1

Introduction

1.1 Duchenne Muscular Dystrophy: A Rare Genetic Disorder

Duchenne muscular dystrophy (DMD) is a serious X-linked recessive disorder which results from mutations in the dystrophin gene, leading to a complete absence of dystrophin protein.[1] The DMD gene, which is the largest known human gene, spans around 2.5 million base pairs and accounts for approximately 0.1% of the human genome and 1.5% of the X chromosome. It encodes dystrophin, a 427-kDa protein comprising of 79 exons and 78 introns, and provides crucial structural support by linking actin filaments to the dystrophin-associated glycoprotein (DAG) complex.[2] DMD affects approximately 1 in 3,500 live male births.[3]

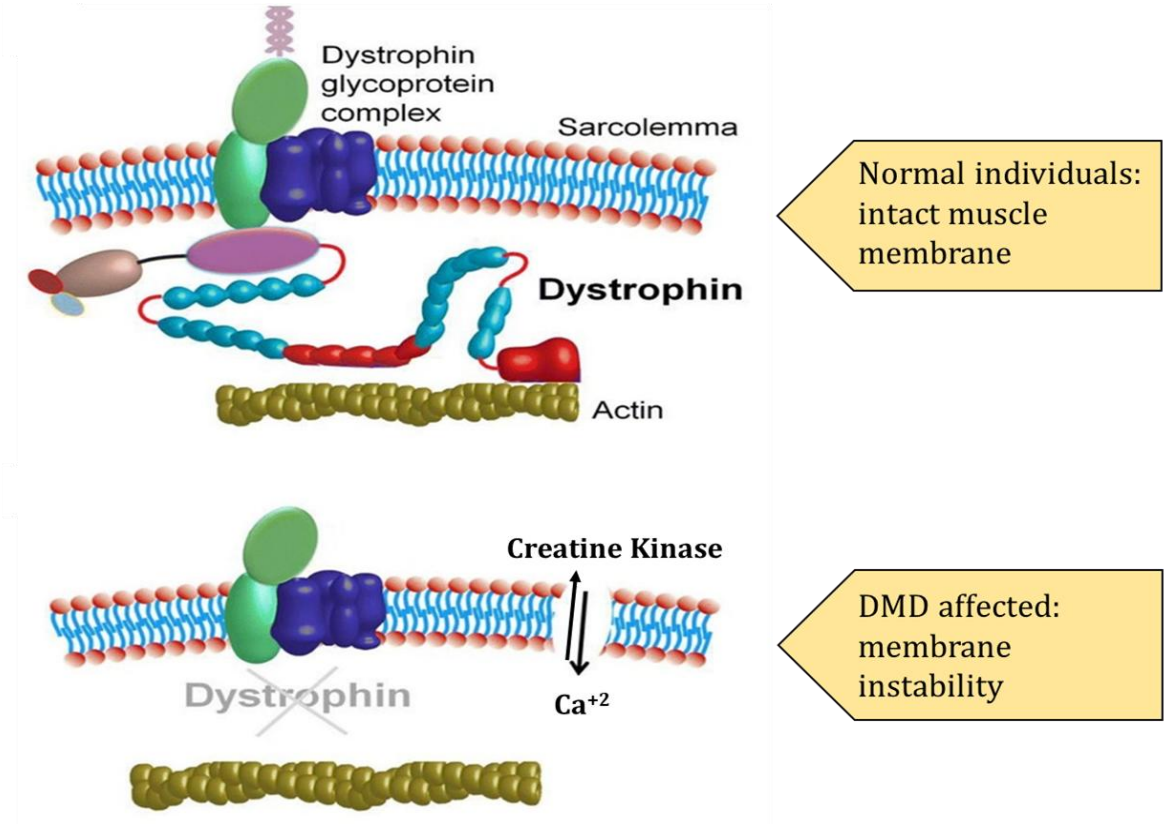


Figure 1.1: Comparative illustration of an intact muscle membrane (top panel) in which dystrophin anchors the dystrophin-glycoprotein complex (DGC) to the actin cytoskeleton, versus a dystrophin-deficient membrane (bottom panel) showing membrane instability associated with DMD. Comparison depicts differences between a healthy muscle cell in a normal boy and a dystrophic muscle cell in a boy with DMD. Image reproduced from Houang *et al.*, Skeletal Muscle, 2018 with copyright permission.

Dystrophin plays a critical role in connection of the cytoskeleton to the extracellular matrix through its interaction with the transmembrane protein dystroglycan and associated proteins, which includes the sarcoglycans. It affix to the actin in the cytoskeleton through its N-terminal actin-binding domain 1 (ABD1) and to β-dystroglycan via its C-terminal domain. The central rod domain, composed of 24 spectrin-like repeats, bridges these two binding sites (Figure 1.1). The loss of dystrophin protein triggers complex pathophysiological processes. Dystrophin is thought to play a critical role in maintaining membrane integrity as part of the dystrophin-associated protein complex. Its rod-like structure suggests that dystrophin protects muscle fibers from contraction-induced mechanical damage by stabilizing the sarcolemma. Additionally, the presence of multiple linkage domains, which anchor structural and signalling proteins, implies that dystrophin's functions extend beyond mechanical stability to include significant roles in cellular signalling as well.[4] The absence of dystrophin leads to increased membrane permeability and a cascade of pathological events that drive muscle necrosis and fibrosis. Elevated calcium influx across the dystrophin-deficient membrane disrupts calcium homeostasis, activating calcium-dependent proteases and eventually leading to cell death.[5] Mislocalization of neuronal nitric oxide synthase (nNOS) impairs hemodynamic responses during exercise, causing further cell damage. Over time, chronic inflammation and upregulation of extracellular matrix genes contribute to extensive muscle necrosis and fibrosis at the end stage of the disease.[6]

1.2 Role of Dystrophin Protein

Dystrophin is a crucial protein responsible for protection against membrane tears which occurs during muscle contractions. Presence of dystrophin in muscle connects the actin cytoskeleton to the sarcolemma, ensuring membrane stability.[7] The dystrophin protein consists of four domains namely N-terminal actin-binding domain, a central rod domain with 24 spectrin-like repeats, a cysteine-rich domain, and a C-terminal domain. Each domains have different functional features such as this domains enable dystrophin to bind actin filaments to the muscle cell membrane, also interact with β-dystroglycan, and connect to the dystrophin-glycoprotein complex (DGC), which forms a linkage between cytoskeleton and the extracellular matrix.[8] The central rod domain, with its elastic hinge regions, functions as a "shock absorber," and thereby supporting repetitive contraction-relaxation cycles.[9] This domain also supports interactions with actin and microtubules network, which results in maintaining muscle fibre integrity. The cysteine-rich region protects the binding with β-dystroglycan, affixing dystrophin to the sarcolemma.[10]

The DGC plays an important role in restoring muscle integrity by connecting the intracellular actin network to the extracellular matrix via relations with proteins such as laminin.[11] DGC consist of both transmembrane and cytoplasmic components, including α- and β-dystroglycan, sarcoglycans, syntrophins, and neuronal nitric oxide synthase (nNOS), and thus DGC confirms stable connections and networks across the sarcolemma.[12] Syntrophins and dystrobrevin bind to dystrophin at the sarcolemma, and resulting in proper positioning of nNOS for suitable cellular signalling and muscle maintenance.[13]

1.3 Diagnosis of DMD

DMD boys are generally first diagnosed between ages 2 and 5, often suffering with gait abnormalities. Boys with growing age frequently shows toe walking toe which reflects abnormalities and raises concern before a DMD diagnosis is performed.[14] Next after clinical concern, serum creatine kinase (CK) assessment is studied as a part of the standard screening for DMD boys with motor delays.[15] In young patients with DMD, clinical diagnosis occurs due to elevated creatine kinase (CK) levels in the bloodstream, which occurs due to leakage of CK outside the muscle membrane because of damaged and ripped muscle membranes which occurs every time whenever muscle contracts or relax in absence of shock prove protein dystrophin. Rise in CK levels are also accompanied by elevated levels of muscle-derived enzymes, such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT). CK levels generally increase by ten times more in comparison to upper normal limit, which rises to its peak in DMD patients between ages 1 to 6.[15] Recent studies shows that impairment of motor function is also prominent during childhood in DMD, alongside with other abnormalities such as development of cognitive challenges and delayed speaking ability.[16] Intelligence quotient (IQ) is also affected with growing age and it is observed that IQ level is generally lower by one standard deviation in comparison to normal, but verbal IQ improvement is observed over time.[17] Additionally, boys with DMD face a higher risk of autism, attention deficit hyperactivity disorder (ADHD), and obsessive-compulsive disorder.[18] To further confirm a suspected diagnosis of DMD, genetic testing should be pursued. Multiplex ligation-dependent probe amplification (MLPA) can quantitatively assess all exons to detect whole exon deletions or duplications, which account for approximately 75% of DMD mutations. Additional sequencing of the DMD gene is recommended to identify point mutations or smaller rearrangements. Muscle biopsy with immunohistochemical staining for dystrophin (using Dys1, Dys2, and Dys3 antibodies) and associated proteins of the transmembrane complex is useful to verify the absence of subsarcolemmal dystrophin or to diagnose less common muscular dystrophies. However, a muscle biopsy is not necessary when clinical suspicion is substantiated by genetic findings.[19]

1.4 Disease Progression of DMD

The initial symptoms of DMD, generally observed between age of 2 and 3, which includes a frequent falls, waddling gait, difficulty in rising from a sitting position (Gower's sign), and challenges with climbing stairs.[20] Affected children showed delay in development of gross motor skills, and require wheelchair support by the age of 10 to 12.[21] As the disease progresses, muscle weakness leads to joint contractures and scoliosis, contributing to restrictive lung disease.[22] By the age of 15 to 20, assisted ventilation becomes necessary, and despite optimal care and support, most individuals suffering from DMD surrender to cardiac or respiratory failure between 20 and 30 years of age (Figure 1.2).[23, 24]

