mild LGE compared to those without LGE. However, cTnI levels were not elevated in patients with moderate-to-severe LGE. Since LGE is representative of cardiac fibrosis on CMR, this finding is likely due to increased cell death and fibrofatty tissue replacement in early disease and decreased enzyme leak at later stages of the disease when fibrofatty tissue has replaced most of the myocardium (Voleti et al.,

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2020). Elevated troponin levels have also been reported in asymptomatic patients prior to development of cardiac disease (Voleti et al., 2020), which may represent the necrosis and fibrosis resulting from subclinical or early cardiac remodeling. Decreased cTnI was observed in dystrophic dogs treated with membrane sealant, which was thought to ameliorate cardiac injury (Townsend et al., 2010). No current recommendations exist for routine monitoring of cTnI in DMD patient and therefore, only the symptomatic patients tend to undergo testing, making unbiased assessment of the distribution of troponin levels difficult (Spurney et al., 2021).

In addition to troponin, B-type natriuretic peptide (BNP) is a well-known serological marker of cardiac dysfunction; however, studies have revealed that BNP levels are inconsistent and thus are not appropriate measurements of cardiomyopathy in DMD patients (Dittrich et al., 2015). Despite this, lower BNP levels have been reported in patients with DMD-induced cardiomyopathy versus those with idiopathic cardiomyopathy (Demachi et al., 2004). Only a mild elevation of BNP levels is seen and typically only in the presence of severe cardiac dysfunction (Mohyuddin et al., 2007; van Bockel et al., 2009).

Other potential biomarkers and indicators include plasma alpha-ANP, CK-MB, and diastolic abnormalities. Elevation of plasma alpha-ANP levels may be an indication of poor prognosis in DMD patients as it is correlated with congestive heart failure and respiratory failure (Yanagisawa et al., 1990). Since CK-MB can be seen during skeletal muscle regeneration, this protein is not a good marker for cardiac involvement in DMD patients as it can also be indicative of damage to other muscles in the body (Wang et al., 2011). Furthermore, DCM and systolic dysfunction are preceded by diastolic dysfunction in DMD and therefore, diastolic abnormalities may serve as early indicators for early cardiac decompensation (Markham et al., 2006).

2.6. Systolic and diastolic dysfunction

Several studies have reported the presence of systolic and diastolic dysfunction in patients with DMD. It is hypothesized that the calcium dysregulation resulting from the absence of dystrophin presents phenotypically as sustained ion-driven myocyte contraction, which appears as underfilling of the LV and impaired relaxation prior to the development of DCM (Su et al., 2016; Markham et al., 2006). Su et al. (2016) described the heart in this phase as "tonic contraction", a term describing the abnormally increased tone without ability to relax (Figure 1). Although the LV appears smaller due to its inability to relax, the mass is unaltered. These changes likely lead to a decrease in stroke volume which may be correlated with the resting tachycardia often present in DMD patients (Su et al., 2016). Furthermore, impaired relaxation (which may be attributed to increased myocardial fibrosis leading to stiffness of the ventricular walls), diminished LV cavity size at the end of diastole (a possible outcome of impaired calcium handling - to be discussed later), and thickened myocardium have been observed in DMD animal models (Greally et al., 2013; Townsend, 2011; Wagner et al., 2012; Su et al., 2016). Prior to the appearance of clinical symptoms and decreased LVEF in DMD patients, early changes in diastolic function were observed through DTI (Cho et al., 2018). Specifically, abnormal measures of impaired diastolic function included altered myocardial velocity E' basal lateral and the mitral valve velocity/myocardial basal lateral E-wave ratio E/E' (Mertens et al., 2008; Ryan et al., 2013). In a recent study utilizing CMR in DMD patients, LV atrioventricular plane displacement (LVAPD), a marker of systolic and diastolic dysfunction, was decreased in patients with DMD, indicating reduced atrioventricular (AV) plane displacement (Batra et al., 2022). Decreased AV displacement correlates to a reduction in contractility and relaxation of the heart, resulting in reduced diastolic filling pressure and diastolic volume (Batra et al., 2022). This study found that LV end-diastolic volume (LVEDV) was significantly lower than controls, an observation well supported by the literature (Amedro et al., 2019; Panovsky et al., 2021; Dual et al., 2021). Other significant findings in the study by Batra et al. (2022) included reduced LV end-diastolic index (LVEDI) and changes in LV mass and LV end-systolic volume (LVESV).

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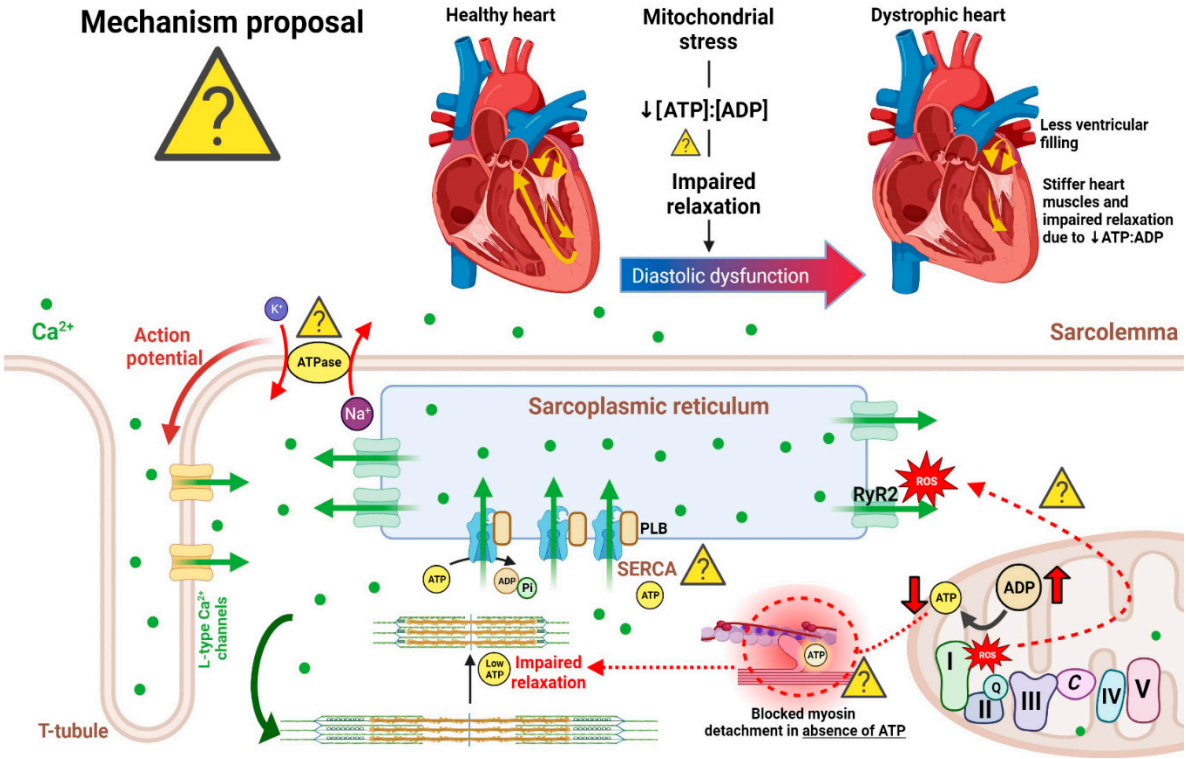

Figure 1. Proposed mechanism demonstrating how dystrophin deficiency-induced cardiac mitochondrial stress contributes to diastolic dysfunction. As an outcome of mitochondrial stress, left ventricular [ATP]:[ADP] is decreased, while mitochondrial reactive oxygen species (mH_2O_2) emission is elevated (Hughes et al., 2020; Dubinin et al., 2020b). Yellow question marks denote pathways where ATP insufficiency and elevated mH_2O_2 provision may precede impaired muscle cross-bridge relaxation, with independent hypotheses broadly reflecting extensive previous literature. Pathways include Na^+/K^+ ATPases along the t-tubule (which require ATP to maintain action potential charge balance and membrane excitability) (Dunn et al., 1995); ATP-dependent SERCA (which requires ATP re-uptake of excess cytosolic Ca^{2+} to allow for cardiac relaxation) (Williams and Allen, 2007; Voit et al., 2017; Rohman et al., 2003); blocked myosin detachment (which requires ATP to drive filament sliding during muscle cross-bridge cycling); and ROS-induced RyR2 oxidative stress (which may aberrantly release Ca^{2+} from its SERCA storage site) (Fauconnier et al., 2010; Bellinger et al., 2009; Voit et al., 2017; Rohman et al., 2003). Potentially as an outcome of elevated mitochondrial stress, and consequent decreased [ATP]:[ADP], impaired relaxation and the corresponding stiffer heart muscles are hypothesized to precede diastolic dysfunction exhibited in various late-stage dystrophic models. Created on BioRender.com.

2.7. Cardiac fibrosis

Deterioration of cardiomyocytes observed during dystrophin deficiency-induced cardiomyopathy activates an inflammatory cascade that results in macrophages clearing damaged cells, and fibroblasts invading the compromised area to form fibrotic (collagenous) scar tissue. In DMD, a prolonged subclinical phase of myocardial fibrosis is typically observed, beginning early in life (Markham et al., 2006).

Death of cardiomyocytes and subsequent development of cardiac fibrosis initially present in the posterobasal segment of the LV (Goodwin and Muntoni, 2005; Finsterer and Stollberger, 2003) and extend to the outer third and ultimately the entire LV and septum (Perloff et al., 1967). The fibrotic lesions are more prominent in the subepicardium and spare the muscle fibers nearest to the chambers in GRMD (Schneider et al., 2022). Additionally, fibrosis of the papillary muscles and posterobasal area contributes to mitral valve regurgitation in DMD (Sanyal et al., 1980). Fibrosis is prevalent from

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a young age, as evidenced by LGE on CMR (Spurney, 2011; Mertens et al., 2008), where cardiac fibrosis was identified in 17% of patients under 10 years, 34% of patients between 10-15 years, and 59% of patients over 15 years (Hor et al., 2013). Fibrosis occurs prior to the onset of decreased systolic function, which has been demonstrated with LGE on CMR (Spurney, 2011; Mertens et al., 2008). Myocardial fibrotic changes typically have a heterogeneous distribution, according to evidence of regional wall motion abnormalities determined via imaging modalities (Buddhe et al., 2016). While widespread scarring leads to stretching and thinning of the myocardial walls, focal fibrosis increases the risk of sudden death (Magrath et al., 2018).

2.8. Dilated cardiomyopathy

As myocardial fibrosis increases with disorder progression, the fibrotic region of the heart gradually stretches and thins, losing its contractility and resulting in DCM (reviewed in Kaspar et al., 2009). This may involve dilation of the LV or both ventricles, although right ventricle (RV) function tends to be relatively preserved in the setting of improved respiratory interventions (Mehmood et al., 2015). Dystrophin deficiency-induced cardiomyopathy is different than other types of cardiomyopathy in that the LV dilatation is less prominent, while the prognosis is worse (Connuck et al., 2008). DCM typically occurs in the second decade of life in DMD patients, although it has been reported in children under six years of age (Nigro et al., 1990). Clinical signs of DCM in DMD patients include increased LV diameter and volume, reduced FS, decreased LVEF, and development of mitral valve regurgitation (Kaspar et al., 2009).

