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Current Advances and Future Directions in AAV Gene Therapy for Duchenne Muscular Dystrophy

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Abstract

Duchenne muscular dystrophy (DMD) is a serious genetic disease that affects muscles, caused by loss-of-function mutations in the *DMD* gene, which encodes the structural protein dystrophin. The absence of dystrophin leads to destabilization of the dystrophin-associated glycoprotein complex (DAPC), compromises sarcolemmal integrity and trigger progressive muscle fiber degeneration. Current therapeutic strategies focus on restoring dystrophin expression using gene therapy approaches. Adeno-associated virus (AAV) vectors have emerged as the leading delivery platform, thanks to their preferential tropism for muscle tissue, favorable immunogenicity profile, and capacity for sustained transgene expression. Nevertheless, the restricted packaging capacity of AAV (~4.7 kb) constrains transgene design to truncated micro-dystrophin (µDys) constructs. Major challenges include immune responses against the viral capsid and transgene products, as well as limited functional gain observed in patients, that might be due to the lack of key domains in the µDys protein. In this review, we examine the inherent limitations of µDys gene therapy, discuss the rationale for incorporating additional dystrophin domains into therapeutic constructs, and explore the functional and structural domain combinations being investigated to advance next-generation treatments for DMD.

Keywords: Duchenne muscular dystrophy, Adeno-associated virus, Dystrophin, *DMD* gene

Duchenne Muscular Dystrophy and Dystrophin

Duchenne muscular dystrophy (DMD) is a rare severe muscle-wasting disease that affects roughly 1 in 3,500-5,000 male births [1,2]. DMD is an X-linked recessive disease that is caused by deleterious mutations in the *DMD* gene, the largest gene found in the human genome. Due to its size, the *DMD* gene exhibits a relatively high mutation rate, with more than 7,000 distinct mutations identified. The majority consist of large deletions or duplications (79%), followed by smaller sequence variants (20%), whereas deep intronic mutations and complex rearrangements occur less frequently [3].

DMD patients show progressive muscle wasting, declining skeletal muscle and cardiac function, and, at late onset, severe cardiac and respiratory impairments, leading to patient death in the second decade of life [4]. Despite significant advancements in care and treatment with corticoids, individuals with DMD generally live between 20 to 40 years, with severely impaired quality of life. Dystrophin is a large sub-sarcolemmal protein that enables the assembly of several cytosolic and transmembrane proteins into the dystrophin-associated glycoprotein complex (also known as DAPC) which anchors muscle cells to the extracellular matrix [5,6]. Dystrophin protein is expressed at the sarcolemma and is enriched at the costameres (assembly of subsarcolemmal proteins that connects the Z-disk of myofibrils to the sarcolemma and the basement membrane) [7] and at the myotendinous junction [8] and neuromuscular junction (NMJ) [9]. Dystrophin's main function is to protect myofibers from mechanical stress due to muscle contraction. In the absence of dystrophin, the sarcolemma is damaged by the shearing force generated during force transmission, leading to membrane tearing and myofiber necrosis [7]. Dystrophin protein can be divided into four structural regions: the actin-binding domain (ABD) at the N-terminus, the central rod domain, the cysteine-rich domain

(CR), and the C-terminal domain (C-term). The central rod domain is formed by 24 spectrin-like regions (from now on referred to as R), each of them composed of a 110-aminoacid motif that forms three antiparallel helix bundles [10] (**Figure 1**). Within the myofiber, dystrophin interacts with structural and signaling proteins which confer dystrophin a central role in the regulation of cell homeostasis [11,12].