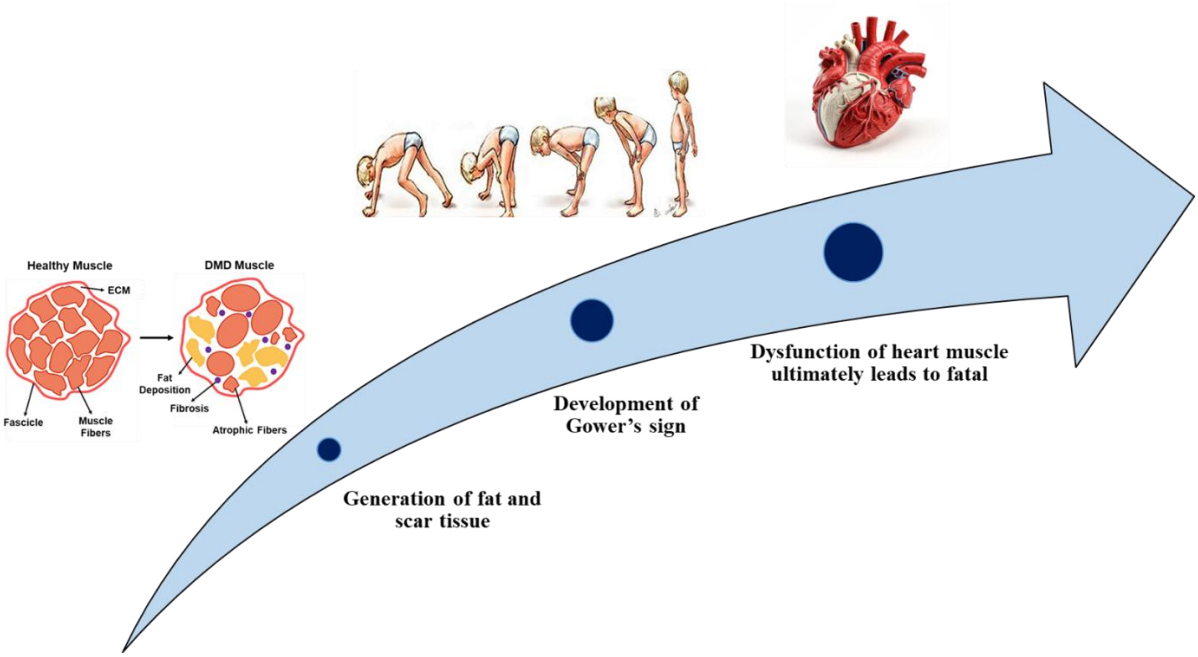


Figure 1.2: Illustrative representation of the progression of DMD disease starting from deposition of fat and scar tissue to dysfunction of heart muscle.

1.4.1 Pathogenesis of DMD

- **Membrane Instability:** In DMD, the absence of dystrophin and associated proteins disrupts the structural link between the sarcolemma, cytoskeleton, and ECM, making the sarcolemma more permeable and prone to injury.[13, 25]. Hallmarks of muscle damage in DMD patients, such as sarcolemmal tears known as "delta lesions," have been observed.[26, 27] These studies express the key role of the dystrophin protein and its associated complex in preventing muscle sarcolemma integrity and focus on its destabilization impact in DMD.

- **Calcium Dysregulation:** In DMD patients, due to increased membrane instability and appearance of ribs in muscle membrane leads to influx of excessive calcium ions thus results in elevated intracellular calcium levels which further results in disrupted calcium homeostasis.[28] Calcium enters muscle fibers through sarcolemmal tears, which results in negative effects on muscle function. Increased calcium levels activates various calcium-dependent proteases which damage healthy muscles apart from damaged muscles. Apart from that elevated calcium levels also promotes several calcium dependent signalling pathways, which includes calmodulin,[29] calpains,[30] and dysferlin,[31] which results in impaired calcium cycling and muscle repair. Further, increased calcium leak from the sarcoplasmic reticulum (SR) and reduced sarco-endoplasmic reticulum calcium ATPase (SERCA) function results in elevated cytosolic calcium.[27, 28] This calcium dysregulation is a key factor in the DMD pathology. Additional damage of the muscle can arise because of mitochondrial dysfunction, which occurs due to disrupted calcium homeostasis, which results in significant decrease in

ATP production and compromise with the membrane potential.[28, 32] Increased cytosolic calcium levels in dystrophic muscle fibers is a significant contributing factor to the disease progression, highlighting the multiple harmful impacts of calcium signalling on muscle functionality.

- **Inflammation:** DMD patients, infiltration of immune cell is a significant factor contributing to disease progression.[33] Inflammatory responses begin before the onset of symptom[34] and multiply with the disease progression, which further damages muscle tissue.[35, 36] Healthy skeletal muscle possesses a delicate immune balance, which allows low MHCI expression with limited inflammatory cell presence, which enables controlled muscle repair on damage with minimum inflammation.[37]

  However in DMD patients, membrane instability due to dystrophin loss causes the continuous release of cytoplasmic components, which further activates toll-like receptors (TLRs) leading to continuous inflammation.[12] This rise in inflammation, along with calcium dysregulation and oxidative stress, altogether promotes a prolonged immune response which includes early infiltration of macrophages and T cells,[37] ultimately resulting in fibrosis and accumulation of adipose tissue.[35] Next, macrophages play a critical role in DMD progression, and their rise is associated with diminished muscle pathology.[36] T cells, in particular CD4+ and CD8+ subsets, further contributes to the muscle damage via cytokine production and rising cytotoxicity.[38]

- **Mitochondrial Energetics:** Mitochondria plays significant role in muscle bioenergetics, and supports oxidative phosphorylation via electron transport chain, where NADH and $FADH_2$ are oxidized in Complexes I and II to produce a proton gradient crucial for ATP synthesis.[39] Proper coordination of electron transport chain components is essential for energy balance. In DMD, however, disruptions in muscle homeostasis compromise mitochondrial stability.[40] Membrane instability in DMD promotes excessive Ca^{2+} influx, leading to mitochondrial Ca^{2+} overload, which can activate the mitochondrial permeability transition pore (mPTP) and trigger damage.[41] Structurally, mitochondria in dystrophic muscle appear smaller, with reduced cristae, likely due to altered fission and fusion cycles. These structural deficits are accompanied by diminished mitochondrial density, reduced ATP synthesis, and impaired respiration.[42] Notably, mitochondrial dysfunction and metabolic disruptions have been observed prior to muscle degeneration in young mdx mice, suggesting a pre-necrotic role in disease progression.[43]

- **Reactive Oxygen Species Dysregulation:** In normal muscle, reactive oxygen species (ROS) are generated at low levels, playing essential roles in cellular activities like contraction.[44] However, in DMD, both patient and animal models show significantly higher ROS production, rendering dystrophic muscles more susceptible to oxidative stress.[45] Mitochondria, particularly through electron leakage in the electron transport chain, are major ROS sources in muscle cells. While a healthy cell maintains a balance

between ROS and antioxidants, excess ROS in DMD leads to oxidative imbalance, contributing to cellular dysfunction and eventual cell death.[46] Elevated ROS levels, particularly H_2O_2 production supported by Complex I, have been observed in the skeletal muscles of mdx mice, a DMD model.[47] Although an adaptive increase in ROS buffering capacity occurs in DMD, but is transient and can temporarily counter ROS generation, and further chronic disease progression leads to accumulating oxidative damage, exacerbated by persistent inflammation from muscle membrane damage.[48] The diaphragm, due to higher ROS emission rates and mitochondrial density, is especially vulnerable to ROS-induced injury, worsening cardiorespiratory decline in DMD patients.[12]

NADPH oxidase 2 (NOX2) further contributes to ROS in DMD muscles. Activated by the microtubule-associated Rac1 protein upon mechanical stress, NOX2-induced ROS production is amplified in dystrophic muscle due to disrupted microtubule architecture from the absence of dystrophin.[49] Additional ROS sources include reactive nitrogen species from aberrant nNOS activation, collectively leading to extensive oxidative damage to DNA, proteins, and lipids.[6] These insights suggest targeting mitochondria and NOX2 as potential strategies for mitigating ROS-related damage in DMD.

- **Fibrosis:** Fibrosis is a reparative response mechanism involving excessive extracellular matrix (ECM) deposition, which ultimately results in scar tissue formation. While ECM typically comprises about 5% of muscle tissue, this fraction increases significantly in response to muscle injury or disease.[50] In DMD patients, muscle damage and regeneration associated with chronic inflammation leads to fibrosis during disease progression, which results in diminished muscle functionality and contractility.[51] The damaged muscle membrane in DMD patients further initiates fibrosis as inflammatory response and accumulation of acidic metabolites occurs which results in deposition of fibronectin, glycosaminoglycans, and proteoglycans in the ECM.[52] Key molecules in this fibrotic response include TGF-β, which activates myofibroblasts, stimulating the excessive production of collagens,[53] and myostatin, which further drives ECM protein build up by encouraging fibroblast proliferation.[54]

  In DMD-affected muscles demonstrate increased stiffness and collagen deposition, particularly in lower limb muscles, as seen in mdx mouse models.[55] Due to the early onset of fibrosis in DMD, therapeutic strategies to limit ECM accumulation have become a focus. Treatments targeting fibrosis—such as those reducing type I collagen—show promise by enhancing utrophin expression and increasing revertant myofibers.[56] Further development of therapies to mitigate fibrosis offers a promising approach to improving muscle function in DMD.

1.5 Different Types of Mutation in DMD Gene

Dystrophin gene is the largest human gene and thus associated with its highest mutation rate, were roughly 1/3rd of the mutations occurs randomly without any prior genetic history and

the remaining 2/3rd of the population is attributed to germline mosaicism or inheritance from carrier mothers.[57] Over 7,000 distinct mutations have been identified in patients with DMD or Becker muscular dystrophy (BMD).[58] Most DMD mutations (around 60%) are deletions, while duplications account for about 11%; the remainder includes smaller mutations that disrupt the coding sequence or splice sites.[58] Mutations predominantly occur in two hotspots within the gene, located at exons 3–9 and 45–55.[59] This high mutation rate and the potential for diverse frameshifts pose significant challenges in developing gene therapies that can address the various mutations in dystrophin. Generally, out-of-frame deletions lead to disrupted reading frames, which results in a lack of functional dystrophin and a severe dystrophic phenotype. In contrast, a milder phenotype of DMD is Becker muscular dystrophy (BMD), is a muscle wasting disorder caused due to mutations in the same gene, yet maintaining the open reading frame and allows the production of an internally truncated, but partially functional dystrophin. BMD presents with a milder, less progressive phenotype, and patients typically have near-normal life expectancies. Rarely, larger genomic rearrangements, such as translocations between an X chromosome and an autosome, can lead to DMD in both male and female patients. In such cases, X-inactivation of the unaffected X chromosome in females prevents dystrophin production, as cells where the mutated X is inactivated are not viable.[60] The mutation spectrum of the dystrophin gene and its affected global population is illustrated in Figure 1.3 (A & B).