2.9. Arrhythmia

Frequent ECG changes detected in DMD after development of cardiac fibrosis consist of ST, short PR interval, inferolateral Q waves, increased R/S ratio in precordial leads with tall R waves, left atrial abnormality, and right axis deviation (Shih et al., 2020). In addition to dystrophin deficiency-induced cardiac fibrosis, calcium transients, and elevated reactive oxygen species (ROS), ECG abnormalities may be secondary to dysregulated sodium, calcium, and potassium channels; kinases; and nitric oxide synthase (nNOS) triggered by the absence of dystrophin (Adams et al., 2018; Koenig et al., 2018). Briefly, dystrophin is thought to play an important role in scaffolding voltage-dependent sarcolemmal ion channels in the heart via syntrophin binding, which is an adaptor protein that binds directly to two sites in dystrophin's carboxyl-terminal region (Adams et al., 2018; Koenig et al., 2018). Dystrophic cardiomyocytes have demonstrated considerable sodium channel loss-of-function, while the activity of some potassium channels may also be reduced (Koenig et al., 2018). It is hypothesized that sarcolemmal ion channel abnormalities may occur prior to onset of cardiomyopathy in the dystrophic heart and may represent a primary effect of the *DMD* mutation (Koenig et al., 2018). QRS duration appears to increase with age, regardless of systolic function (Segawa et al., 2017). The onset of resting ST is typically seen in DMD patients by 5 years of age and conduction changes are observed by 10 years of age (Kaspar et al., 2009). Cellular mechanisms, including altered calcium homeostasis and elevated ROS, are hypothesized to cause arrhythmias in these patients and will be discussed in more detail in following sections. More serious arrhythmias, such as atrial fibrillation, AV block, ventricular tachycardia, and ventricular fibrillation, have been reported in the presence of advanced fibrosis (Corrado et al., 2002; Kaspar et al., 2009). One study estimated that approximately 44% of DMD patients had arrhythmias, including frequent atrial (3%) or ventricular premature contractions (22%), atrial (16%) or ventricular couplets (32%), supraventricular tachycardia (SVT) (9%), and ventricular tachycardia (VT) (13%) (Chiang et al. 2016). It has also been noted that DMD patients exhibit Wolff Parkinson White (WPW) patterns in their ECGs (Takami et al., 2008; reviewed in Fayssoil et al., 2017). Arrhythmias have been shown to be significantly correlated with decreased systolic function (Chiang et al., 2016; Villa et al., 2015) and an age older than 17 years was significantly associated with development of SVT or VT (Chiang et al., 2016). Sudden death does occur in DMD patients, but the percentage due to arrhythmias is unknown (Groh, 2012). Continuous Holter recordings have shown resting ST, loss of cardiac circadian rhythm, and heart rate variability (Santos et al., 2010). Although one study showed that ECG abnormalities frequently precede cardiac

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dysfunction by several years (Shah et al., 2010), no differences were noted on ECG of DMD patients with cardiomyopathy compared to those in the subclinical stages (Thrush et al., 2009). These inconsistencies suggest that ECG is not a useful diagnostic tool; however, periodic Holter monitoring is recommended in this population (Birkrant et al., 2018).

3. Mechanisms of Cardiomyopathy in DMD

3.1. Inflammatory signaling and immune response

Although inflammation is necessary for healing, it can also be damaging if it occurs aberrantly. Inflammatory and immune infiltrate are responsible for clearing damaged muscle cells and are often present prior to the onset of symptoms. The immune infiltrate primarily consists of macrophages and T cells in young (2 to 8 years of age) DMD patients (Spencer et al., 1997; reviewed in Evans et al., 2009). Since the heart has a limited capacity to regenerate, macrophages secrete chemokines, such as transforming growth factor beta (TGF-β), to activate resident fibroblasts and endothelial cells in response to injury, thus increasing the ratio of nascent myofibroblasts to quiescent fibroblasts. The fibroblasts are activated into collagen-secreting myofibroblasts and enhance ECM deposition at the injury site, forming fibrocollagenous scar tissue within the wall of the ventricular myocardium (reviewed in Kanisicak et al., 2016; Borthwick et al., 2013). Muscle biopsies from DMD patients demonstrated that TGF-β expression is associated with skeletal muscle fibrosis (Bernasconi et al., 1995). A high concentration of macrophages and T lymphocytes have been reported in pooled dystrophic muscles (quadriceps, hamstrings, and gastrocnemius) beginning early in disorder progression, suggesting that these cells play a crucial role in the pathology of dystrophic muscle (Spencer et al., 2001; reviewed in Evans et al., 2009). Sarcolemmal lesions in dystrophic skeletal muscles initiate calcium leakage and inflammatory cytokine upregulation; produce and release tumour necrosis factor alpha (TNF-α) and histamine; and contribute to mast cell degranulation and elevations to eosinophils (Gorospe et al., 1994; Cai et al., 2000; reviewed in Evans et al., 2009). A combination of these cytokines contributes to a proinflammatory environment and consequently promotes muscle necrosis (Evans et al., 2009). While inflammatory mechanisms have been discovered in dystrophic skeletal muscle, these pathways have not been extensively examined in the dystrophic heart, thus representing a future avenue of research.

Two main inflammatory pathways are involved in DMD: the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway and the nucleotide-binding oligomerization domain (NOD)-like receptor family pyrin domain containing 3 (NLRP3) pathway. NF-κB, a transcription factor that regulates the expression of chemokines and cytokines, may be activated by dystrophin deficiency-induced mechanical stretch. Increased activation of NF-κB is seen in dystrophic skeletal muscle (Kumar and Boriek, 2003; reviewed in Evans et al., 2009). Overexpression of chemokines is hypothesized to occur prior to initial disorder onset (defined as the initial mechanical damage that results from the absence of the membrane-stabilizing effects of dystrophin) and induce macrophage and T-cell infiltration (Wehling et al., 2001; reviewed in Evans et al., 2009). It has been previously determined that inhibition of NF-κB in utrophin/dystrophin-deficient mice improves cardiac contractile function (Delfin et al., 2011).

Before NLRP3 can be activated, it must be primed, which can be accomplished through activation of NF-κB and subsequent transcription of NLRP3 components and pro-inflammatory cytokines (reviewed in Reid and Alexander, 2021). Stressed mitochondria in dystrophin deficient models exhibit elevated mitochondrial hydrogen peroxide (mH_2O_2) release during attenuated oxidative phosphorylation (Hughes et al., 2019; Hughes et al., 2020; Ramos et al., 2020, Bellissimo et al., 2023), and it has been proposed that elevated ROS (Naik and Dixit, 2011) and possibly mitochondrial cardiolipin release (Iyer et al., 2013) can activate the NF-κB pathway through the NLRP3 inflammasome (reviewed in Elliot and Sutterwala, 2015). The degree to which such mitochondrial stress contributes to cellular degeneration and inflammation versus a reciprocal inflammation-induced mitochondrial stress requires extensive research.

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3.2. Calcium handling dysregulation and cell death

In healthy cardiac muscle, contraction and relaxation are driven by the cycling of calcium between the sarcoplasmic reticulum (SR) and cytoplasm. Briefly, this process involves plasma membrane depolarization that subsequently activates the L-type calcium channels (LTCC), which allows calcium to flow into the cell and subsequently causes a larger calcium flux from the SR through the ryanodine receptor 2 (RyR2), resulting in muscle contraction. This process is termed calcium-induced calcium release (CICR). It is imperative to note that the relationship between muscular dystrophy-induced elevations to net intracellular influx of calcium and muscle-cell necrosis was first proposed 40 years ago (Wrogemann and Pena, 1976; reviewed in Zulian et al., 2016). It was hypothesized that this increased intracellular calcium concentration triggers a vicious cycle of downstream mitochondrial calcium overload and eventual insufficient ATP synthesis, which further perpetuates cytoplasmic calcium levels by impairing calcium pumps, leading to hypercontraction of muscle fibres and subsequent cell necrosis (Wrogemann and Pena 1976). Interestingly, Wrogemann and colleagues were among the first to determine that calcium chelators such as EDTA could ameliorate the depressed respiration in dystrophic mitochondria, at least from skeletal muscle, by lowering mitochondrial calcium concentrations (Wrogemann et al., 1973). Although this finding was not originally established in dystrophic cardiac tissue, it is still a seminal finding that highlights the importance of intracellular calcium balance in dystrophic tissue and how calcium handling can be targeted in pursuit of a therapy.

Multiple mechanisms are believed to contribute to calcium overload and disrupted calcium homeostasis. Increased calcium influx and resting levels of mitochondrial calcium have been observed in myocytes from C57BL/10 *mdx* mice (Viola et al., 2013). In addition to the extracellular calcium that leaks through the sarcolemmal membrane tears, ion channels and calcium handling proteins likely contribute to the excessive calcium found in dystrophic myocytes. Stretch-activated calcium influx differences, increased diastolic calcium levels, altered calcium transient kinetics, and changes in calcium-handling protein expression, activation, or post-translational modifications have been observed in cardiomyocytes of C57BL/10 *mdx* mice (Yasuda et al., 2005; Williams et al., 2007; reviewed in Vallejo-Illarramendi et al., 2014; reviewed in Meyers and Townsend, 2019) (Figure 2).