Structural role of dystrophin: DAGC assembly

In striated muscle, dystrophin serves as a key structural linker between the sarcolemma, the cytoskeleton, and the dystroglycan complex (**Figure 1**). Its association with the sarcolemma has been demonstrated by Zhao *et al*., who showed that sarcolemmal localization of dystrophin fragments was restored only when the R1–R3 and R10–R12 regions were present [13]. This interaction is essential for maintaining membrane stability and protecting myofibers from contraction-induced damage. Dystrophin also interacts with the cytoskeleton through multiple binding sites, including F-actin, microtubules, and intermediate filaments. Two actin-binding regions have been described: an N-terminal domain predicted by Hammond Jr *et al*. and subsequently confirmed by several studies [14–18], and an internal actin-binding site located within R11–R17 [14]. Together, these domains promote lateral association with actin and contribute to filament stabilization. In parallel, dystrophin binds microtubules

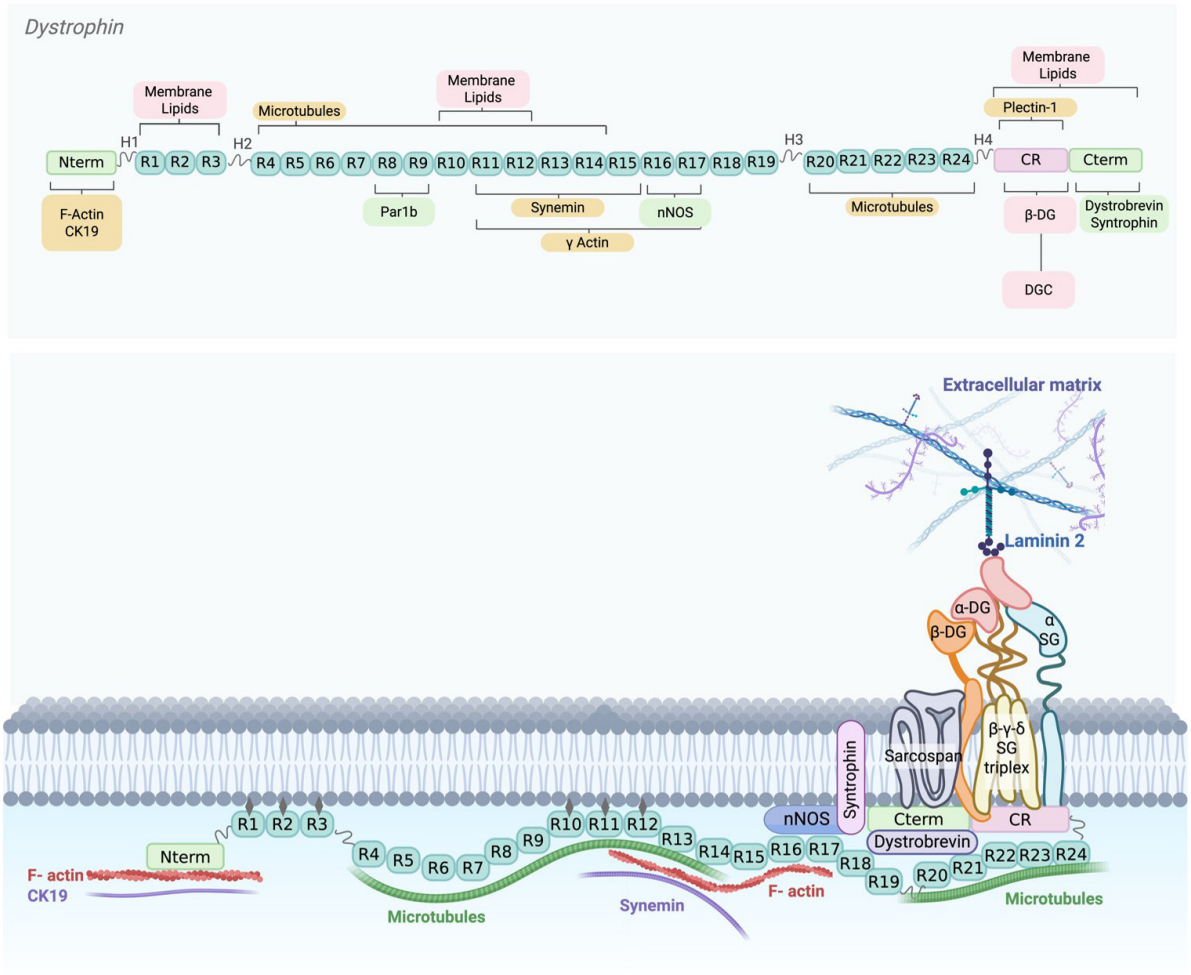

Figure 1. Dystrophin interacts with several structural and signaling proteins in the myofiber. **Top panel**: Schematic representation of dystrophin protein and its interactors. Sarcolemmal bindings are highlighted in pink, while cytoskeletal proteins (actin, microtubules and intermediate filaments) are indicated in yellow. Additionally, signaling proteins (Par1b, nNOS, dystrobrevin and syntrophin) are highlighted in green. **Lower panel**: Schematic representation of dystrophin in the skeletal muscle cell. Abbreviations used: Nterm: N-terminal; γ SG: γ Sarcoglycan; α SG: α Sarcoglycan; β-SG: β-Sarcoglycan; δ SG: δ Sarcoglycan; β-DG: β-Dystroglycan; α DG: α Dystroglycan; CR: Cystein Rich; Cterm: C terminal. Created in BioRender. Palmieri L. (2026) https://BioRender.com/ztj6ipq

through R4–15 and R20–23, helping preserve microtubule lattice organization and resistance to force loss during eccentric contractions [19–21]. Dystrophin also associates with intermediate filaments via the N-terminal ABD1, R11–R15, and the CR domain, thereby contributing to costamere organization and overall cytoskeletal integrity [22–24]. Finally, through its interaction with the dystroglycan complex, mediated by the C-terminal and CR domains, dystrophin links the intracellular cytoskeleton to the extracellular matrix and thereby plays a central role in preserving muscle structure during contraction [25–28].

Signaling role of dystrophin