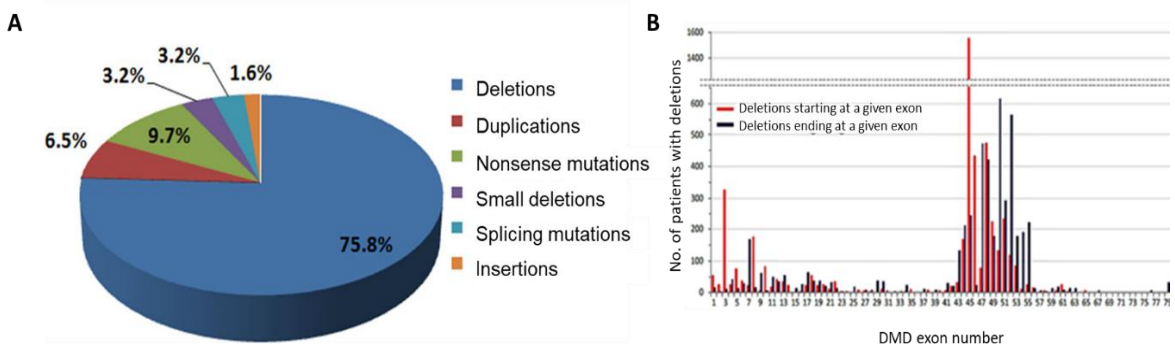


Figure 1.3: Different mutations in DMD gene. A) Mutation spectrum of DMD gene in the cohort. B) Deletion mutations in the DMD gene exhibits two hotspot regions: Proximal (exons 1–22) and distal (exons 43–55).

1.6 Therapeutic Strategies for DMD

Despite significant progress in understanding the underlying mechanisms of DMD and the development of innovative therapeutic strategies targeting its molecular causes, a definitive cure remains elusive. Nevertheless, an integrated approach combining medical, surgical, and rehabilitative interventions, along with long-term corticosteroid therapy and ventilatory support, has significantly influenced the disease's progression and improved life expectancy.(Neuromuscular disorders: NMD 2007;17:470-5). Guidelines for coordinated multidisciplinary care, established by the DMD Care Considerations Working Group, are widely circulated

through international collaborative networks to enhance patient care and quality of life. (Lancet neurology 2010; 9, 177-89)This section provides an overview of current practices and recommendations in the primary areas of DMD medical management.

Treatment with Corticosteroids: In DMD, glucocorticoids such as prednisone and deflazacort are the only medications proven to positively influence muscle function. As glucocorticosteroids, such as prednisone or deflazacort are employed as gold standard care of treatment to manage the secondary pathological mechanisms of DMD. These medications are a basis of DMD management because they help to mitigate some of the disease's downstream effects, including:

- **Reducing Inflammation**: Glucocorticosteroids suppress chronic inflammation caused by muscle degeneration, which is a hallmark of DMD. This helps slow muscle damage and loss.

- **Improving Muscle Strength and Function:** By reducing inflammation, these drugs help improve or maintain muscle strength and delay the progression of motor function decline.

- **Delaying Onset of Respiratory and Cardiac Complications:** Prolonged glucocorticosteroids use has been associated with delayed onset of complications like respiratory failure and cardiomyopathy, which are common secondary pathological issues in DMD.

- **Mitigating Fibrosis:** They can slow the replacement of muscle tissue with fibrotic or fatty tissue by deposition of excessive collagen, preserving muscle structure.

- **Regulating Immune Response:** By modulating the immune system, glucocorticosteroids can prevent overactive immune responses that impair tissue damage.

Although their precise mechanism of action in DMD remains unclear, numerous studies have validated their beneficial effects. Prednisone, administered at a standard dose of 0.75 mg/kg/day, has been shown to enhance muscle strength after six months of treatment.[61] Similarly, deflazacort, given at 0.9 mg/kg/day, provides comparable efficacy and is associated with fewer side effects, including less significant weight gain.[62] Multiple clinical trials support these findings, with evidence indicating that corticosteroid therapy can extend the period of ambulation by 1 to 3 years compared to no treatment.[63]

Corticosteroid therapy is, however, associated with various side effects, including reduced height, weight gain (attributable to fat deposition and increased appetite), osteoporosis, cataract formation, delayed puberty, and behavioural disturbances. These adverse effects necessitate close monitoring.[22] Nutritional management is crucial, with an emphasis on

maintaining bone health through vitamin D supplementation and regular bone density assessments. Current guidelines recommend initiating glucocorticoid therapy between the ages of 2 and 5 years, particularly if a child's strength is in decline. It is then highly recommended to begin treatment in such cases.[22] However, there is no consensus on whether to discontinue corticosteroids following the loss of ambulation. Thus there is an urgent need to replace corticosteroid treatment with alternative therapeutics strategy to address the secondary pathophysiological mechanism associated with the disease progression.

1.6.1. Therapeutic Approaches in Clinical Stage of Development

Deeper understanding of molecular genetics and the disease mechanisms underlying DMD have led to the development of novel therapeutic strategies. Some of these approaches are being evaluated in clinical trials or are nearing the stage of clinical application. Among the most promising strategies targeting the genetic defect responsible for DMD are exon skipping and stop codon mutation suppression. However, these therapies are mutation-specific, restricting their use to DMD patients with particular genetic mutations. In addition to these targeted approaches, other pharmacological strategies hold potential for broader application across the DMD patient population, regardless of genetic profile. These include methods to upregulate utrophin, enhance muscle growth, and address downstream pathophysiological consequences of dystrophin deficiency. Such approaches may not only benefit all DMD patients but could also complement gene-based therapies by providing additional therapeutic effects.

1.6.1.1 Dystrophin Restoration Therapies via Antisense Mediated Exon Skipping Strategy

Exon skipping therapy is based on the principles of the reading frame rule observed in clinical settings. This approach utilizes antisense oligonucleotides (ASOs) designed to bind to exon designation elements within a target exon, effectively inducing its exclusion during pre-mRNA splicing. ASOs are sequence-specific, single-stranded oligonucleotides chemically modified to resist degradation by nucleases. They bind to partially accessible pre-mRNA structures within target exons, disrupting interactions with splicing regulatory factors. Although this process generates an mRNA with a larger deletion, the adjusted sequence is restored to an in-frame configuration, enabling translation through the C-terminus. By targeting specific exons, this technique has the potential to address multiple mutations associated with DMD. For instance, skipping exon 45 could theoretically correct deletions of exons 46–47 and exons 46–48. Targeting a small set of exons, including 44, 45, 51, and 53, could benefit approximately 35% of DMD patients. Moreover, skipping two exons simultaneously could potentially provide therapeutic benefits to as many as 83% of patients.[64, 65]

Two ASO chemistries aimed at skipping exon 51 have been tested in clinical trials involving DMD patients 2′-O-methyl phosphorothioate ASOs and phosphorodiamidate morpholino

oligomers. Initial findings from these trials are promising.[66] In a recent small-scale trial (n = 12), morpholino treatment demonstrated that after 48 weeks, patients who received immediate therapy exhibited a significantly greater improvement in walking distance compared to those initially given a placebo.[66] Furthermore, the absence of notable side effects highlights the potential safety of this approach.

1.6.1.2 Antisense Oligonucleotides (ASOs)

Two main types of ASOs are currently under clinical investigation for DMD: 2′-O-methyl-ribo-oligonucleoside-phosphorothioates (2′OMe) and phosphorodiamidate morpholino oligomers (PMOs). These ASOs differ in backbone chemistry and physicochemical properties. (Figure 1.4)

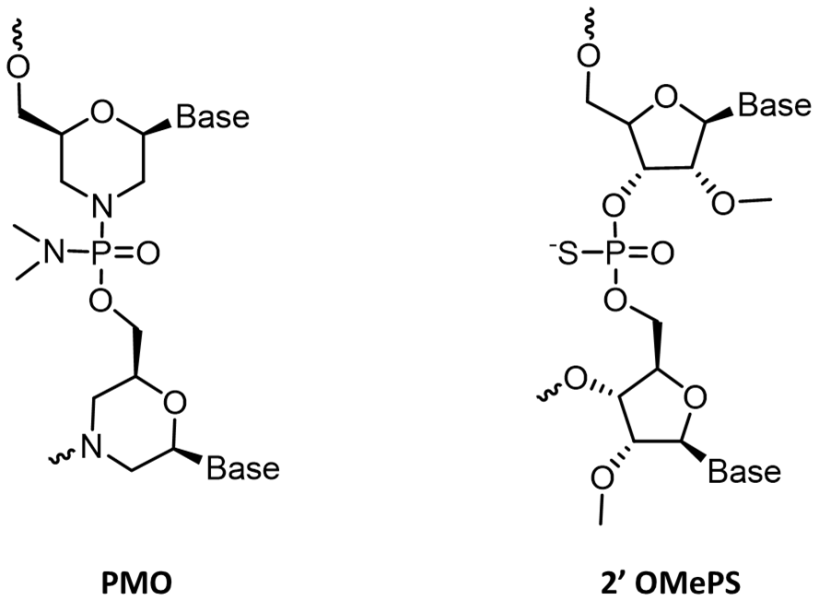


Figure 1.4: Chemical structures of PMO and 2′OMe-PS subunits.