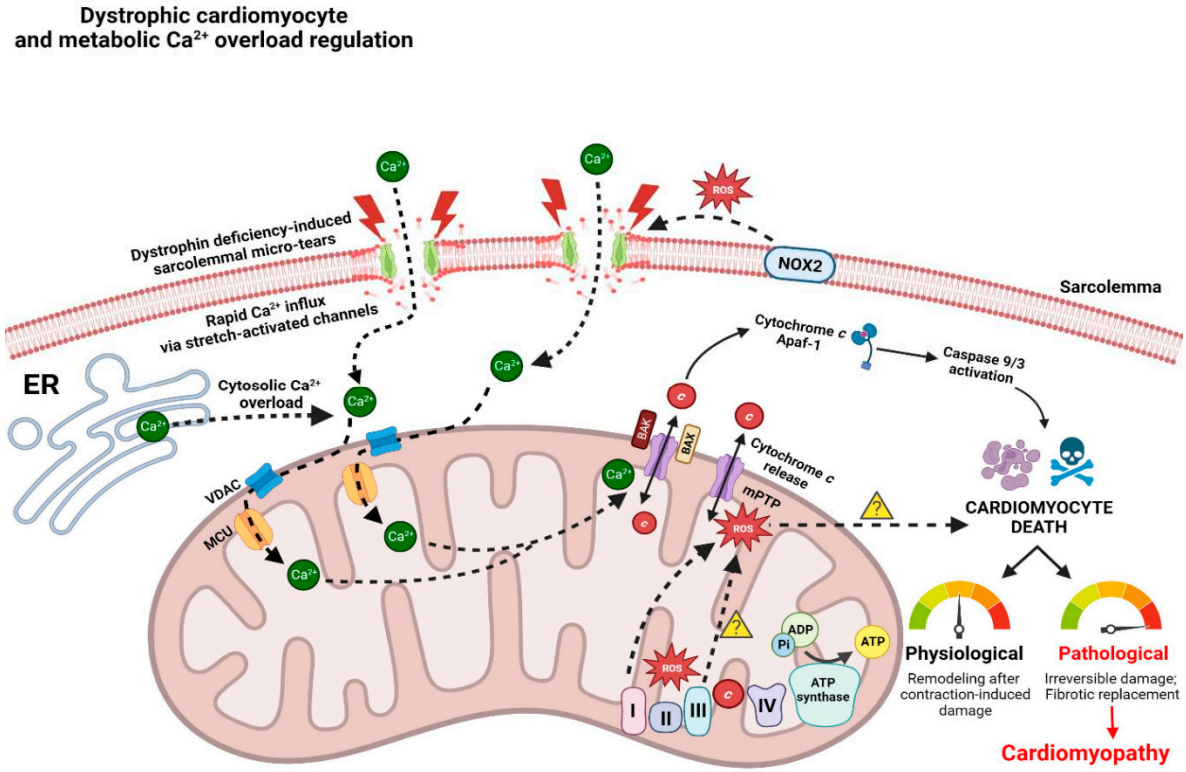

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Figure 2. Role of pathological Ca^{2+} overload in perpetuating cardiomyocyte death, and mPTP as a prospective therapeutic target for attenuating cardiomyopathic symptoms in DMD. Dystrophin deficiency-induced sarcolemmal tears lead to rapid cytosolic Ca^{2+} influx via stretch-activated channels (SACs) along the membrane (Yasuda et al., 2005; Williams et al., 2007; reviewed in Vallejo-Illarramendi et al., 2014). NOX2-derived oxidative damage to SACs further damages these channels, thus forcing additional Ca^{2+} to enter the cytosol (Allen and Whitehead, 2010). During cytosolic Ca^{2+} overload, the mitochondrion takes up excess Ca^{2+} via voltage-dependent anion channel (VDAC) and mitochondrial calcium uniporter (MCU) channels along the OMM and IMM, respectively. Subsequent mitochondrial Ca^{2+} overload results in mPTP opening, which expels cytochrome c into the cytosol (Burelle et al., 2010). It is also hypothesized that complex I and complex III (yellow question mark denotes insufficient data)-derived reactive oxygen species (ROS) damage stimulates mPTP opening, however, further research is required to characterize the presence of complex III-derived ROS in mdx models (Burelle et al., 2010; Hughes et al., 2020). It is also presently unknown if mitochondrial ROS directly triggers cardiomyocyte death in dystrophic hearts (denoted by yellow question mark). Cytochrome c release from the OMM interacts with the apoptosome-containing adaptor Apaf-1 and other initiator caspases (9/3) (Ascah et al., 2011; Budihardjo et al., 1999), which induce cardiomyocyte death downstream. In healthy physiological systems, regulated control of cardiomyocyte death is required for remodeling events following contraction-induced damage; however, in pathological states, excessive cardiomyocyte death leads to irreversible damage and fibro-fatty tissue replacement, also known as cardiomyopathy. Created on BioRender.com.

Excessive cytoplasmic calcium may activate calcium-dependent proteases, such as calpains, contributing to apoptosis (Feng et al., 2001). A study investigating single ventricular myocytes from C57BL/10 *mdx* proposed that calcium-activated calpains degrade troponin I, resulting in contractile dysfunction and decreased calcium sensitivity (Williams and Allen, 2007). A separate study assessing calpains in 4-week-old C57BL/10 *mdx* mice determined that total concentrations of calpains were elevated in necrotic hind limb muscle from dystrophic mice compared to controls, however, further research on the heart is required (Spencer et al., 1995).

Another possible source of calcium overload in dystrophic cardiomyocytes is through transient receptor potential channels (TRPC) and the LTCC $Ca_V1.2$ (Johnstone et al., 2017; Esposito et al., 2019; Koenig et al., 2018). TRPCs are hypothesized to engage in the augmented stretch-activated cation influx seen in C57BL/10 *mdx* cardiomyocytes and overexpression of these channels has been observed in dystrophic cardiomyocytes (Johnstone et al., 2017; Allen and Whitehead, 2010; Lorin et al., 2015; Allen et al., 2005). A decrease in excessive calcium was demonstrated in C57BL/10 *mdx* cardiomyocytes using inhibitors of TRPC, stretch activated channels and transient receptor potential vanilloid (TRPV2) (Williams and Allen, 2007; Lorin et al., 2015). Redox modifications of $Ca_V1.2$ alpha1 subunit that occur during periods of elevated oxidative stress leads to increased channel-mediated calcium influx (Cserne et al., 2017; Muralidharan et al., 2017; Koenig et al., 2018). Resultantly, ROS may partly explain the gain-of-function calcium channel abnormalities observed in dystrophic cardiomyocytes (Koenig et al., 2018). Indeed, communication between LTCC and mitochondria has proven to be crucial in healthy cells for metabolic function and this communication was disrupted in C57BL/10 *mdx* cardiomyocytes (Viola et al., 2009; Viola et al., 2014). Studies in mice lacking either dystrophin alone or dystrophin and utrophin demonstrated increased calcium influx through the LTCC in cardiomyocytes (Williams and Allen, 2007; Lorin et al., 2015). The LTCC pathway to calcium overload is also suspected to play a role in the calcium-dependent arrythmias seen in DMD (Koenig et al., 2014).

RyR2 and sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA2a) may enhance the release of store-operated calcium in cardiomyocytes of DMD patients (Andersson and Marks, 2010; Fauconnier et al., 2010; Bellinger et al., 2009). It has been observed that in C57BL/10 *mdx* hearts, RyR2 levels are 2 to 3 times greater compared to those of wild-type (WT) mice (Williams and Allen, 2007). Also, protein kinase A (PKA)- or calcium/calmodulin-dependent protein kinase II (CaMKII)-mediated hyperphosphorylation and S-nitrosylation of RyR2 have been demonstrated in dystrophic cardiomyocytes and lead to dissociation from the stabilizing-protein calstabin2 and subsequent

increased release of calcium from the SR (Andersson and Marks, 2010; Fauconnier et al., 2010; Bellinger et al., 2009; Shirokova et al., 2013). This is supported by studies demonstrating that agents blocking RyR2 phosphorylation result in normalization of cytosolic calcium levels as well as improvement in cardiac pathology and arrythmias (Fauconnier et al., 2010; Bellinger et al., 2009; Voit et al., 2017; Rohman et al., 2003). SERCA2a activity is significantly reduced in the C57BL/10 *mdx* dystrophic hearts due to lower SERCA2a expression and impaired regulation by inhibitory proteins phospholamban (PLN) and sarcolipin (Williams and Allen, 2007; Voit et al., 2017; Rohman et al., 2003). Phosphorylation of PLN via PKA or CaMKII is mostly regulated by β-adrenergic signaling (reviewed in Kranias and Hajjar, 2012; MacLennan and Kranias, 2003). Inositol trisphosphate (IP_3) receptors, which are calcium release channels on the SR, are activated by the downstream product of phospholipase C (PLC) (Johnstone et al., 2017). PLC inhibitors have demonstrated a normalization of intracellular calcium levels in C57BL/10 *mdx* cardiomyocytes to WT levels (Mijares et al., 2014).

Sodium ions can also affect mitochondrial-cytoplasmic calcium exchange through the Na^+-Ca^{2+} exchanger (NCX), which drives calcium out of the mitochondria in exchange for sodium when cytosolic sodium begins to accumulate as a result of dystrophin deficiency-induced microtears. A significant increase of cytosolic sodium levels (Kyrychenko et al., 2015) and elevated levels of NCLX ($Na^+/Li^+/Ca^{2+}$) have been observed in cardiomyocytes of C57BL/10 *mdx* mice (Dubinin et al., 2020b). To decrease intracellular sodium and calcium overload caused by pathologic reversal of the NCX, a potent and selective sodium-proton exchanger isoform 1 (NHE-1) inhibitor known as rimeporide has been in development (Porte-Thomé et al., 2015; Previtali et al., 2020). NHE-1 is a membrane transporter responsible for catalyzing the electroneutral counter transport of sodium and hydrogen ions through the plasma membrane (Porte-Thomé et al., 2015). Interestingly, when rimeporide was tested on *mdx* mice, protective effects against inflammation and accumulation of fibrosis were observed in the heart, but additional work in this field to identify potential side-effects and dose-response curves is still required (Porte-Thomé et al., 2015). Additionally, previous work in gastrocnemius and longissimus dorsi muscles from 5- to 6-month-old C57BL/10 *mdx* mice demonstrated elevations in Na^+/K^+ ATPase content (Dunn et al., 1995). Albeit not in the heart, this further suggests that sodium regulation is abnormal in dystrophic muscle, and potentially precedes cell death via abnormal regulation of cell volume (Dunn et al., 1995) (Figure 1). Collectively, these findings highlight opportunities for further investigation into the potential role of ion (e.g. Na^+, Ca^{2+}, Li^+, K^+, H^+) dysregulation as a prospective driver of mitochondrial stress responses.

Cardiac mitochondria do not typically act as a significant dynamic buffer of cytosolic calcium in healthy hearts; however, prolonged elevations of intracellular calcium substantially enhance mitochondrial calcium uptake (Boyman et al., 2014; Williams et al., 2013). Recently, increased calcium uptake has been observed in the mitochondria of C57BL/10 *mdx* skeletal muscle likely due to enhanced expression of the channel-forming MCU subunit and reduced expression of dominant-negative MCUb subunit (Dubinin et al., 2020b). While the precise mechanisms regulating excess mitochondrial calcium uptake requires more investigation, current theories posit that calcium overload triggers cell death through mitochondrial permeability transition pore activity in conjunction with impaired oxidative phosphorylation and elevated ROS production as described below.

3.3. Mitochondrial permeability transition pore and apoptosis

Mitochondrial permeability transition pore (PTP) formation is an event that fuses the inner (IMM) and outer mitochondrial membrane (OMM) while permeabilizing the inner membrane. Recent evidence supports a model whereby ATP synthase components dimerize to form the mPTP in response to reactive oxygen species and excess calcium stress (Bernardi et al., 2022; Reviewed in Giorgio et al., 2018) which then leads to depolarization, a loss of ATP synthesis, and rapid mitochondrial calcium release to the cytosol through an event known as permeability transition (PT). Following subsequent release of cytochrome *c*, caspases 9/3 are activated which activate proteases that contribute to apoptosis (Budihardjo et al., 1999) (Figure 2).

Myotubes cultured from C57BL/10 *mdx* demonstrate ~10-fold higher calcium in the matrix compared to the cytosol (Robert et al., 2001). Calcium-induced PT was elevated following ischaemia reperfusion in young C57BL/10 *mdx* hearts and was accompanied by the release of proapoptotic factors (including cytochrome *c* into the cytosolic fraction) as well as enhanced activities of caspases 9/3 prior to the onset of cardiac dysfunction (Ascah et al., 2011; reviewed in Burelle et al., 2010) (Figure 2). In LV of 4-week-old D2.*mdx* mice, no differences in calcium-induced PT or caspases 9/3 activities were observed (Hughes et al., 2020) in contrast to increases in both measures seen in certain skeletal muscles (Hughes et al., 2019). As there were no reductions in ejection fraction at this young age, the study concluded that mitochondria do not demonstrate PT in LV in early-stage disease. A separate study conducted on 7-month-old C57BL/10 *mdx* mice using mitochondria isolated from whole hearts demonstrated a greater propensity for calcium-induced PT (Stevens et al., 2024). Collectively, these findings suggest that introducing physiological stressors such as ischaemia reperfusion or assessing more advanced disease states may be required to reveal an underlying mitochondrial propensity for PT, although the degree to which PT exists across specific regions of the heart is unknown. The contributions of PT to cardiomyopathy at specific stages of disease require further research.

It has been proposed that different analytical interpretations can be associated with the specific technique that is used to determine and quantify PT. Techniques include quantifying the concentration of calcium required to trigger its opening via bolus titrations in a permeabilized fiber system, versus the elapsed time that is required to trigger this opening in response to a single bolus of calcium. To this end, differing conclusions from studies have led to the belief that disease effects on time may not be mirrored by changes in total calcium uptake by PT (proposed and reviewed in Bellissimo et al., 2022). In one such study, both approaches were compared and demonstrated no change in total calcium uptake despite lower time for calcium to trigger PT in C57BL/10 *mdx* mice (Ascah et al., 2011). This finding implies that a greater propensity for calcium-induced PT may be due to greater velocities of calcium uptake independent of potential differences in total uptake. Such methodological considerations could be considered when assessing PT in relation to cardiomyopathy in *mdx* models.