PMOs are uncharged molecules with a morpholino ring in place of the ribose sugar and a phosphorodiamidate linkage instead of the natural phosphodiester bond. This unique backbone makes PMOs highly stable and resistant to nuclease degradation. Whereas, 2'-OMePS are ribose-based oligonucleotides with a methyl group added to the 2'-hydroxyl position of the sugar and a phosphorothioate bond replacing one of the non-bridging oxygen atoms in the phosphate group. The negative charge of the phosphorothioate linkage enhances binding to plasma proteins and extends circulation time. In contrast, the uncharged backbone of PMOs leads to rapid renal clearance and a shorter plasma half-life. Both PMOs and 2'-OMePS have significant utility in exon-skipping therapies, with the choice of chemistry being designed with respect to the therapeutic context.

The widespread application of ASOs is limited by challenges associated with their delivery to target tissues, primarily due to poor bioavailability. Current ASO-based therapies requires weekly dosing to attain and sustain clinical improvement in DMD, thus focusing the need for reduced dosing frequency is a desirable aspect. In this regard two drugs firstly Eteplirsen for Exon 51 and other Golodirsen for Exon 53 are currently approved by FDA. Another peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO) targeting exon 51, developed by Sarepta Therapeutics, was discontinued following Phase II clinical trials. Oligonucleotide-based drugs currently in clinical trials, along with their approval numbers, are presented in Table 1.1.

Drug Name	Description	Company	Delivery Route	Current Stage	Clinical Trial Number
Stop Codon Read-Through					
Ataluren	Nonsense Suppression	PTC Therapeutics	Oral	Phase III	NCT01557400
Exon skipping					
Etiplersen	PMO morpholino targeting exon 51	Sarepta Therapeutics	Intravenous	Phase II Phase II Phase III Phase III	NCT03218995 NCT04179409 NCT03992430 NCT03985878
Golodirsen	PMO morpholino targeting exon 53	Sarepta Therapeutics	Intravenous	Phase II Phase III Phase III	NCT04179409 NCT02500381 NCT03532542
Casimersen	PMO morpholino targeting exon 45	Sarepta Therapeutics	Intravenous	Phase II	NCT04179409
Viltolarsen	PMO morpholino targeting exon 53	Nippon Shinyaku Co. Ltd	Intravenous	Phase II	NCT03167255
Drisapersen	2'-o-Me PS Targeting exon 51	BioMarin Pharmaceutical Inc	Intravenous	Extension	NCT02636686
SRP-5051	PPMO morpholino targeting exon 51	Sarepta Therapeutics	Intravenous	Phase-I Phase-II (Discontinued)	NCT03375255 NCT04004065

Table 1.1: Clinical trials utilizing exon skipping and ston codon read through for DMD therapy in 2023. Image reproduced from Angulski et al., Frontiers in Physiology, 2023 with copyright permission.

1.6.1.3 Cell Penetrating Peptides (CPPs)

To deal with the challenge associated with the bioavaibility of the ASOs as therapeutics, Cell-penetrating peptides CPPs have emerged as one of the most promising delivery strategies among the various methods investigated were a CPPs is linked to the ASO, aiming to enhance cellular uptake, exon skipping, and dystrophin levels.

The successful systemic administration of a drug involves multiple steps, including prolonged circulation, crossing biological barriers, cellular uptake, and endosomal escape into the cytosol following endocytosis. Each of these stages presents specific challenges. Many bioactive molecules face difficulties in reaching their target sites by cell membrane penetration

to exert therapeutic effects. Specifically for oligonucleotides, which have been widely utilized in therapeutic studies, often exhibits low transmembrane efficiency. This limitation makes it challenging to achieve sufficient drug concentrations at the desired site of action. To address this issue, CPPs have emerged as a promising tool. CPPs are short sequences of peptides, generally less than 30 amino acids in length, and have been broadly studied in fundamental and preclinical research over the past few decades. These peptides have potential to translocate into cells independently and enables smooth delivery of therapeutic leads or cargo complexes across the plasma membrane, making them a potential therapeutic tool for targeted drug delivery in disease diagnosis and treatment.A schematic illustration of the cellular uptake mechanisms for CPPs through various pathways is presented in Figure 1.5.

- **Cellular Uptake Mechanisms of CPPs:** CPPs promotes the intracellular delivery of a wide range of cargoes. However, the underlying mechanisms of their cellular uptake remain a topic of discussion. But, there is a growing accord regarding the internalization mechanisms of CPPs and CPP/cargo complexes.[67] These mechanisms are broadly characterised based on whether energy is essential during their internalization process. Specifically, cellular uptake occurs via two primary pathways, firstly direct translocation, which is energy independent process, and endocytosis, which is energy-dependent process.[67]

- **Direct Translocation:** Direct translocation, also known as the non-endocytic uptake pathway, occurs without requirement of energy.[68] This process initiates with electrostatic interactions orelse hydrogen bonding between the phospholipid bilayer and the CPPs or their cargo complexes.[69] These interactions results in entry of CPP/cargoes inside the cellular compartment through pore formation or membrane destabilization.[70] Based on the entry mode, direct translocation can be categorized into three main models which are as follows "Barrel-Stave" model, the "Carpet-like" model, and the Inverted-Micelle model.

  - In the **Barrel-Stave** model, translocation of CPP cargoes occurs via pore formation. CPPs or CPP/cargoes containing amphipathic units insert into the cellular membrane, with their hydrophobic units interacting with the phospholipids in the membrane and simultaneously their hydrophilic regions interacts with the hydrophilic head groups of the lipids. This creates "barrel-like" pores, augmenting transmembrane movement and results in delivery of the cargoes into the cytoplasm.[67, 71]

  - The **Inverted-Micelle** model involves the invagination of the phospholipid bilayer and the formation of inverted micelles. Charged CPPs interact with the phospholipids present within the cell surface, and thus hydrophobic units of CPPs interacts with the cell membrane to form inverted micelles. These structures supports the transport of CPPs or CPP/cargoes into the cell. This mechanism is mainly applicable for CPPs or CPP/cargoes containing hydrophobic amino acid residues.[72]

- In the **Carpet-like model**, CPPs or CPP/cargoes affix to the surface of the cell membrane via charge interactions, and spread across in a "carpet-like" fashion. The hydrophobic units of the CPPs interact with the hydrophobic core of the membrane. At higher concentrations, these regions flip into the hydrophobic membrane core, and ultimately leads to the membrane disruption, thus allowing entry of CPPs or CPP carrying cargoes.[73]

❖ **Endocytosis:** Apart from direct translocation, CPPs can also enter cells through energy-dependent process known as endocytosis, which is particularly effective for large CPPs.[74] Endocytosis is generally characterised into four main pathways which includes macropinocytosis, caveolin-mediated endocytosis, clathrin-mediated endocytosis, and clathrin- and caveolin-independent endocytosis. These pathways differes in mechanism and specificity.

- **Macropinocytosis**, is a receptor-independent and lipid raft-dependent process, which is commonly used by CPPs carrying large cargos. In this process growth factor initiates and actin stimulation enables vesicle formation, which facilitates CPP/cargo complexes to enter into cells. The process starts with CPP-cargo interaction with cellular membrane proteoglycans, provoking Rac protein activation in the cytoplasm. This results in activation of F-actin organization, which further triggers to contraction of actin filaments and deformation of cellular membrane, creating protrusions that develop into endocytic vesicles, allowing the internalization of CPPs carrying cargoes. [75]

- **Clathrin-mediated endocytosis**, also known as receptor-mediated endocytosis, which involves the specific uptake of extracellular molecules. In this case, CPPs first interacts with membrane receptors, resulting in membrane curvature through binding with epsin proteins. This utilizes clathrin and adaptor proteins such as AP-2, forming clathrin-coated vesicles that supports internalization of the CPP-Cargo complexes. These vesicles subsequently transforms into endosomes in the cytoplasm.[76]

- **Caveolin-mediated endocytosis** operates in a similar fashion as clathrin-mediated pathways but encompasses caveolin proteins. In this case CPPs bind to receptors located in lipid raft regions. Caveolin interacts with cavin-1, which transforms into caveolin-coated vesicles and later matures to endosomes.[77]

- **Clathrin & caveolin-independent endocytosis** is less common and specifically occurs in specialized cells, such as macrophages. In this case, CPP-Cargo complexes are affixed by opsonins and thus bind to Fc receptors on the cell membrane. This interaction promotes actin, which results in vesicle formation and supports internalization of the CPP-Cargo complexes into the cytoplasm.[78]

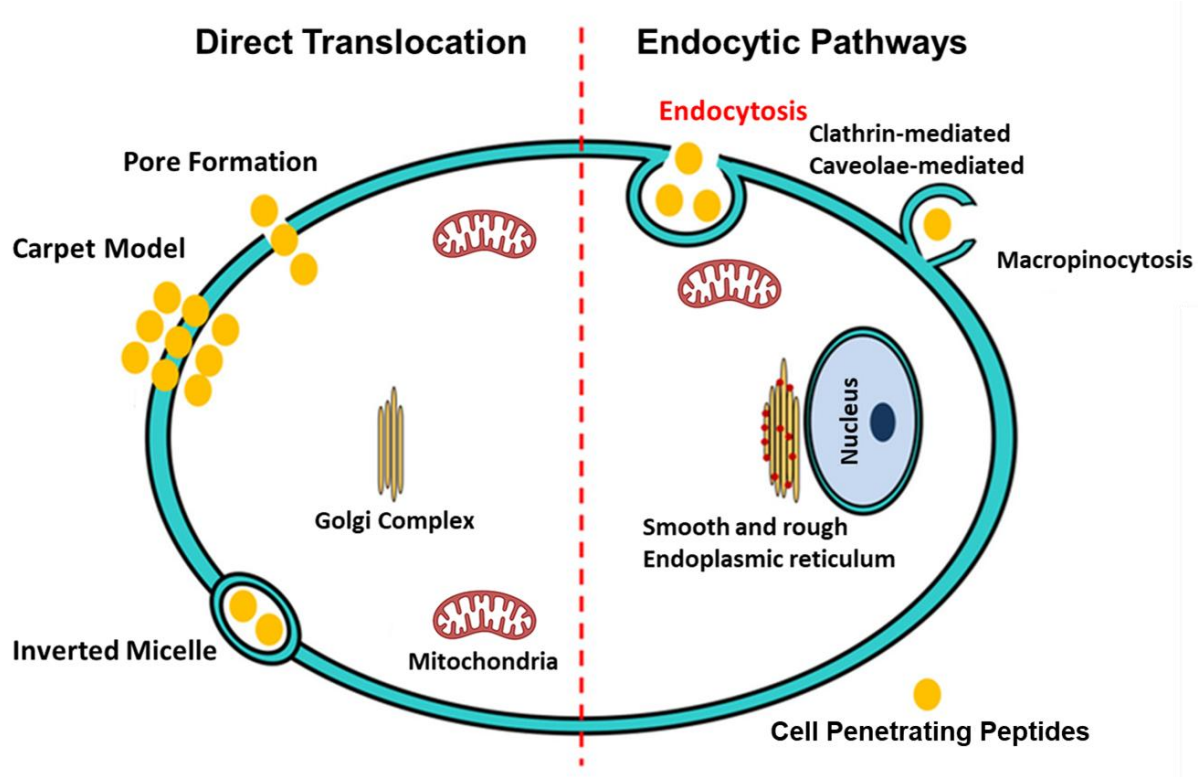


Figure 1.5: Schematic representation of the cellular uptake mechanisms for CPPs or CPP-Cargo complexes. Two main pathways of internalization are illustrated: direct translocation and endocytosis. Image reproduced from Cell-Penetrating Peptides - Creative Peptides Blog.

While endocytosis is the major route for cellular uptake of large CPP-Cargo complexes, these complexes often remain trapped within endosomes, limiting their therapeutic potential. For effective role, CPPs must escape the endosomes to avoid lysosomal degradation. Research indicates that endosomal escape is promoted by mechanisms such as pH gradient formation, vesicle crowding, and electrostatic interactions between CPPs and endosomal membranes which results in membrane destabilization and rupture.[74]

1.6.1.4 CPP Conjugated ASOs

To improve oligonucleotides delivery inside the cell membranes, CPP conjugated oligonucleotides have been developed. This CPP conjugated oligonucleotide construct are synthesized by chemically linking short arginine-rich CPP fragments (Pip peptides) to the 5′ or 3′ end of the oligonucleotides. Compared to unmodified ASOs, CPP conjugated ASOs exhibits enhanced bioavaibility and significantly improved cellular delivery, in particular to

cardiac muscle cells. Among various Pip peptides, Pip5 and Pip6 have shown effective targeting of cardiac myofibers. Despite their advantages, Peptide tagged ASOs exhibit higher toxicity than ASOs due to their high positive charge, which is due to the presence of arginine residues. At high doses in animal studies, peptide conjugated ASOs have caused lethargy, weight loss, and renal toxicity. Recent developments in Pip design (series 7, 8, and 9) have reduced the number of arginine residues (from 10 to 6–9), significantly decreasing toxicity, but simultaneously this modification reduced splice-switching efficiency.[79]

CPP conjugated ASO remain a promising approach to overcoming delivery challenges associated with traditional ASOs. For example, Sarepta Therapeutics has reengineered eteplirsen as a Peptide conjugated ASO (SRP-5051) containing two arginine-rich domains separated by a hydrophobic core. Therefore, the development of a novel CPP with minimal cytotoxicity, efficient cellular internalization, and precise nuclear localization is critically needed to advance oligonucleotide-based therapeutics for DMD.

1.6.1.5 Utrophin Modulation

Utrophin, is a large cytoskeletal protein (395 kDa) structurally and functionally analogous to dystrophin.[80] During early human development, utrophin is abundantly expressed at the sarcolemma of developing muscle, compensating for the absence or low levels of dystrophin. However, as muscle fibers mature, utrophin expression declines and becomes localized primarily at the neuromuscular junction.[80, 81] A schematic representation of the transition from utrophin to dystrophin protein during human development in the mother's womb is shown in Figure 1.6. In patients with DMD or Becker Muscular Dystrophy (BMD), utrophin levels are upregulated at the sarcolemma as a compensatory response to dystrophin deficiency.[82, 83] Preclinical studies have demonstrated that increased utrophin levels can mitigate dystrophin-deficient muscle pathology. Transgenic mdx mice shows elevated utrophin expression and reduced muscle damage, with the extent of protection directly related with utrophin expression levels.[84] Utrophin pay off for dystrophin deficiency by stabilizing the sarcolemma and providing muscle protection against membrane instability.[85] Utrophin, being an endogenous protein, is less likely to trigger an immune response, and thereby offering an advantageous position in its therapeutic applications.[86]

Strategies to elevate utrophin levels include direct gene or protein replacement and indirect transcriptional upregulation. Utrophin is transcribed from two promoters, A and B, were promoter A found active in muscle tissue and promoter B in endothelial cells.[87] Pharmacological stimulus of promoter A has been an attention of drug development. Direct protein replacement using recombinant utrophin variants, have proven potential in animal models but requires further development.[88] Gene therapy approaches utilizing micro-utrophin constructs have also shown success in preclinical studies, thereby improving

sarcolemma stability and muscle function in dystrophic models.[89] High-throughput screening identified several small molecules, such as heregulin, nabumetone, L-arginine, and okadaic acid that enhance utrophin expression in preclinical studies.[90] Ezutromid (SMTC1100), the first orally bioavailable utrophin regulator, showed encouraging preclinical results but failed to clear Phase 2 clinical trial.[85, 91]

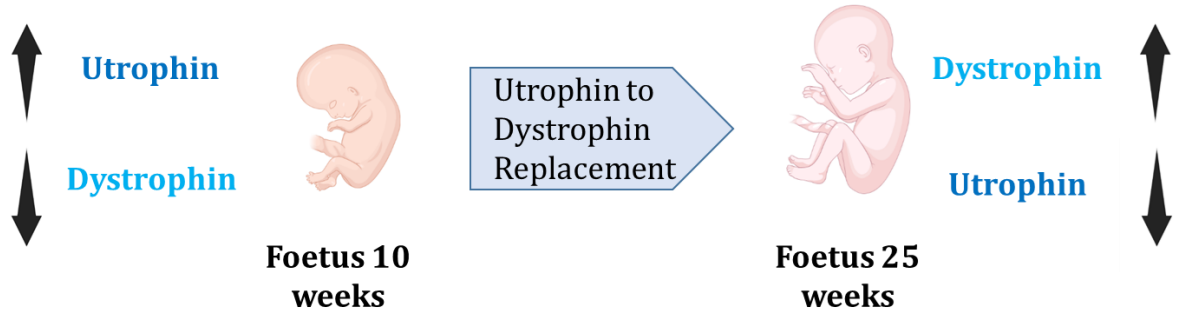


Figure 1.6: Replacement of utrophin with dystrophin during early human development, from 10 to 25 weeks of fetal development.

These findings highlight the therapeutic potential of utrophin upregulation. Thus there is a dire need for new utrophin modulator which could be beneficial for entire DMD population irrespective of the type of mutation.

1.7 Research Objectives

In this thesis I like to focus on addressing the current limitations of existing therapeutics in treating Duchenne Muscular Dystrophy. Multimodal approach has been executed to find a cure for these devastating rare genetic disorder. Out of several therapeutic strategy I focused on four major objectives to overcome the current limitations. Overall, my research endeavours provides a multifaceted therapeutic approach aimed at addressing the needs of the broad DMD population and providing cost-effective supportive care. My doctoral studies offers a comprehensive framework towards transformative DMD management strategies. Key objectives of this approach are as follows:

a) To design and develop novel Quinazoline and Quinoline based small molecules to enhance utrophin expression via AhR antagonism to treat global DMD population.

b) To design and develop novel short non-cationic cell penetrating peptide and its further conjugation to oligonucleotides employing click chemistry for advancing delivery of antisense oligonucleotides precisely to the nucleus in the pursuit of DMD.

c) To design and develop novel mitochondrial targeted peptide based therapeutics for the treatment of DMD pathophysiology via enhancing myogenesis and mitigating fibrosis.

1.8 Thesis Overlay

This thesis is structured into four chapters. Chapter I provides an introduction and background study, laying the foundation for the research. From Chapter II onward, each chapter is designed to present a detailed account of the findings addressing the specific objectives of the study. Each chapter critically examines existing limitations in the field and highlights the advancements achieved through this research. Chapter II focuses on the discovery of novel utrophin modulators aimed at addressing the global DMD population, regardless of mutation type. Chapter III highlights the development of a novel non-cationic cell-penetrating peptide for the efficient delivery of oligonucleotides precisely to the nuclear compartment, representing a significant advancement in oligonucleotide-based gene delivery therapeutics.

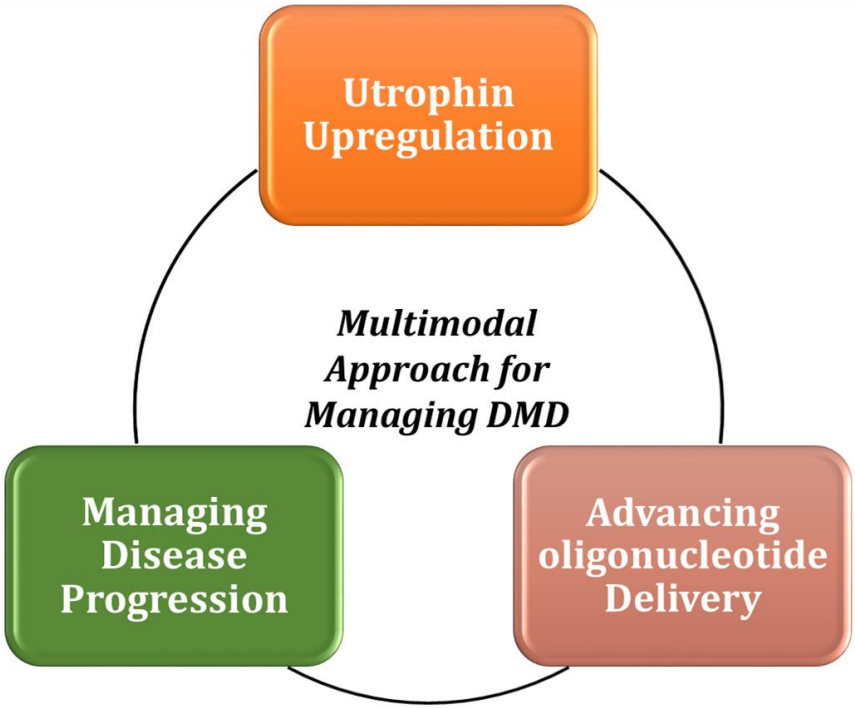


Figure 1.7: Research Overlay illustrating multifaceted approach to find cure for Duchenne Muscular Dystrophy.