Although this has not been studied extensively in the heart, pharmacological inhibitors of mPTP in skeletal muscle have shown promise by improving indices of muscle function in C57BL/10 *mdx* mice (reviewed in Bellissimo et al., 2022; Millay et al., 2008; De Luca et al., 2005). Whether this can be attributed to the inhibitory effects on PT itself or the indirect benefit of immunosuppression is still a contentious topic. This is important given that immunosuppressive glucocorticoids have been known to suppress PT in C57BL/10 *mdx* skeletal muscle (Dubinin et al., 2020a).

Overall, the specific importance and role of PT-induced cell death in dystrophic cardiomyopathy is still not fully understood and requires more research. Insight may be gained by considering the effects of mPTP inhibitors in skeletal muscle of *mdx* mouse models. For example, Debio 025 (D-MeAla^{3}EtVal^4-cyclosporin), an inhibitor of the mPTP regulator cyclophilin, showed beneficial roles in locomotor and respiratory muscles of 3-week-old *mdx*^{5Cv} mice (Reutenauer et al., 2008; Wissing et al., 2010) but had no effect on echocardiograph assessments of heart function or fibrosis in 18-week-old D2.*mdx* mice (Hayes et al., 2022). Alisporivir, another cyclophilin inhibitor, is a cyclosporin A derivative that desensitizes the PTP without inhibiting calcineurin (Schiavone et al., 2017). Albeit not in the heart, treatment of alisporivir in primary cultures obtained from muscle biopsies of DMD patients demonstrated that this compound could restore the maximal respiratory capacity in dystrophic muscle cells without interfering with basal oxygen consumption, therefore restoring physiological respiratory reserve (Schiavone et al., 2017). In addition, treatment with alisporivir also recovered respiratory function, which matched improvements to muscle ultrastructure and survival in the *sapje* zebrafish model of DMD (Schiavone et al., 2017). TR001, which is a metabolically stable triazole analog and inhibitor of mPTP improved markers of motility 6 days post-fertilization when administered in *sapje* zebrafish (Stocco et al., 2021). In addition to improving muscle structure and function recovery, as well as mitochondrial respiration and survival in these zebrafish, TR001 also improved respiration in skeletal muscle-derived myoblasts and myotubes from DMD patients (Stocco et al., 2021). The promise and efficacy of mPTP inhibitor treatment modalities on dystrophic

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locomotor and respiratory muscle in various pre-clinical DMD models supports the need for these compounds to be tested on cardiac tissue and/or cardiomyocytes.

3.4. Oxidative phosphorylation and substrate catabolism

Mitochondrial ATP synthesis occurs predominantly through oxidative phosphorylation within the electron transport chain (ETC), a process vital for the energy-demanding heart. In healthy mitochondria, this process depends upon the governance of membrane potential across the IMM and is stimulated by temporary elevations to mitochondrial matrix calcium caused by contraction (Glancy et al., 2012; Nicholls and Ferguson, 2013). Early identification of mitochondrial deficits in muscular dystrophies using direct assessments of respirometry in isolated mitochondria in people with DMD were reported in 1967 albeit in skeletal muscle (Ionăsescu et al., 1967). Other reports around this time focused on skeletal muscle animal models of undefined muscular dystrophies (Wrogemann and Blanchaer 1967; Wrogemann and Blanchaer 1968; Wrogemann et al., 1970; Wrogemann et al., 1973) prior to the identification of the *mdx* mouse (Bulfield et al., 1984). Numerous factors need to be considered when assessing the regulation of mitochondrial oxidative phosphorylation in muscle, including phenotype severity (due to the progressive nature of dystrophin deficiency), and that not all mitochondrial protein markers uniformly change across a spectrum of muscle-types, models, or age ranges (reviewed in Bellissimo et al., 2023). This is important to note because mitochondrial protein content should not always be interpreted as being reflective of mitochondrial function (discussed in Bellissimo et al., 2023).

While no study has performed functional assessments of cardiac mitochondrial oxidative phosphorylation in people with DMD, one study found iPSC-derived cardiomyocytes from adults with DMD had significant reductions oxygen consumption coupled to ATP synthesis (Willi et al., 2022). Attenuations in the phosphocreatine (PCr) energy system indicated by lower PCr concentrations were also observed in these cells (Willi et al., 2022). In the C57BL/10 *mdx* mouse, studies have shown significant reductions in the ratio of cytoplasmic PCr to ATP ratio which reflects a compromised ability to maintain energy homeostasis prior to the development of cardiac fibrosis (Cui et al., 2015). This observation is consistent with many reports of attenuated mitochondrial respiration in dystrophin deficient heart (see Bellissimo et al., 2022for review).

Of interest, mitochondria can also export phosphocreatine as an alternative to ATP (reviewed in Schlattner et al., 2018; Guzun et al., 2012). Regulated by mitochondrial creatine kinase, this model posits that PCr is recycled back to ATP by local ATP-hydrolyzing proteins through cytoplasmic creatine kinases. As PCr diffuses at a rate several fold faster than ATP, with the creatine product returning to mitochondria ~2,000 fold faster than ADP, this fast phosphate shuttling mechanism may be advantageous to maintaining energy homeostasis in cells that can experience very high rates of demand for ATP, such as the heart (Meyer et al., 1984; reviewed in Schlattner et al., 2018; and Wallimann et al., 2011). One study found that this faster creatine-dependent phosphate shuttling mechanism was more attenuated than the direct ATP export system when assessed with respirometry in permeabilized muscle fibres from the LV of 4-week-old D2.*mdx* mice (Hughes et al., 2020) which has also been reported in skeletal muscle (Hughes et al., 2019; Bellissimo et al., 2023). Such 'creatine insensitivity' occurring early in the disease was suggested to be a potential unique mechanism preceding the eventual development of cardiomyopathy.

Additional work is required to address the fates of glucose and other substrates being utilized in dystrophic cardiac tissue. Whether reductions in mitochondrial oxidative phosphorylation and elevations to fat oxidation occur concurrently, and should be considered 'mitochondrial dysfunctions', are still questions remaining to be answered. For this reason, when assessing new therapeutic avenues, careful consideration should be given to designing experiments that consider the role of mitochondrial oxidative phosphorylation as part of an integrated perspective on substrate fates (reviewed in Bellissimo et al., 2022).

A variety of other metabolic changes occur in the dystrophic heart. For example, a shift in metabolism from fatty acid to carbohydrate oxidation was observed *in vivo* in C57BL/10 *mdx* hearts prior to cardiomyopathy, suggesting that altered mitochondrial metabolism is one of the first

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metabolic changes to occur (Khairallah et al., 2007). In *ex vivo* perfused C57BL/10 *mdx* hearts, a shift in substrate selection from long chain fatty acids (LCFA) to carbohydrate (glucose) oxidation in C57BL/10 *mdx* hearts was demonstrated by a ~30% lower oleate flux ratio, ~120% higher pyruvate decarboxylation flux ratio, and ~80% increased glycolysis rate (Khairallah et al., 2007). This shift is supported by evidence from cardiac positron emission tomography (PET) studies in DMD patients using ^{18}F-deoxyglucose or a radioiodinated branched fatty acid (Krumenacker et al., 2001; Momse et al., 2001; Naruse et al., 2004; Perloff et al., 1984). On the note of carbohydrate oxidation, glucose transporter type 4 (GLUT4) is the main transporter of glucose in cardiomyocytes (Zorzano et al., 1997; Fischer et al., 1997) and glucose transport into muscle cells in response to insulin or contraction occurs via translocation of GLUT4 from the cytoplasm to the sarcolemma/T-tubules (Birnbaum, 1989; Fischer et al., 1997). GLUT4 was observed to be abnormally localized in the sarcolemmal membrane and a trend toward elevated GLUT4 expression was observed in skeletal muscle of GRMD, but not in the LV (Schneider et al., 2018). Primary cardiac insulin resistance was also observed in the LV of GRMD (Nikolaidis et al., 2004). Together, this data suggests that glucose/carbohydrate metabolism can potentially be used as a preclinical biomarker of the dystrophic phenotype in skeletal muscle of GRMD dogs, but whether this can be implemented in cardiac tissue remains to be determined (Schneider et al., 2018).

Interventions that correct the substrate imbalance may lead to improvements or recovery of the cardiac contractile dysfunction (reviewed in Glatz et al., 2020). Improved contractile performance was observed in C57BL/10 *mdx* hearts perfused with multiple substrates, including carbohydrates (glucose, lactate, and pyruvate) and a LCFA (oleate), in comparison to C57BL/10 *mdx* hearts with glucose as the sole substrate (Burelle et al., 2010; Danialou et al., 2001). Another potential explanation for substrate shift is the p38 mitogen-activated protein kinase (MAPK) pathway, which is involved through the nuclear receptor peroxisome proliferator-activated receptor (PPARα), a transcriptional regulator of fatty acid oxidation enzyme expression (Khairallah et al., 2007). A 2-fold decrease in p38 MAPK phosphorylation may account for the decrease in LCFA oxidation observed in the C57BL/10 *mdx* heart (Burelle et al., 2010). This emphasizes the importance of increased and balanced substrate supply to maintain adequate cellular energy in the C57BL/10 *mdx* heart.

It is also possible that certain metabolic stress responses are compensatory in nature. For instance, a shift from fat to carbohydrate oxidation could be beneficial given carbohydrate oxidation produces more adenosine triphosphate (ATP) for a given amount of oxygen consumption (Burkhoff et al., 1991; reviewed in Stanley et al., 2005). However, in DMD, competition for glucose, fatty acids, and proteins may exist for uses unrelated to bioenergetics, such as for membrane repair, as reviewed elsewhere (Bellissimo et al., 2023). Interestingly, greater levels of fatty acid biosynthesis enzymes that convert fatty acid constituents to acetyl-CoA for the tricarboxylic (TCA) cycle were observed in skeletal muscle from people with DMD (Capitanio et al., 2020). Despite this finding, decreases in TCA pool size and lower aconitase activity were found in C57BL/10 *mdx* hearts (Khairallah et al., 2007, Griffin et al., 2009) which could limit oxidative phosphorylation. Likewise, lower mitochondrial respiratory sensitivity to ADP, reduced mitochondrial creatine metabolism, and attenuated complex I activity were reported in LV of 4-week-old D2.*mdx* mice (Hughes et al., 2020). Some of these metabolic changes (Khairallah et al., 2007, Griffin et al., 2009, Hughes et al., 2020) preceded the development of overt cardiac dysfunction, suggesting metabolic stress could be an initial event in the disorder. This will be explored extensively in section 3.6.

The relationship of such mitochondrial reprogramming and/or deficiencies to impaired nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) signaling is relatively understudied in DMD, but several reports supporting continued investigation in this area are warranted. NO/cGMP is believed to regulate nuclear gene expression supporting mitochondrial biogenesis (Nisoli et al, 2004). Increased expression of the atrial natriuretic factor (*anf*) gene, an activator of the NO/cGMP signaling pathway and marker of cardiac remodeling, was observed in C57BL/10 *mdx* hearts at 10-12 weeks of age (Khairallah et al., 2007). Increased levels of the alpha unit of soluble guanylate cyclase (*sgcα*$_1$) gene were observed in hearts of 25-week-old mice, a gene which typically negatively correlates with NO/cGMP (Krumenacker et al., 2004). These results suggest that an increase in *anf* expression may

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compensate for a defective NO/cGMP pathway in young mice, but this adaptive mechanism appears to diminish with age (Khairallah et al., 2007; Krumenacker et al., 2004). However, despite lower nNOS protein content, a study examining skeletal muscle in C57BL/10 *mdx* reported that nitrate supplementation (an approach used to increase NO) did not rescue mitochondrial oxidative phosphorylation, increased mitochondrial peroxynitrite, and promoted muscle damage. Collectively, these data suggest that a defect in the NO/cGMP signaling pathway potentially contributes to the metabolic abnormalities in the heart (Khairallah et al., 2007; Burelle et al., 2010) but negative effects of nitrate supplementation in skeletal muscle identify an incomplete understanding of this pathway's role in contributing to mitochondrial dysfunction in DMD.