Finally, Chapter IV addresses the limitations of the current gold-standard treatment for DMD, particularly the adverse side effects associated with prolonged corticosteroid use. As an alternative, it proposes peptide-based therapeutics as a promising strategy to address critical hallmarks of DMD pathogenesis, mainly impaired myogenesis, excessive fibrosis, and calcium dysregulation through a single therapeutic platform. Collectively, this doctoral thesis adopts a multifaceted approach to find an affordable cure for DMD management.

1.9 Research Contribution

Through this doctoral research, I believe that my research contribution towards the advancement of the field in discovery of therapeutics solutions for fatal genetic discover DMD will significantly pave the way for future. In this doctoral thesis I worked on multifaceted approach to find out the cure for DMD.

- **Firstly,** my research focused on addressing the critical gap in the therapeutic landscape for utrophin upregulation, where no approved drug currently exists. I successfully identified therapeutic lead 'SG-02' as potent utrophin upregulator, entirely distinct from the previously discovered 'Ezutromid' family, which later failed from clinical trial phase 2.

- **Secondly,** I worked in the advancement of the oligonucleotides delivery, addressing a current limitation in exon-skipping strategies. To overcome these limitations, I designed a novel short, non-cationic CPP and further conjugated it with ASO. This newly build CPP-ASO construct serves as an ideal candidate possessing all the necessary characteristics for effective delivery of ASOs.

- **Thirdly,** my research area addressed the secondary pathophysiological conditions of DMD, aiming to reduce reliance on corticosteroids, which remain the current gold standard of care despite their long-term side effects. To achieve this, I designed a novel mitochondrial-targeted peptide that offers a promising alternative for managing disease progression. This innovative approach has the potential to redefine the standard of care for DMD.

1.10 Discussion and Future Prospect

From this research work, multiple future prospects have opened. This work will definitely pave a path for future DMD drug discovery and will make it footprint in the DMD drug discovery journey.

From **chapter II** outcome 'SG-02' the lead candidate identified from the study has presented itself as an efficient oral bioavailable drug candidate to significantly upregulate utrophin at nanomolar doses. Therefore, in future 'SG-02' holds potential as an utrophin modulator drug candidate for the treatment of the broader DMD population. Similarly newly designed CPP-ASO construct from **chapter III** construct hold potential for efficient delivery of ASOs and become a milestone in targeted gene therapy, not only for DMD but also for a broader spectrum of genetic disorders. The mitochondrial-targeted peptide introduced in **Chapter IV** represents a transformative approach to managing the secondary pathophysiological conditions of DMD. By reducing reliance on corticosteroids, this strategy could emerge as a future gold standard in DMD care, providing a safer and more effective alternative for disease progression management.

1.11 References

1. Hoffman, E. P., Brown, R. H., Jr., Kunkel, L. M., Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell* **1987,** *51* (6), 919-28.
2. Cohn, R. D., Campbell, K. P., Molecular basis of muscular dystrophies. *Muscle & nerve,* **2000,** *23* (10), 1456-71.
3. Emery, A. E.; Duchenne muscular dystrophy-Meryon's disease. *NMD,* **1993,** *3* (4), 263-6.
4. Svegliati-Baroni, G., Ridolfi, F., Di Sario, A., Saccomanno, S., Bendia, E., Benedetti, A., Greenwel, P., Intracellular signaling pathways involved in acetaldehyde-induced collagen and fibronectin gene expression in human hepatic stellate cells. *Hepatology (Baltimore, Md.)* **2001,** *33* (5), 1130-40.
5. Hopf, F. W., Turner, P. R., Denetclaw, W. F., Jr., Reddy, P., Steinhardt, R. A., A critical evaluation of resting intracellular free calcium regulation in dystrophic mdx muscle. *Am. J. Physiol. Cell Physiol.,* **1996,** *271* (4 Pt 1), C1325-39.
6. Grounds, M. D., Terrill, J. R., Al-Mshhdani, B. A., Duong, M. N., Radley-Crabb, H. G., Arthur, P. G., Biomarkers for Duchenne muscular dystrophy: myonecrosis, inflammation and oxidative stress. *DMM,* **2020,** *13* (2).
7. Ervasti, J. M., Campbell, K. P., Membrane organization of the dystrophin-glycoprotein complex. *Cell,* **1991,** *66* (6), 1121-31.
8. Petrof, B. J., Shrager, J. B., Stedman, H. H., Kelly, A. M., Sweeney, H. L., Dystrophin protects the sarcolemma from stresses developed during muscle contraction. *PNAS,* **1993,** *90* (8), 3710-3714.
9. Koenig, M., Monaco, A. P., Kunkel, L. M., The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell,* **1988,** *53* (2), 219-28.
10. Ahn, A. H.; Kunkel, L. M., The structural and functional diversity of dystrophin. *Nat. Genet.,* **1993,** *3* (4), 283-91.

11. Campbell, K. P., Kahl, S. D., Association of dystrophin and an integral membrane glycoprotein. *Nature,* **1989,** *338* (6212), 259-62.
12. Bez Batti Angulski, A., Hosny, N., Cohen, H., Martin, A. A., Hahn, D., Bauer, J., Metzger, J. M., Duchenne muscular dystrophy: disease mechanism and therapeutic strategies. *Front. Physiol.,* **2023,** *14*, 1183101.
13. Guiraud, S., Aartsma-Rus, A., Vieira, N. M., Davies, K. E., van Ommen, G. J., Kunkel, L. M., The Pathogenesis and Therapy of Muscular Dystrophies. *Annu. Rev. Genomics Hum. Genet.*, **2015,** *16*, 281-308.
14. Aartsma-Rus, A., Hegde, M., Ben-Omran, T., Buccella, F., Ferlini, A., Gallano, P., Howell, R. R., Leturcq, F., Martin, A. S., Potulska-Chromik, A., Saute, J. A., Schmidt, W. M., Sejersen, T., Tuffery-Giraud, S., Uyguner, Z. O., Witcomb, L. A., Yau, S., Nelson, S. F., Evidence-Based Consensus and Systematic Review on Reducing the Time to Diagnosis of Duchenne Muscular Dystrophy. *J Pediatr.*, **2019,** *204*, 305-313.e14.
15. Ciafaloni, E., Fox, D. J., Pandya, S., Westfield, C. P., Puzhankara, S., Romitti, P. A., Mathews, K. D., Miller, T. M., Matthews, D. J., Miller, L. A., Cunniff, C., Druschel, C. M., Moxley, R. T., Delayed diagnosis in duchenne muscular dystrophy: data from the Muscular Dystrophy Surveillance, Tracking, and Research Network (MD STARnet). *J Pediatr.*, **2009,** *155* (3), 380-5.
16. Cyrulnik, S. E., Fee, R. J., Batchelder, A., Kiefel, J., Goldstein, E., Hinton, V. J., Cognitive and adaptive deficits in young children with Duchenne muscular dystrophy (DMD). *J Int Neuropsychol Soc.,* **2008,** *14* (5), 853-61.
17. Cotton, S. M., Voudouris, N. J., Greenwood, K. M., Association between intellectual functioning and age in children and young adults with Duchenne muscular dystrophy: further results from a meta-analysis. *Dev. Med. Child Neurol.,* **2005,** *47* (4), 257-265.
18. Hendriksen, J. G.; Vles, J. S., Neuropsychiatric disorders in males with duchenne muscular dystrophy: frequency rate of attention-deficit hyperactivity disorder (ADHD), autism spectrum disorder, and obsessive--compulsive disorder. *JCN.,* **2008,** *23* (5), 477-81.
19. Abbs, S.; Tuffery-Giraud, S.; Bakker, E.; Ferlini, A.; Sejersen, T.; Mueller, C. R., Best practice guidelines on molecular diagnostics in Duchenne/Becker muscular dystrophies. *NMD,* **2010,** *20* (6), 422-7.
20. Emery, A. E. H., The muscular dystrophies. *The Lancet* **2002,** *359* (9307), 687-695.
21. Tsuda, T., Clinical Manifestations and Overall Management Strategies for Duchenne Muscular Dystrophy. *Methods Mol Biol. (Clifton, N.J.)* **2018,** *1687*, 19-28.
22. Bushby, K., Finkel, R., Birnkrant, D. J., Case, L. E., Clemens, P. R., Cripe, L., Kaul, A., Kinnett, K., McDonald, C., Pandya, S., Poysky, J., Shapiro, F., Tomezsko, J., Constantin, C., Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and pharmacological and psychosocial management. *Lancet Neurol.*, **2010,** *9* (1), 77-93.
23. Mendell, J. R., Shilling, C., Leslie, N. D., Flanigan, K. M., al-Dahhak, R., Gastier-Foster, J., Kneile, K., Dunn, D. M., Duval, B., Aoyagi, A., Hamil, C., Mahmoud, M., Roush, K., Bird, L., Rankin, C., Lilly, H., Street, N., Chandrasekar, R., Weiss, R. B., Evidence-based path to newborn screening for Duchenne muscular dystrophy. *Ann. Neurol.*, **2012,** *71* (3), 304-13.
24. Mercuri, E.; Bönnemann, C. G.; Muntoni, F., Muscular dystrophies. *Lancet.,* **2019,** *394* (10213), 2025-2038.
25. Houang, E. M.; Sham, Y. Y.; Bates, F. S.; Metzger, J. M., Muscle membrane integrity in Duchenne muscular dystrophy: recent advances in copolymer-based muscle membrane stabilizers. *Skelet Muscle* **2018,** *8* (1), 31.

26. Mokri, B., Engel, A. G., Duchenne dystrophy: electron microscopic findings pointing to a basic or early abnormality in the plasma membrane of the muscle fiber. *Neurol.,* **1975,** *25* (12), 1111-20.
27. Duan, D., Goemans, N., Takeda, S. i., Mercuri, E., Aartsma-Rus, A., Duchenne muscular dystrophy. *Nat Rev Dis Primers.*, **2021,** *7* (1), 13.
28. Mareedu, S., Million, E. D., Duan, D., Babu, G. J., Abnormal Calcium Handling in Duchenne Muscular Dystrophy: Mechanisms and Potential Therapies. *Front. Physiol.*, **2021,** *12*, 647010.
29. Pertille, A., de Carvalho, C. L., Matsumura, C. Y., Neto, H. S., Marques, M. J., Calcium-binding proteins in skeletal muscles of the mdx mice: potential role in the pathogenesis of Duchenne muscular dystrophy. *Int. J. Exp. Pathol.*, **2010,** *91* (1), 63-71.
30. Gailly, P., New aspects of calcium signaling in skeletal muscle cells: implications in Duchenne muscular dystrophy. *BBA.,* **2002,** *1600* (1-2), 38-44.
31. Bansal, D., Miyake, K., Vogel, S. S., Groh, S., Chen, C. C., Williamson, R., McNeil, P. L., Campbell, K. P., Defective membrane repair in dysferlin-deficient muscular dystrophy. *Nat.,* **2003,** *423* (6936), 168-72.
32. Kyrychenko, V., Kyrychenko, S., Tiburcy, M., Shelton, J. M., Long, C., Schneider, J. W., Zimmermann, W. H., Bassel-Duby, R., Olson, E. N., Functional correction of dystrophin actin binding domain mutations by genome editing. *JCI insight* **2017,** *2* (18).
33. Rosenberg, A. S.; Puig, M.; Nagaraju, K.; Hoffman, E. P.; Villalta, S. A.; Rao, V. A.; Wakefield, L. M.; Woodcock, J., Immune-mediated pathology in Duchenne muscular dystrophy. *Sci. Transl. Med.,* **2015,** *7* (299), 299rv4.
34. Pescatori, M., Broccolini, A., Minetti, C., Bertini, E., Bruno, C., D'Amico, A., Bernardini, C., Mirabella, M., Silvestri, G., Giglio, V., Modoni, A., Pedemonte, M., Tasca, G., Galluzzi, G., Mercuri, E., Tonali, P. A., Ricci, E., Gene expression profiling in the early phases of DMD: a constant molecular signature characterizes DMD muscle from early postnatal life throughout disease progression. *FASEB J.,* **2007,** *21* (4), 1210-26.
35. Vidal, B., Serrano, A. L., Tjwa, M., Suelves, M., Ardite, E., De Mori, R., Baeza-Raja, B., Martínez de Lagrán, M., Lafuste, P., Ruiz-Bonilla, V., Jardí, M., Gherardi, R., Christov, C., Dierssen, M., Carmeliet, P., Degen, J. L., Dewerchin, M., Muñoz-Cánoves, P., Fibrinogen drives dystrophic muscle fibrosis via a TGFbeta/alternative macrophage activation pathway. *Genes Dev.*, **2008,** *22* (13), 1747-52.
36. Mojumdar, K.; Liang, F.; Giordano, C.; Lemaire, C.; Danialou, G.; Okazaki, T.; Bourdon, J.; Rafei, M.; Galipeau, J.; Divangahi, M.; Petrof, B. J., Inflammatory monocytes promote progression of Duchenne muscular dystrophy and can be therapeutically targeted via CCR2. *EMBO Mol. Med.,* **2014,** *6* (11), 1476-92.
37. Evans, N. P., Misyak, S. A., Robertson, J. L., Bassaganya-Riera, J., Grange, R. W., Immune-mediated mechanisms potentially regulate the disease time-course of duchenne muscular dystrophy and provide targets for therapeutic intervention. *PM & R : the journal of injury, function, and rehabilitation* **2009,** *1* (8), 755-68.
38. Lozanoska-Ochser, B., Benedetti, A., Rizzo, G., Marrocco, V., Di Maggio, R., Fiore, P., Bouche, M., Targeting early PKCθ-dependent T-cell infiltration of dystrophic muscle reduces disease severity in a mouse model of muscular dystrophy. *J. Pathol.* **2018,** *244* (3), 323-333.
39. Ahmad, I., Goel, D., Ghosh, A., Kapoor, H., Kumar, D., Ramesh, K, Ashley, B., Deepika, K., Shastry, A., Faruq, M., Generation of two induced pluripotent stem cell (iPSC) lines from

patients with Duchenne muscular dystrophy (IGIBi006-A and IGIBi008-A) carrying exonic deletions in the dystrophin gene. *Stem Cell Res.,* **2022,** *64*, 102927.
40. Cole, M. A., Rafael, J. A., Taylor, D. J., Lodi, R., Davies, K. E., Styles, P., A quantitative study of bioenergetics in skeletal muscle lacking utrophin and dystrophin. *NMD.,* **2002,** *12* (3), 247-257.
41. García-Castañeda, M., Michelucci, A., Zhao, N., Malik, S., Dirksen, R. T., Postdevelopmental knockout of Orai1 improves muscle pathology in a mouse model of Duchenne muscular dystrophy. *JGP.,* **2022,** *154* (9).
42. Percival, J. M.; Siegel, M. P.; Knowels, G.; Marcinek, D. J., Defects in mitochondrial localization and ATP synthesis in the mdx mouse model of Duchenne muscular dystrophy are not alleviated by PDE5 inhibition. *Hum. Mol. Genet.* **2013,** *22* (1), 153-67.
43. Townsend, E. L.; Bibeau, C.; Holmes, T. M., Supported Standing in Boys With Duchenne Muscular Dystrophy. *APTA.,* **2016,** *28* (3), 320-9.
44. Powers, S. K.; Nelson, W. B.; Hudson, M. B., Exercise-induced oxidative stress in humans: cause and consequences. *Free Radic. Biol. Med.* **2011,** *51* (5), 942-50.
45. Fridovich, I., Fundamental aspects of reactive oxygen species, or what's the matter with oxygen? *Ann. N. Y. Acad. Sci.*, **1999,** *893*, 13-8.
46. Powers, S. K.; Ji, L. L.; Kavazis, A. N.; Jackson, M. J., Reactive oxygen species: impact on skeletal muscle. *Compr. Physiol.,* **2011,** *1* (2), 941-69.
47. Hughes, M. C.; Ramos, S. V.; Turnbull, P. C.; Rebalka, I. A.; Cao, A.; Monaco, C. M. F.; Varah, N. E.; Edgett, B. A.; Huber, J. S.; Tadi, P.; Delfinis, L. J.; Schlattner, U.; Simpson, J. A.; Hawke, T. J.; Perry, C. G. R., Early myopathy in Duchenne muscular dystrophy is associated with elevated mitochondrial H(2) O(2) emission during impaired oxidative phosphorylation. *JCSM.,* **2019,** *10* (3), 643-661.
48. Terrill, J. R., Duong, M. N., Turner, R., Le Guiner, C., Boyatzis, A., Kettle, A. J., Grounds, M. D., Arthur, P. G., Levels of inflammation and oxidative stress, and a role for taurine in dystropathology of the Golden Retriever Muscular Dystrophy dog model for Duchenne Muscular Dystrophy. *Redox Biol.,* **2016,** *9*, 276-286.
49. Khairallah, R. J., Shi, G., Sbrana, F., Prosser, B. L., Borroto, C., Mazaitis, M. J., Hoffman, E. P., Mahurkar, A., Sachs, F., Sun, Y., Chen, Y. W., Raiteri, R., Lederer, W. J., Dorsey, S. G.; Ward, C. W., Microtubules underlie dysfunction in duchenne muscular dystrophy. *Sci. Signal.,* **2012,** *5* (236), ra56.
50. Lieber, R. L., Ward, S. R., Cellular mechanisms of tissue fibrosis. 4. Structural and functional consequences of skeletal muscle fibrosis. *Am. J. Physiol. Cell Physiol.*, **2013,** *305* (3), C241-52.
51. Kharraz, Y., Guerra, J., Pessina, P., Serrano, A. L., Muñoz-Cánoves, P., Understanding the process of fibrosis in Duchenne muscular dystrophy. Biomed Res. Int., **2014,** *2014*, 965631.
52. Klingler, W., Jurkat-Rott, K., Lehmann-Horn, F., Schleip, R., The role of fibrosis in Duchenne muscular dystrophy. *Acta Myol.*, **2012,** *31* (3), 184-95.
53. Meng, X. M.; Nikolic-Paterson, D. J.; Lan, H. Y., TGF-β: the master regulator of fibrosis. Nat. Rev. Nephrol., **2016,** *12* (6), 325-38.
54. Li, B., Wang, J. H., Fibroblasts and myofibroblasts in wound healing: force generation and measurement. *J. Tissue Viability,* **2011,** *20* (4), 108-20.
55. Lacourpaille, L., Gross, R, Hug, F., Guével, A., Péréon, Y., Magot, A., Hogrel, J. Y., Nordez, A., Effects of Duchenne muscular dystrophy on muscle stiffness and response to electrically-induced muscle contraction: A 12-month follow-up. *NMD,* **2017,** *27* (3), 214-220.