Collectively, the regulation of mitochondrial substrate oxidation is altered in the heart during DMD. However, the degree to which such stress responses contribute to cardiomyopathy or serve as an intentional reprogramming to permit alternative fates of glucose or fatty acid substrates requires considerable attention given the latter possibility has rarely been considered (proposed in Bellissimo et al., 2022).

3.5. Reactive oxygen species (ROS) and mitochondrial H_2O_2 emission

Many studies measuring ROS in *mdx* models used relatively non-specific fluorophores that do not permit definitive conclusions regarding the precise subcellular origin or type of ROS assessed (reviewed in Bellissimo et al., 2022). In this regard, very few studies have used methodologies that isolated the source of ROS from mitochondria or other sources.

Increased pyruvate-stimulated, complex I-supported mH_2O_2 emission was observed during attenuated oxidative phosphorylation in permeabilized LV cardiac muscle fibres of dystrophin-deficient mice due to a reduced ability of ADP to suppress mH_2O_2, as normally occurs during oxidative phosphorylation (Nicholls and Ferguson, 2013). As creatine normally accelerates mitochondrial ADP/ATP cycling (reviewed in Schlattner et al., 2018), and has been shown to enhance ADP's ability to attenuate mH_2O_2 (Meyer et al., 2006), the additional finding that mitochondrial ADP attenuation of mH_2O_2 was no longer sensitive in D2.*mdx* LV fibres suggests that mitochondrial creatine kinase may represent a unique mechanism of mitochondrial dysfunction in dystrophin deficient hearts (Hughes et al., 2020). The precise mechanisms by which this elevation in mH_2O_2/O_2 may contribute to cardiac dysfunction remains unknown, particularly given there were no changes in the glutathione equilibrium (Hughes et al., 2020 or the ability of mitochondria to scavenge H_2O_2 in C57BL/10 *mdx* hearts (Ascah et al., 2011), but such elevations in mH_2O_2/O_2 occurred during a time of apparent cardiac compensations as mice showed slight elevations in ejection fraction albeit before overt cardiac dysfunction (Hughes et al., 2020).

Similar observations were made in isolated mitochondria from mixed hearts (rather than a specific chamber) in the C57BL/10.*mdx* mouse also at 4 weeks of age (Dubinin et al., 2020b) as well as skeletal muscle of D2.*mdx* mice (Hughes et al., 2019; Ramos et al., 2020; Bellissimo et al., 2023). While various antioxidants that do not target mitochondria *per se* have been tested in C57BL/10 *mdx* mice for their potential cardioprotective properties, and have demonstrated discordant results (Gartz et al., 2022), to our knowledge, there are no studies assessing the effects of mitochondrial-targeted antioxidants on mitochondrial bioenergetics and cardiac function in *mdx* models.

The precise signaling mechanisms downstream of mH_2O_2 that may mediate cardiac dysfunction in dystrophin deficient hearts have not been explored extensively in *mdx* mice or DMD patients. Understanding how mitochondrial-derived ROS contributes to cardiac dysfunction with consideration of ROS-sensitive targets mediating cell membrane injury, metabolic dysfunction, calcium dysregulation, and cardiomyocyte degeneration could guide the development of ROS-specific targeted therapies.

The superoxide-generating enzyme Nicotinamide Adenine Dinucleotide Phosphate (NADPH) is a major source of non-mitochondrial ROS in skeletal and cardiac muscle (Prosser et al., 2011; Gonzalez et al., 2014), especially during mechanical stress and at early stages of disorder progression (Shirokova et al., 2013; Kyrychenko et al., 2015) (Figure 2). NOX2-mediated ROS generation is significantly increased in C57BL/10*mdx* hearts (Prosser et al., 2011; Khairallah et al., 2012; Williams

and Allen, 2007; Gonzalez et al., 2014), which has been attributed to altered microtubule association with NOX2 (Khairallah et al., 2012; Prosser et al., 2013). Inhibiting NOX2-derived ROS (Jung et al., 2007; Kyrychenko et al., 2013) or using the non-specific ROS scavenger N-acetylcysteine (NAC) (Prosser et al., 2011; Williams and Allen, 2007) restored calcium homeostasis in dystrophin-deficient muscle. NOX2 inhibition with the drug apocynin improved single sarcomeric shortening in isolated cardiomyocytes (Gonzalez et al., 2014) while NAC improved fractional shortening (Williams and Allen, 2007). However, the therapeutic potential of NAC is uncertain given it also reduces muscle weights in C57BL10/*mdx* mice (Pinniger et al., 2017) although the degree to which this occurred because of attenuated superoxide is not clear.

3.6 Altered mitochondrial autophagy (mitophagy)

In healthy tissue, defective mitochondria can be removed through mitochondrial autophagy, also known as mitophagy, in a process intended to uphold quality control of the healthy mitochondrial pool. This process mitigates damage that may be caused by dysfunctional mitochondria (Reid and Alexander, 2021) to maintain energy metabolism stability (Li et al., 2020; reviewed in Eisner et al., 2017). Mitophagy is regulated by PTEN-induced kinase 1 (PINK1) and Parkinson juvenile disease protein 2, (PARKIN) (reviewed in Bellissimo et al., 2023). In a damaged mitochondrion, PINK1 is not degraded and is able to phosphorylate mitofusin 2 (Mfn2), which subsequently signals PARKIN to tag the mitochondria for degradation (Reid and Alexander, 2021; Kang et al., 2018). Important mitophagy-related genes, including *PINK1*, *PARK2*, and *BNIP3* were decreased in DMD patients (dystrophic quadriceps) and dystrophin-deficient rodent models (12-month-old C57BL/10 *mdx* mice) (Luan et al., 2021; Kang et al., 2018). Improving mitophagy has shown promise in the diaphragm of C57BL/10 *mdx* mice (Pauly et al., 2012), but further research on the heart is required to elucidate if PINK/PARKIN pathways represent potential therapeutic targets.

Given that loss of PINK1 increases the vulnerability of the heart to IR-injury, it appears to possess some cardioprotective properties (Kang et al., 2018). A study by Kuno et al., (2018) revealed that the co-localization of LC3 dots with fragmented mitochondria was significantly increased in hearts of 22-week-old C57BL/10 *mdx* mice compared to healthy controls, indicating that the elimination of damaged mitochondria via mitophagy was impaired and subsequent accumulation of damaged mitochondria persisted. Other studies in skeletal muscle from C57BL/10 *mdx* mice and DMD patients demonstrated significantly reduced levels of autophagy (De Palma et al., 2012), suggesting that the suppression of autophagy may be muscle-type dependent. Further, mitophagy is a dynamic process and therefore, changes to protein content and gene expression of common mitophagic markers may not always be proportional to mitophagic activity and/or flux. It is essential to understand phenotype severity, muscle-type, and age when considering mitophagy as a therapeutic target. The majority of research to date investigating the role of mitophagy in dystrophin deficient models has been conducted in skeletal muscle, thus, advancements in cardiac muscle remain to be addressed.

3.7. Altered mitochondrial content and structure

iPSC-cardiomyocytes from DMD patients possess increased mitochondria with abnormal morphologies (Willi et al., 2022). Degeneration of mitochondrial structure (swelling and loss of cristae) has been identified in dystrophic cardiomyocytes from hearts of 1-month-old and 3-4-month-old *mdx* mice prior to disorder onset (Kyrychenko et al., 2015). Furthermore, a significant increase in structurally abnormal mitochondria were observed in hearts of 12-month and older C57BL/10 *mdx* mice after developing DCM (Kang et al., 2018) which has also been reported as early as 4 weeks of age in this model (Dubinin et al., 2020b). Various mitochondrial proteins contents have been assessed in mdx mouse models with divergent responses observed depending on the pathway, age and model suggesting that altered control of oxidative phosphorylation could occur through post-translational modifications that have yet to be fully identified, and that compensations in the content of certain pathways may occurs (Hughes et al., 2020; Ascah et al., 2011 as reviewed in Bellissimo et al., 2022).

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4. Current interventional targets and approved non-genetic therapies for cardiomyopathy in DMD

No cure exists for DMD or its associated cardiac dysfunction, and therefore, most treatments are aimed at treating the symptoms and delaying the progression of dystrophic cardiomyopathy. Unfortunately, cardiac transplantation is generally contraindicated in DMD patients due to muscular weakness and respiratory insufficiency (Connuck et al., 2008). Left ventricular assist devices (LVAD), if implemented as a destination therapy rather than a bridge to transplantation, can be considered as a therapeutic option in DMD patients (Rose et al., 2001). While novel therapies have been in development to specifically address defects resulting from dystrophin deficiency, their effectiveness with respect to cardiac function has been limited due to the diverse range of genetic mutations in DMD and the complex risks of each prospective therapy (reviewed in Shah and Yokota 2023). For example, certain small molecule-based therapies used in practice or under development target secondary cellular dysfunctions that are common in most if not all DMD patients and therefore have potential for widespread use. Such therapies tend to focus on symptom management and delaying development of cardiomyopathy and heart failure. Other gene-based treatments focus on restoring dystrophin expression in a truncated or complete sequence must be customized to each unique mutation that occurs in DMD.

4.1. Glucocorticoid therapy

The most widely used intervention in DMD is corticosteroids, which have proven to reduce inflammation, prolong strength, and delay loss of ambulation by 1 to 3 years in DMD patients (Escolar et al., 2011). However, beneficial effects of chronic steroids in the heart are less established. Therapy most often involves daily dosing of either prednisone or deflazacort initiated around 2 to 5 years of age (Flanigan, 2017). Although it is unknown how steroids delay DMD progression, the following mechanisms are suspected to contribute: reduced cytokine production, activation of insulin-like growth factors, increased myoblast proliferation, decreased lymphocyte reaction, and upregulation of synergistic molecules (Angelini and Peterle, 2012). Cardioprotective mechanisms in boys with DMD may include cell membrane stabilization, decreased myocardial inflammation and fibrosis, and improvement in skeletal muscle function leading to secondary cardiac effects (Barber et al., 2013). Steroids also activate mineralocorticoid receptors which likely causes adverse effects, including reduced bone density, obesity, hypertension, adrenal insufficiency, and increased muscle catabolism (Hoffman et al., 2012; Bylo et al., 2020; Liu et al., 2013). This will be discussed in detail later. Recent studies have suggested that pulsed steroid regimens may limit the adverse effects while maintaining the benefits of daily steroid dosing in DMD patients (Connolly et al., 2019; Quattrocelli et al., 2019). Preclinical studies have demonstrated that steroids may worsen the progression of cardiomyopathy in the heart of C57BL/10 *mdx* mice with findings including decreased cardiac function, increased dilation, and increased cardiac fibrosis (Janssen et al., 2014; Bauer et al., 2009; Guerron et al., 2010), consistent with similar findings in the diaphragm (Howard et al., 2022). It should be noted that these studies used a more continuous method of drug delivery than is equivalent to the single daily dose therapies administered in DMD patients (Spurney et al., 2011).