56. Levi, O., Genin, O., Angelini, C., Halevy, O., Pines, M., Inhibition of muscle fibrosis results in increases in both utrophin levels and the number of revertant myofibers in Duchenne muscular dystrophy. *Oncotarget.,* **2015,** *6* (27), 23249-60.
57. Nallamilli, B. R. R., Chaubey, A., Valencia, C. A., Stansberry, L., Behlmann, A. M., Ma, Z., Mathur, A., Shenoy, S., Ganapathy, V., Jagannathan, L., Ramachander, V., Ferlini, A., Bean, L., Hegde, M., A single NGS-based assay covering the entire genomic sequence of the DMD gene facilitates diagnostic and newborn screening confirmatory testing. *Hum. Mutat.*, **2021,** *42* (5), 626-638.
58. Bladen, C. L., *et al.*, The TREAT-NMD DMD Global Database: analysis of more than 7,000 Duchenne muscular dystrophy mutations. *Hum. Mutat.,* **2015,** *36* (4), 395-402.
59. Min, Y. L., Bassel-Duby, R., Olson, E. N., CRISPR Correction of Duchenne Muscular Dystrophy. *Annu. Rev. Med.*, **2019,** *70*, 239-255.
60. Monaco, A. P.; Neve, R. L.; Colletti-Feener, C.; Bertelson, C. J.; Kurnit, D. M.; Kunkel, L. M., Isolation of candidate cDNAs for portions of the Duchenne muscular dystrophy gene. *Nat.,* **1986,** *323* (6089), 646-50.
61. Mendell, J. R.; Moxley, R. T.; Griggs, R. C.; Brooke, M. H.; Fenichel, G. M.; Miller, J. P.; King, W.; Signore, L.; Pandya, S.; Florence, J.; et al., Randomized, double-blind six-month trial of prednisone in Duchenne's muscular dystrophy. *NEJM.,* **1989,** *320* (24), 1592-7.
62. Bonifati, M. D., Ruzza, G., Bonometto, P., Berardinelli, A., Gorni, K., Orcesi, S., Lanzi, G., Angelini, C., A multicenter, double-blind, randomized trial of deflazacort versus prednisone in Duchenne muscular dystrophy. *Muscle & nerve.,* **2000,** *23* (9), 1344-7.
63. Escolar, D. M., Hache, L. P., Clemens, P. R., Cnaan, A., McDonald, C. M., Viswanathan, V., Kornberg, A. J., Bertorini, T. E., Nevo, Y., Lotze, T., Pestronk, A., Ryan, M. M., Monasterio, E., Day, J. W., Zimmerman, A., Arrieta, A., Henricson, E., Mayhew, J., Florence, J., Hu, F., Connolly, A. M., Randomized, blinded trial of weekend vs daily prednisone in Duchenne muscular dystrophy. *Neurol.,* **2011,** *77* (5), 444-52.
64. Aartsma-Rus, A., Janson, A. A., Kaman, W. E., Bremmer-Bout, M., den Dunnen, J. T., Baas, F., van Ommen, G. J., van Deutekom, J. C., Therapeutic antisense-induced exon skipping in cultured muscle cells from six different DMD patients. *Hum. Mol. Genet.,* **2003,** *12* (8), 907-14.
65. Aartsma-Rus, A., Antisense-mediated modulation of splicing: therapeutic implications for Duchenne muscular dystrophy. *RNA Biol.,* **2010,** *7* (4), 453-61.
66. Cirak, S., Arechavala-Gomeza, V., Guglieri, M., Feng, L., Torelli, S., Anthony, K., Abbs, S., Garralda, M. E., Bourke, J., Wells, D. J., Dickson, G., Wood, M. J., Wilton, S. D., Straub, V., Kole, R., Shrewsbury, S. B., Sewry, C., Morgan, J. E., Bushby, K., Muntoni, F., Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study. *Lancet,* **2011,** *378* (9791), 595-605.
67. Kauffman, W. B., Fuselier, T., He, J., Wimley, W. C., Mechanism Matters: A Taxonomy of Cell Penetrating Peptides. *Trends Biochem. Sci.* **2015,** *40* (12), 749-764.
68. Reissmann, S., Cell penetration: scope and limitations by the application of cell-penetrating peptides. *J. Pept. Sci.,* **2014,** *20* (10), 760-84.
69. Pescina, S., Ostacolo, C., Gomez-Monterrey, I. M., Sala, M., Bertamino, A., Sonvico, F., Padula, C., Santi, P., Bianchera, A., Nicoli, S., Cell penetrating peptides in ocular drug delivery: State of the art. *JCR.,* **2018,** *284*, 84-102.

70. Guidotti, G., Brambilla, L., Rossi, D., Cell-Penetrating Peptides: From Basic Research to Clinics. *TIPS,* **2017,** *38* (4), 406-424.
71. Habault, J.; Poyet, J. L., Recent Advances in Cell Penetrating Peptide-Based Anticancer Therapies. *Molecules (Basel, Switzerland)* **2019,** *24* (5).
72. Xie, J.; Bi, Y.; Zhang, H.; Dong, S.; Teng, L.; Lee, R. J.; Yang, Z., Cell-Penetrating Peptides in Diagnosis and Treatment of Human Diseases: From Preclinical Research to Clinical Application. *Frontiers in pharmacology* **2020,** *11*, 697.
73. Palm-Apergi, C.; Lönn, P.; Dowdy, S. F., Do cell-penetrating peptides actually "penetrate" cellular membranes? *Molecular therapy : the journal of the American Society of Gene Therapy* **2012,** *20* (4), 695-7.
74. Borrelli, A.; Tornesello, A. L.; Tornesello, M. L.; Buonaguro, F. M. Cell Penetrating Peptides as Molecular Carriers for Anti-Cancer Agents *Molecules (Basel, Switzerland)* [Online], 2018.
75. Xu, J.; Wang, F.; Ye, L.; Wang, R.; Zhao, L.; Yang, X.; Ji, J.; Liu, A.; Zhai, G., Penetrating peptides: Applications in drug delivery. *Journal of Drug Delivery Science and Technology* **2023,** *84*, 104475.
76. Richard, J. P.; Melikov, K.; Vives, E.; Ramos, C.; Verbeure, B.; Gait, M. J.; Chernomordik, L. V.; Lebleu, B., Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *The Journal of biological chemistry* **2003,** *278* (1), 585-90.
77. Zhao, X.-L.; Chen, B.-C.; Han, J.-C.; Wei, L.; Pan, X.-B., Delivery of cell-penetrating peptide-peptide nucleic acid conjugates by assembly on an oligonucleotide scaffold. *Scientific Reports* **2015,** *5* (1), 17640.
78. Ruseska, I.; Zimmer, A., Internalization mechanisms of cell-penetrating peptides. *Beilstein Journal of Nanotechnology* **2020,** *11*, 101-123.
79. Moulton, H. M.; Moulton, J. D., Morpholinos and their peptide conjugates: Therapeutic promise and challenge for Duchenne muscular dystrophy. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2010,** *1798* (12), 2296-2303.
80. Love, D. R.; Hill, D. F.; Dickson, G.; Spurr, N. K.; Byth, B. C.; Marsden, R. F.; Walsh, F. S.; Edwards, Y. H.; Davies, K. E., An autosomal transcript in skeletal muscle with homology to dystrophin. *Nature* **1989,** *339* (6219), 55-8.
81. McNally, E. M.; Kaltman, J. R.; Benson, D. W.; Canter, C. E.; Cripe, L. H.; Duan, D.; Finder, J. D.; Groh, W. J.; Hoffman, E. P.; Judge, D. P.; Kertesz, N.; Kinnett, K.; Kirsch, R.; Metzger, J. M.; Pearson, G. D.; Rafael-Fortney, J. A.; Raman, S. V.; Spurney, C. F.; Targum, S. L.; Wagner, K. R.; Markham, L. W., Contemporary cardiac issues in Duchenne muscular dystrophy. Working Group of the National Heart, Lung, and Blood Institute in collaboration with Parent Project Muscular Dystrophy. *Circulation* **2015,** *131* (18), 1590-8.
82. Blake, D. J.; Weir, A.; Newey, S. E.; Davies, K. E., Function and genetics of dystrophin and dystrophin-related proteins in muscle. *Physiological reviews* **2002,** *82* (2), 291-329.
83. Blake, D. J.; Tinsley, J. M.; Davies, K. E., Utrophin: a structural and functional comparison to dystrophin. *Brain pathology (Zurich, Switzerland)* **1996,** *6* (1), 37-47.
84. Tinsley, J.; Deconinck, N.; Fisher, R.; Kahn, D.; Phelps, S.; Gillis, J. M.; Davies, K., Expression of full-length utrophin prevents muscular dystrophy in mdx mice. *Nature medicine* **1998,** *4* (12), 1441-4.
85. Soblechero-Martín, P.; López-Martínez, A.; de la Puente-Ovejero, L.; Vallejo-Illarramendi, A.; Arechavala-Gomeza, V., Utrophin modulator drugs as potential therapies for Duchenne and Becker muscular dystrophies. *Neuropathology and applied neurobiology* **2021,** *47* (6), 711-723.

86. Mendell, J. R.; Campbell, K.; Rodino-Klapac, L.; Sahenk, Z.; Shilling, C.; Lewis, S.; Bowles, D.; Gray, S.; Li, C.; Galloway, G.; Malik, V.; Coley, B.; Clark, K. R.; Li, J.; Xiao, X.; Samulski, J.; McPhee, S. W.; Samulski, R. J.; Walker, C. M., Dystrophin immunity in Duchenne's muscular dystrophy. *The New England journal of medicine* **2010,** *363* (15), 1429-37.
87. Burton, E. A.; Tinsley, J. M.; Holzfeind, P. J.; Rodrigues, N. R.; Davies, K. E., A second promoter provides an alternative target for therapeutic up-regulation of utrophin in Duchenne muscular dystrophy. *Proceedings of the National Academy of Sciences of the United States of America* **1999,** *96* (24), 14025-30.
88. Sonnemann, K. J.; Heun-Johnson, H.; Turner, A. J.; Baltgalvis, K. A.; Lowe, D. A.; Ervasti, J. M., Functional substitution by TAT-utrophin in dystrophin-deficient mice. *PLoS medicine* **2009,** *6* (5), e1000083.
89. Cerletti, M.; Negri, T.; Cozzi, F.; Colpo, R.; Andreetta, F.; Croci, D.; Davies, K. E.; Cornelio, F.; Pozza, O.; Karpati, G.; Gilbert, R.; Mora, M., Dystrophic phenotype of canine X-linked muscular dystrophy is mitigated by adenovirus-mediated utrophin gene transfer. *Gene therapy* **2003,** *10* (9), 750-7.
90. Moorwood, C.; Lozynska, O.; Suri, N.; Napper, A. D.; Diamond, S. L.; Khurana, T. S., Drug discovery for Duchenne muscular dystrophy via utrophin promoter activation screening. *PloS one* **2011,** *6* (10), e26169.
91. Ricotti, V.; Mandy, W. P.; Scoto, M.; Pane, M.; Deconinck, N.; Messina, S.; Mercuri, E.; Skuse, D. H.; Muntoni, F., Neurodevelopmental, emotional, and behavioural problems in Duchenne muscular dystrophy in relation to underlying dystrophin gene mutations. *Developmental medicine and child neurology* **2016,** *58* (1), 77-84.