Studies in DMD patients collectively revealed the protective benefits of steroid treatment in dystrophic hearts, including decreased fibrosis, preserved ventricular function, and better survival (Schram et al., 2013; Markham et al., 2005; Barber et al., 2013; Silversides et al., 2003; Raman et al., 2015; Spurney et al., 2011). When steroid therapy is initiated prior to the onset of cardiomyopathy, delayed development of cardiac dysfunction has been observed (Markham et al., 2008). In fact, a delay in the onset of cardiomyopathy by 4% for each year of steroid use has been observed in the setting of chronic steroid use (Barber et al., 2013). Data from a surveillance program demonstrated that longer duration of steroid use correlates with a greater improvement in LV function (Barber et al., 2013). Furthermore, the duration of steroid treatment has been proven to be inversely correlated with the incidence of cardiomyopathy in DMD patients (Barber et al., 2013). Long-term steroid treatment beyond loss of ambulation in DMD led to improvement in all-cause mortality secondary to improved cardiac outcome (Schram et al., 2013). Improvement in LVEF and FS was demonstrated

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in DMD patients treated with steroids (Markham et al., 2008; Houe et al., 2008). On a molecular level, a study in 7-week-old C57BL/10 *mdx* mice demonstrated that glucocorticoids prevented calcium-induced mPTP opening and restored ADP-stimulated respiration as the result of enhanced expression of Complex III, Complex IV, and IV protein content markers in skeletal muscle (Dubinin et al., 2020a), but further work on cardiac muscle is required in this area.

4.2. Angiotensin-inhibiting therapies

The goal of blocking the renin-angiotensin system is to improve the adverse remodeling that results from cardiomyocyte loss. Angiotensin II and angiotensin II type 1 receptor induce many harmful cardiac effects, including increased fibrosis, remodeling, ROS production, and cardiomyocyte death (Dikalov and Nazarewicz, 2013; Dasgupta and Zhang, 2011; Kawai et al., 2017). The two main angiotensin-inhibiting drug classes used in heart failure and DMD patients are angiotensin receptor blockers (ARBs) and angiotensin converting enzyme inhibitors (ACEIs). ACEIs prevent an enzyme from converting angiotensin I to angiotensin II, which decreases the activation of genes that enhance fibrosis and scarring of the myocardium (Greenberg et al., 1999), and block the action of angiotensin II, allowing veins and arteries to dilate.

Based on findings that the first clinical signs of cardiac involvement appear around 6 to 10 years of age, current guidelines advise that DMD patients begin an angiotensin-inhibiting agent by age 10 or earlier in the asymptomatic patient (Birnkrant et al., 2018). Since ACEIs are used as the first line of therapy for general heart failure and have been studied more extensively, these agents are typically the first recommended drug for the treatment of cardiac dysfunction in DMD patients (Kaspar et al., 2009). In fact, ACEIs were the first class of drugs to improve cardiac function in DMD patients in clinical trials (Duboc et al., 2005; Duboc et al., 2007). For example, the onset and progression of LV dysfunction was delayed, and survival rate improved in DMD patients when treated early with the ACEI perindopril (Duboc et al., 2005; Duboc et al., 2007). In addition, improvement in LV function was reported with use of ACEI alone or in combination with a beta-blocker in DMD patients (Viollet et al., 2012).

Despite being equally as effective and better tolerated than ACEIs (Allen et al., 2013), ARBs are an alternative treatment for cardiac dysfunction in DMD patients and typically reserved for patients who do not tolerate ACEIs (Birnkrant et al., 2018). In C57BL/10 *mdx* mice with pharmacologically induced myocardial injury, treatment with the ARB, Losartan, ameliorated cardiac injury (Meyers Heitzman, Krebsbach et al., 2019). Losartan reduced cardiac damage 2.8-fold in C57BL/10 *mdx* hearts without injury on WT hearts, but ACEI treatment had no effect on the myocardial injury (Meyers, Heitzman, and Townsend, 2019). This reveals that direct blockade of the angiotensin II type 1 receptor may be necessary in the dystrophic heart (Meyers, Heitzman, and Townsend, 2019).

4.3. Beta-adrenergic receptor blockers

The cardiac dysfunction in DMD is typically characterized by tachycardia (Perloff et al., 1967). Activation of β-adrenergic receptors enhances heart rate elevations and increases contractility through its action on the calcium transients in the cardiomyocyte (Wagner and Maier, 2015). Beta-blockers may limit these adverse effects through inhibition of β-receptor binding and subsequent catecholamine binding (Wagner and Maier, 2015). The latest guidelines recommend considering use of a beta-blocker at the onset of tachycardia or ventricular dysfunction (Birnkrant et al., 2018). Beta-blockers are considered a second-line candidate for cardiac-related therapy in DMD patients, typically administered in addition to an angiotensin-inhibiting agent (Viollet et al., 2012; reviewed in McNally et al., 2015). Its benefit in this patient population is unclear and not all beta-blockers have a similar efficacy in DMD (Schultz et al., 2022; reviewed in McNally et al., 2015). For example, administration of metoprolol to C57BL/10 *mdx* mice with early cardiomyopathy led to worsening right ventricular ejection fraction (RVEF) with no effect on myocardial calcium influx (Blain et al., 2013). Carvedilol administered for 6 months improved LVEF and decreased the incidence of ventricular tachycardia in DMD patients (Rhodes et al., 2008). Significantly higher survival rates and reduction in severe cardiac events were observed in DMD patients treated with carvedilol over 5

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years (Matsumura et al., 2010). Furthermore, a prospective open study demonstrated no difference between subjects receiving ACEIs alone versus ACEIs with beta-blockers (Viollet et al., 2012). However, other trials have shown that this combination therapy improves LV function (Jefferies et al., 2005; Kajimoto et al., 2006), prevention of major cardiac events (Matsumura et al., 2010), and long-term survival (Ogata et al., 2009) in comparison to ACEIs alone. Beta-blockers administered to DMD human iPSC-derived cardiomyocytes decreased the incidence of arrhythmogenesis (Kamdar et al., 2020). Since beta-blockers are second-line agents, there is difficulty in determining their benefit in DMD patient as their use is often delayed and used in combination with a first-line therapy (Viollet et al., 2012; reviewed in McNally et al., 2015).

Systolic blood pressure is typically lower in DMD patients, which complicates the use of these and other standard heart failure medications (McNally et al., 2015). There is, however, a cross-sectional study which showed increased systolic and diastolic BP, and heart rate in adolescents with DMD in response to an active sitting test that promoted reduced autonomic modulation (Rodrigues et al., 2021). Of note, it was observed that infusion of the β-1 adrenergic receptor agonist dobutamine in 7-month-old C57BL/10 *mdx* Langendorff perfused hearts resulted in significantly lower peak systolic pressure, developed pressure, and dP/dt_{MAX} compared to control mice (Stevens et al., 2024).

4.4. Mineralocorticoid receptor antagonists

Aldosterone activates the mineralocorticoid receptor (MR), resulting in cardiomyocyte death, hypertrophy, and fibrosis in DMD cardiomyopathy (Raman et al., 2015). MR antagonists, such as eplerenone and spironolactone, are treatments for heart failure with reduced LVEF and are used in some cases of DMD cardiomyopathy (Kamdar and Garry, 2016). Myeloid cells that infiltrate the heart in dystrophic mice express aldosterone synthase, which may increase the injury and subsequent remodeling (Chadwick et al., 2016). Corticosteroids, previously mentioned as common first-line interventions among DMD patients, activate MR as well as their target receptor (Heier et al., 2019). Administration of spironolactone and ACEIs together demonstrated a protective effect in mice models of DMD cardiomyopathy. However, this effect lessened with age and reiterated the importance of early initiation of treatment (Rafael-Fortney et al., 2011; Janssen et al., 2014). Spironolactone and eplerenone have been observed to be of equal efficacy in the DMD population (Raman et al., 2019). Clinical trials involving patients with preserved ejection fraction (EF) on an angiotensin-limiting drug prior to and during the trial revealed marked improvements in myocardial strain, EF, and chamber dilation with eplerenone treatment (Raman et al., 2015b). The open label extension of this trial demonstrated preservation of the myocardial circumferential strain over 36 months while being treated with an angiotensin-limiting drug and eplerenone (Raman et al., 2017). Vamorolone, an MR antagonist that matches the potency of eplerenone and possesses anti-inflammatory benefits similar to corticosteroids, was recently approved in the USA for DMD (Guglieri et al., 2022).

4.5. Gene-targeted therapies

Gene-targeted therapies for DMD aim to restore normal myocyte function through production of a functional dystrophin gene product (Shah and Yokota, 2023). Although gene therapy is an attractive technology, these complex therapies pose many challenges to achieving appropriate and lasting correction. While the impact of many of these therapies has been examined in skeletal muscle, assessing the benefits of these approaches on cardiac outcomes has proven difficult (reviewed in Shah and Yokota, 2023, and Meyers and Townsend, 2019). Restoring dystrophin in skeletal muscle without addressing the cardiomyopathy could lead to increased cardiac demand, exacerbate cardiomyopathy, and accelerate development of heart failure (reviewed in Meyers and Townsend, 2019; Townsend et al., 2008). Gene-targeted therapies, including stop codon readthrough, antisense oligonucleotides (AONs), viral gene therapy, and clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) gene editing, have been thoroughly reviewed elsewhere (reviewed in Shah and Yokota 2023; and Meyers and Townsend, 2019) and will therefore only be briefly discussed here.

 doi:10.20944/preprints202401.2158.v1

Stop codon readthrough therapy, such as Ataluren which is approved in Europe, involves binding to ribosomal RNA and bypassing the premature stop codon, typically resulting in partial dystrophin expression in skeletal muscle (Namgoog and Bertoni, 2016). However, this type of treatment would only be useful in about 10% of DMD cases (i.e. those with nonsense mutations) (Bladen et al., 2015) and studies have demonstrated no effect on cardiac function with Ataluren administration (Ebrahimi-Fakhari et al., 2018).

In turn, AONs, which can restore the reading frame to generate a truncated yet functional protein (Piga et al., 2019), are useful in the case of deletions. Although approximately 80% of DMD patients have mutations that may be amenable to this approach, AONs can only target one exon at a time. In addition, while AONs were observed to restore dystrophin expression in skeletal muscles in preclinical studies, negligible effects on dystrophin levels were demonstrated in the heart (Lu et al., 2005; Yokota et al., 2009). Peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO) delivery of AONs, which facilitates the delivery of AONs across the plasma membrane, has demonstrated substantial expression of dystrophin in the hearts of DMD animal models (Yin et al., 2008). The AONs Eteplirsen, Golodirsen, Viltolarsen, and Casimersen are FDA-approved based on modest levels of dystrophin restoration rather than on functional outcomes (Duan et al., 2021; Johnston et al., 2021).

Viral gene therapy in DMD patients aims to replace or repair defective dystrophin with micro-dystrophin, which is one-third the size of the full-length dystrophin protein (Chamberlain and Chamberlain, 2017; Hakim et al., 2018). The adeno-associated virus (AAV) is currently the only gene therapy vector that has been considered due to the vector's capability for increasing dystrophin in cardiac and skeletal muscles (Hakim et al., 2018; Duan, 2016). Elevidys, the first and only FDA-approved gene therapy for DMD, uses a MHCK7 promoter and has demonstrated expression of micro-dystrophin in both skeletal and cardiac muscle in animal models (Potter et al., 2021). The MHCK7 promoter has been shown to have higher cardiac expression in mice (Potter et al.,2021; Salva et al., 2007). This is due to the inclusion of the alpha myosin heavy chain enhancer. However, to our knowledge, there is no data demonstrating that the MHCK7 promoter is expressed in human ventricles and thus the efficacy of this therapy has not been fully established. Human atria do express the MHCK7 promoter, but it is unknown how this expression pattern of micro-dystrophin affects conductivity and cardiac function. Multiple micro-dystrophin constructs are also in development, including the Pfizer vector, which employs a skeletal muscle promoter, and the CK8 promoter in the Solid Biosciences construct (Duan, 2018; Salva et al., 2007; Hakim et al., 2017). The impact of both Elevidys and the constructs in development on cardiac muscle is still unknown clinically and some findings suggest that higher doses may have potentially toxic implications (Duan et al., 2018; Hart et al., 2022).

CRISPR-Cas9 gene editing aims to restore muscle physiology by producing truncated dystrophin constructs (Johnston et al., 2021) and involves a guide RNA (gRNA) that targets a genomic sequence as well as an endonuclease that produces a double-stranded break in the DNA at targeted sites (Ran et al., 2013). The subsequently activated DNA repair pathways include homology-directed repair (HDR) and nonhomologous end-joining (NHEJ), which is used to restore the reading frame in non-dividing cells, such as cardiomyocytes (Duan et al., 2021). Several studies showed significant expression of dystrophin and improvement of cardiac pathology in mouse and GRMD models treated using CRISPR-Cas9 technology with a cardiac-expressing promoter (Nelson et al., 2016; Amoasii et al., 2018; Amoasii et al., 2017; Bengtsson et al., 2017; El Refaey et al., 2017). This correction persisted long-term (18 months) in mice, suggesting that permanent gene repair could be possible with this approach (Hakim et al., 2018; Xu et al., 2019; Nelson et al., 2019). Although exciting, gene editing for DMD is in the preclinical stage and challenges must be overcome to apply this technology to humans.

4.6. Other therapies

Tamoxifen, which is a first-generation selective estrogen receptor modulator (SERM), demonstrates potent antiestrogenic activity on the mammary gland and is generally used for the

 doi:10.20944/preprints202401.2158.v1

prevention and treatment of breast cancer (Dorchies et al., 2013). In a study orally administering Tamoxifen on dystrophic *mdx*^{5Cv} mice starting at 3 weeks of age for 15 months at 10 mg/kg/day, remarkable improvements to cardiac structure were noted, with cardiac fibrosis concurrently being diminished by ~50%, compared to WT mice (Dorchies et al. 2013).

5. Chamber-specific cardiomyopathy in DMD

To date, the majority of research in the hearts of DMD patients and dystrophin-deficient animal models has been conducted specifically in the LV or, more commonly, in the unspecified 'whole heart'. Emerging data now suggests that dystrophic cardiomyopathy may affect the heart heterogeneously, with differing data on fibrosis, calcification, function (e.g. echocardiography, hemodynamics, etc.), and other related parameters observed across ventricles.

5.1. Human Duchenne muscular dystrophy

A study by Mehmood et al., which was the first to use CMR to define RV systolic function and size in DMD patients, determined that in DMD patients with reduced LVEF, the RVEF was still relatively unchanged (Mehmood et al., 2016; Bosser et al., 2004). The relative preservation of RV function is thought to be attributed to the advancements that have been made in respiratory care, including improvements to ventilatory support. Furthermore, in a study by Bosser et al., normal resting RVEF (>45%) was observed in 95% of DMD patients, whereas only 79% of patients demonstrated a normal LVEF (50%) (Bosser et al. 2004). This has largely led to the inaccurate belief that the RV is generally less impacted, and therefore less crucial to the outcomes of cardiomyopathy in DMD patients.

Despite these findings, other studies have indicated that RV insults do persist in DMD. Electrical right ventricular hypertrophy (RVH) is frequent in DMD patients, reaching 37% without correlating to LV dysfunction (Takami et al., 2008; reviewed in Fayssoil et al., 2017). Dual et al. demonstrated that pre-contrast RV-T1 (via CMR), which indicates presence of diffuse myocardial fibrosis, is elevated in boys with DMD and that this is negatively correlated with RVEF, compared to age- and sex-matched healthy boys (Dual et al., 2021). This finding is particularly important because boys with DMD develop respiratory impairments prior to heart failure, and the respiratory dysfunction and resulting hypoxia are predictors of increased afterload as seen through the RV due to constriction of pulmonary arteries (Dual et al., 2021). This study also demonstrated abnormal RV function in DMD-affected boys, which was indicated by significantly decreased tricuspid annular excursion (TAE) (a measure of RV longitudinal contraction) and RV mass (Dual et al., 2021). Additionally, the group examined the role of the septum in DMD-induced cardiomyopathy. Given that no differences in pre-contrast septal T1 were determined when compared between groups, it was concluded that when classifying RV mass and BMI as covariates, RV-T1 best predicts DMD status relative to the septum (Dual et al., 2021). It was thus determined that in DMD patients, the septum remains relatively unchanged as cardiomyopathy worsens (Dual et al. 2021). The study by Dual et al. (2021) also reported higher RV-T1 compared to LV-T1 in both healthy patients and those with DMD. This chamber-specific elevation is thought to be attributed to naturally increased collagen content of the RV wall.

Further exploring the RV in DMD, a study by Subramanian et al. investigated the relationship between pulmonary function and RV functional parameters in young patients with DMD (Subramaniam et al., 2015). They determined that abnormal pulmonary function was correlated with reduced RV stroke volume and preserved LV systolic function. Work comparing the LV to the RV has demonstrated that young DMD patients with preserved EF and normal FS present with subclinical regional myocardial dysfunction (Mertens et al., 2008). This work established that during the earlier stages of disorder progression, function of the interventricular septum and RV free wall were better preserved than the LV, as indicated by longitudinal measurements.

It has been theorized that RV lesions in DMD patients are more difficult to assess with LGE (which is commonly used to assess LV fibrosis) because the RV has a smaller mass, potentially smaller-sized lesions, and a more complex geometry (Meyers and Townsend, 2015). Also, it has been

hypothesized that sympathetic activation or hypoxia-induced constriction of the pulmonary artery leads to elevated afterload, resulting in the increased RV (Meyers and Townsend, 2015). Refer to the *cardiac fibrosis* section above for more detail.

5.2. Dystrophin-deficient rodent models

While much of what is known about RV functional parameters has been learned from human DMD patients, there are several known studies that examined RV function in *mdx* mice, including one which tested the effects of early beta-blocker (metoprolol) use on chamber-specific cardiomyopathy (Blain et al., 2013; Stuckey et al., 2012). Blain et al. compared ventricular function in 24-week-old C57BL/10 *mdx* mice without beta-blocker treatment and determined that there was general ventricular hypertrophy, reduced LV stroke volume with a small LV cavity size, and normal LVEF, despite reduced RVEF compared to WTs (Blain et al., 2013). This study concluded that in the untreated, aged C57BL/10 *mdx* mouse model, RV dysfunction potentially preceded LV dysfunction (Blain et al., 2013; Stuckey et al., 2012). Based on their findings, the authors cautioned against early beta-blocker usage due to the lasting effects they may have on the RV. It has been postulated that the apparent improvements in LV function and deterioration in RV function may be linked through a mechanism whereby untreated hearts have a relatively low stroke volume, which may protect the RV from increased volume overload. Given that beta-blockers improve cardiac output and stroke volume to normal levels, greater dilatation of the RV can result from the increased flow to the right heart (Blain et al., 2013). In a separate study, Hayes et al. (2022) demonstrated that 18-week-old D2.*mdx* hearts exhibit thick epicardial fibrotic-calcinosis layers that are limited and restricted to the RV, but such changes are not detectable in the LV or septum (Meyers and Townsend, 2015). While systolic dysfunction is evident in both ventricles of the D2.*mdx* heart, it was determined that RV function was slightly better than LV despite the elevated fibrosis (Meyers and Townsend, 2015). Together, this data suggests that RV damage potentially precedes onset of significant cardiac complication (Meyers and Townsend, 2015). Hemodynamic output is similar between ventricles in both the D2.*mdx* mouse model and in DMD patients (Meyers and Townsend, 2015). Stuckey et al. (2012) determined that C57BL/10 *mdx* mice exhibited normal RV resting function at 1 month of age, but this was noted to be abnormal beginning at 3 months of age, elevated RV-ESV and decreased RVEF. These responses were noted to occur prior to LV dysfunction, with LVEF being reduced at a later time point (1 year of age).

5.3. Limitations and future directions of chamber-specific cardiomyopathy

Historically, the role of the RV in dystrophin deficiency-induced cardiomyopathy has been underappreciated in place of the larger, more physiologically demanding LV. For this reason, literature comparing pathology across ventricles is limited. Although, to date, some work has been conducted to elucidate RV function and its role among the respiratory impairments associated with dystrophin deficiency, further research is required to investigate parameters such as hemodynamics and echocardiography in young dystrophin deficient rodent models (mice aged >24 weeks). Additionally, there is an abundance of conflicting data with regards to ventricle-specific disorder progression due to the use of different dystrophin-deficient models and time points. Future research should seek to track time-course progression of ventricular abnormalities in an established dystrophin-deficient rodent model with cardiomyopathy (*mdx* mice) to determine the order in which these pathologies arise.

Furthermore, researchers should consider conducting four-chamber histopathological assessments of the heart, which can provide valuable insight towards early- and late-stage cardiomyopathy (at least in dystrophin deficient rodent models due to logistical limitations encountered in human DMD models). Exploring these parameters across chambers can help guide therapy development beyond just LV impairments, such that lung and diaphragm abnormalities can be addressed concurrently using RV and atrial data. To date, most papers omit collection of this data due to restrictions brought on by the complex geometry of the RV, coupled with the extremely small free wall size, which is a limitation that can be overcome by repeating experiments to obtain multiple

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phases for various measures. Future work in both human and dystrophin-deficient rodent models should seek to explore chamber-specific cardiomyopathy holistically- from histopathological and functional assessments to molecular and metabolic underpinnings of the disorder using a time-course experimental design.

6. Mitochondria as a potential therapeutic target

6.1. Targeting calcium handling

Given mitochondrial dysfunctions are thought to arise, in part, by elevated calcium uptake, preventing mitochondrial dysfunction could be achieved by improving cytoplasmic handling itself. For example, due to the hypersensitivity to excitation-contraction coupling in cardiomyopathy of DMD patients, targeting RyR2 may offer therapeutic benefit. PKA phosphorylation of RyR2 appears to contribute to increased calcium release from the SR, and therefore, PKA could be a potential therapeutic target (Figure 1). In dystrophic mice, inhibition of PKA-mediated phosphorylation of RyR2 reduced SR calcium leak and in turn prevented cardiomyopathy (Sarma et al., 2010). Oxidative stress can lead to the nitrosylation of RyR2 and subsequent disassociation of calstabin2 from RyR2, increasing SR calcium leak (Fauconnier et al., 2010). Treatment of C57BL/10 *mdx* mice with either NAC to inhibit RyR2 nitrosylation, or the RyR2 stabilizer Rycal, prevented depletion of calstabin2 and subsequent SR calcium leak, aberrant depolarization in cardiomyocytes, and arrhythmias (Fauconnier et al., 2010).

The stretch-sensitive channel TRPV2 is another calcium channel target to consider, as increased TRPV2 has been documented in the cytoplasmic membrane of *mdx* cardiac and skeletal cells and the sarcolemmal membrane of DMD patients (Iwata et al., 2013). Treatment of DMD patients with the antiallergy drug Tranilast, which has anti-TRPV2 activity, resulted in reduction of heart failure biomarkers (Matsmura et al., 2018).

Enhancing SR calcium uptake through overexpression of SERCA or targeting its inhibitors can be considered in restoring calcium homeostasis. In C57BL/10 *mdx* mice 12 months of age, administration of AVV9-SERCA2a gene therapy significantly improved cardiac electrophysiology (Shin et al., 2011), whereas in 3-month-old *mdx* mice a similar therapy ameliorated DCM for at least 18 months (Wasala et al., 2020). Decreasing sarcolipin expression in *mdx* mice restored cardiac SERCA function and calcium cycling, thus preventing cardiomyopathy. Decreased LV internal diameter in diastole as well as decreased fibrotic and necrotic tissue likely contributed to the improved cardiac function (Voit et al., 2017). While targeting sarcolipin may be a promising approach, cardiac function worsened in phospholamban (PLN) knockout C57BL/10 *mdx* mice (Law et al., 2018).

6.2. Mitochondrial calcium overload

As described previously, frequent PT activity may contribute to calcium-dependent mitochondrial dysfunction and therefore, prevention of this opening could be beneficial. As discussed in Section 3, inhibition of a key regulator of mPTP, cyclophilin, may impede opening of the pore and subsequent mitochondrial dysfunction (Millay et al., 2008; Reutenauer et al., 2008).

6.3. Targeting cellular/mitochondrial antioxidant systems

Since cardiac mH_2O_2 is elevated early in DMD (Hughes et al., 2020; Dubinin et al., 2020b), mitochondrial-targeted antioxidant therapies could provide therapeutic benefit in DMD patients. Several general antioxidants, including MitoQ and SkQ1, are conjugated with the lipophilic cation triphenylphosphonin (TTP) to target the mitochondria (reviewed in Murphy, 2008). The small 'SS' peptides are cell permeable agents that bind to cardiolipin on the IMM and can preserve oxidative phosphorylation, attenuate mH_2O_2, and prevent oxidative damage (Szeto and Birk, 2014; Zhao et al., 2003; Birk et al., 2013). Furthermore, SS peptides can inhibit the dissociation of cytochrome *c* from cardiolipin, hindering the activation of cell death pathways (Zhao et al., 2004) (Figure 3). To our knowledge, no publication has investigated the effects of any mitochondrial-ROS lowering compound in models of DMD.

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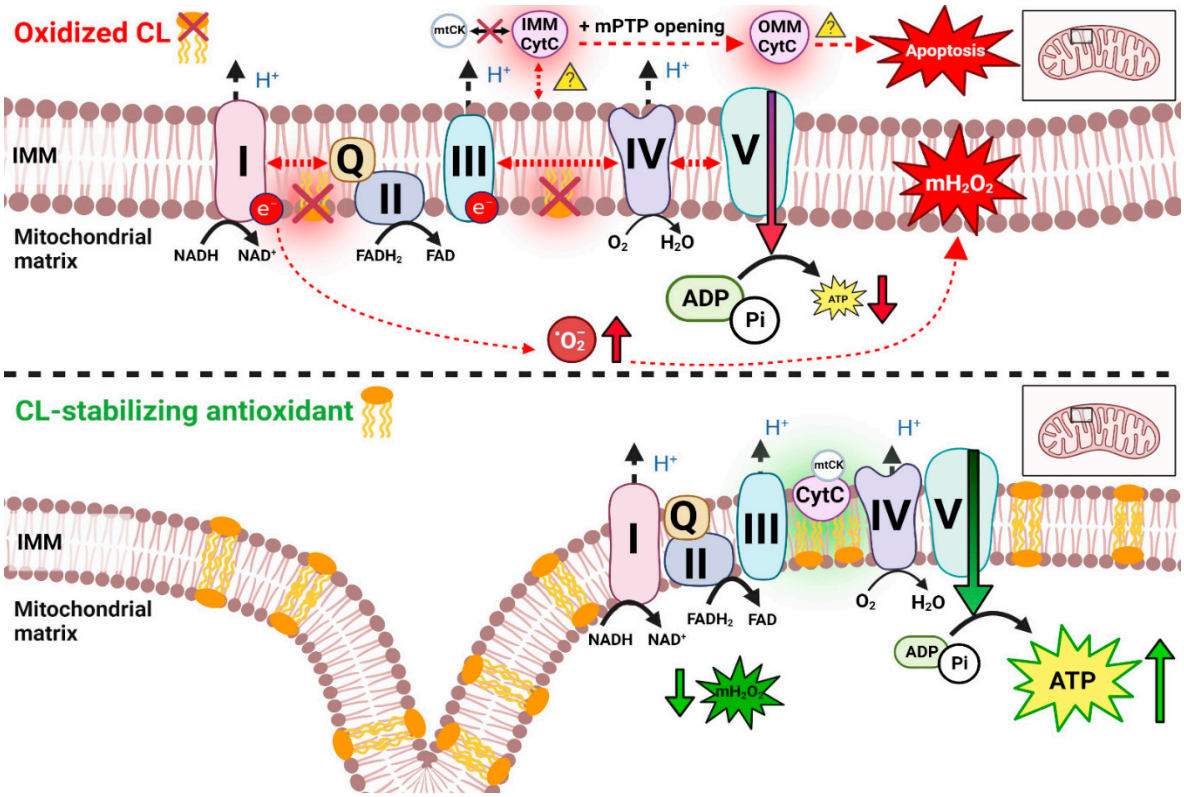

Figure 3. Cardiolipin as a prospective therapeutic target for treating mitochondrial stress in dystrophic cardiac tissue. Since cardiolipin is highly redox-sensitive (attributed to its high structural content of unsaturated fatty acids), and mitochondrial reactive oxygen species (mH_2O_2) provision is elevated in dystrophic cardiac tissue (Hughes et al., 2020), targeting cardiolipin may be beneficial for stabilizing mitochondrial membranes. **(Top panel)** Herein, we propose that when cardiolipin is oxidized, electron transport chain supercomplexes are unevenly distributed and mitochondrial membranes are abnormally linear, thus resulting in elevated mH_2O_2 production and reductions to [ATP]:[ADP] (Koufen et al. 1999; Dolder et al. 2001). Additionally, cytochrome *c* is released into the cytosolic fraction, thus impairing its association with mitochondrial creatine kinase (mtCK) (Schlattner et al. 2018; Koufen et al. 1999; Dolder et al. 2001). Although elevations to cytochrome *c* in the cytosolic fraction of dystrophic hearts have been previously characterized (Burelle et al., 2010), the yellow question mark denotes a lack of causal data linking this elevation to oxidized cardiolipin. The release of cytochrome *c* from the IMM, and eventually OMM (yellow question mark once again denotes a lack of data linking this to oxidized cardiolipin), may also lead to pathological cytosolic cytochrome *c* accumulation, thus perpetuating apoptotic events (Burelle et al. 2010). **(Bottom panel)** In the presence of a cardiolipin-stabilizing agent such as elamipretide, electron transport chain (ETC) supercomplexes can be tethered to each other in a less linear mitochondrial membrane, while cytochrome *c* remains tethered to the ETC and mtCK. This results in lower mH_2O_2 emissions and a higher [ATP]:[ADP] ratio, akin to healthy physiological systems. Created on BioRender.com.

6.4. Cardiolipin and membrane stability

Cardiolipin, a phospholipid enriched with unsaturated fatty acids located in the IMM, is essential to maintain mitochondrial structure and function. Through its binding to key modulators of energy exchange and subsequent proteolipid complex formation, cardiolipin plays a key role in mitochondrial bioenergetics (Schlattner et al., 2018) (Figure 3). This complex can regulate energy exchange, reduce mitochondrial ROS generation, and prevent opening of mPTP (Schlattner et al., 2018). In the presence of oxidative stress, cardiolipin and the key regulator mtCK, lose their binding capacity, leading to dissociation of the proteolipid complex (Koufen et al., 1999; Dolder et al., 2001) (Figure 3). Since mH_2O_2 generation is elevated in dystrophin deficient heart (Hughes et al., 2020;

 doi:10.20944/preprints202401.2158.v1

Dubinin et al., 2020b), a cardiolipin targeting agent, such as elamipretide, may be appropriate for targeting impaired mitochondrial bioenergetics. Although data on cardiolipin physiology in *mdx* is limited, the altered mitochondrial cristae structure reported in section 3.7 serves as a foundation for continuing the investigation on cardiolipin in various dystrophic models.

Albeit not published in a dystrophin deficient model, SS-31, or elamipretide, is an agent that has demonstrated improvements to mitochondrial stress including reduction of mitochondrial ROS across several pathologies (Siegel et al., 2013; Szeto and Schiller, 2011; Szeto, 2014). Elamipretide has also shown evidence of direct cardioprotective mechanisms including amelioration of apoptosis and fibrosis in preclinical models of heart failure (Dai et al., 2011; Eirin et al., 2014) (Figure 3).

Idebenone, a synthetic analogue of Coenzyme Q_{10} with electron shuttling activity, can alter respiratory chain activity (Zs-Nagy, 1990; Gillis et al., 1994), although this may require very high micromolar doses (Suno and Nagaoka, 1984; Suno and Nagaoka, 1989). Long-term idebenone therapy improved cardiac diastolic dysfunction, reduced cardiac inflammation and fibrosis, improved running performance, and limited mortality from cardiac pump failure induced by dobutamine stress testing *in vivo* in *mdx* mice (Buyse et al., 2009). Early clinical trials in patients with DMD revealed improved cardiac and respiratory function with idebenone treatment (Buyse et al., 2011; Buyse et al., 2015; Buyse et al., 2017; McDonald et al., 2016). Specifically, a trend for increased peak systolic radial strain in the LV was seen in these patients (Buyse et al., 2011). However, no measures of mitochondrial bioenergetics were performed to verify that the drug was acting through a mitochondrial mechanism. As reviewed elsewhere, idebenone has a variety of effects on other organelles which may not involve antioxidant properties of the compound (Gueven et al., 2021). This suggests that the beneficial effects in some studies may not be due to alterations in mitochondrial functions despite claims to this effect. These factors might be consistent with a subsequent clinical trial in people with DMD that was unsuccessful (NCT02814019). As such, no study to date has definitively tested the potential of mitochondrial-targeted therapies on cardiac dysfunction in pre-clinical models of dystrophin deficiency or in clinical studies.

Mitochondria are hypothesized to modulate phosphorodiamidate morpholino oligomer (PMO) efficacy by mediating membrane repair (Brown et al., 2022). In fact, mitochondria are known to hone to the site of sarcolemmal injury as well as contribute to the production of membrane phospholipids (Brown et al., 2022). However, PMO uptake by the cells requires ATP (Brown et al., 2022). Elamipretide localizes to the IMM where it binds to cardiolipin to improve membrane stability, enhance ATP synthesis, optimize cristae architecture, and attenuate ROS generation (Szeto and Birk, 2014). This agent has also been observed to improve mitochondrial ultrastructure (Allen et al., 2020) and restore cellular bioenergetics (Obi et al., 2022), which are known to be altered in dystrophin deficient models. In a preclinical study, elamipretide significantly increased dystrophin protein expression evoked by an exon-skipping PMO in *mdx* mice (Brown et al., 2022). When elamipretide was administered alone, trends to reduction of inflammation were observed (Brown et al., 2022). These data suggest that a potential synergy exists between elamipretide and exon-skipping PMOs which may represent a beneficial treatment strategy in patients with DMD (Brown et al., 2022). Given many of these mitochondrial abnormalities exist in cardiac tissue from *mdx* models, there is sufficient rationale to investigate whether such compounds preserve mitochondrial bioenergetics and treat cardiomyopathy in DMD.

7. Summary and future directions

Since emerging therapies are thus far unable to completely restore the *DMD* gene, there remains a need to develop additional therapies treating secondary contributors to cardiomyopathy. While curative approaches customized to individual mutations are actively pursued, developing new combinatorial therapies targeting a variety of cellular and physiological stressors could complement existing conventional approaches in a manner that benefits the majority of people with DMD. In this regard, the collective evidence warrants the development of new approaches targeting partial dystrophin restoration, inflammation, calcium and other ion dysregulation, membrane tearing, oxidative stress, cytoskeletal disorganization, metabolic stress and other cellular dysfunctions. Many

of these stressors are related to mitochondrial-specific stress responses linked to disrupted energy homeostasis, redox balance and calcium-induced cell death. The degree to which each mitochondrial stress response contributes to cardiomyopathy in DMD requires careful consideration of the mechanisms of specific mitochondrial targeting compounds, the age and type of animal model used in pre-clinical investigations, and cardiac chamber-specific dysfunctions. The overview provided in this article serves as a basis for guiding experimental design in a way that captures the specific mechanisms by which mitochondria contribute to cardiac dysfunction and histopathology in DMD.

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