One of the less explored but increasingly recognized roles of dystrophin is its contribution to myofiber signaling (**Figure 1**). This function is mediated by its interaction with several cytosolic signaling and scaffolding proteins, placing dystrophin at the center of key pathways regulating muscle homeostasis. Among these, the R8–R9 spectrin-like repeats of the central rod domain are required for regulation of cell polarity and division of satellite cells [29]. In parallel, the R16–R17 repeats recruit neuronal nitric oxide synthase (nNOS) to the sarcolemma, an interaction further stabilized by the association of nNOS with α-dystrobrevin/syntrophin at the dystrophin C-terminal domain [30,31]. This signaling axis is essential for perfusion control, satellite cells activation, muscle repair, and contractility, as well as for NO-dependent regulation of HDAC2 and metabolic pathways such as GLUT4 trafficking and phosphofructokinase (PFK) activity [32–35]. Finally, the C-terminal domain recruits syntrophins and dystrobrevin, which organize additional signaling components and contribute to neuromuscular junction maturation and maintenance [36,37].

Genetic Restoration Strategies for Dystrophin Expression

Therapeutic approaches for DMD fall into five main categories: (i) pharmacological, (ii) immunological (e.g. use of membrane-stabilizers antibodies, CART to target fibrosis) (iii) exon-skipping, (iv) stop-codon readthrough, (v) genetic intervention. Genetic interventions for DMD include CRISPR-Cas9–mediated genome editing and AAV-mediated gene delivery. CRISPR-Cas9 genome editing aims to correct the mutated *DMD* gene by restoring the open reading frame, thereby re-establishing proper dystrophin expression. While this approach holds considerable promises, its clinical translation remains challenging due to the limited progress made so far, the still preliminary nature of available clinical data [38,39], and its dependency on specific mutation types [40]. These factors currently place CRISPR-Cas9-based strategies at an earlier stage of clinical readiness compared to other genetic interventions such as AAV-mediated gene delivery.

The most developed and advanced genetic intervention is AAV-mediated gene delivery of a mini-gene encoding a shortened, partially functional dystrophin protein, a strategy that bypasses the need for mutation-specific correction and is therefore applicable to all DMD patients. Recombinant adeno-associated virus (rAAV) vectors are widely used experimentally and in clinical trials due to their ability to mediate long-term transgene expression in skeletal and cardiac muscle [41–45]. The primary limitation remains AAV's 4.7 kb packaging capacity versus dystrophin's 11.2 kb coding sequence, necessitating truncated micro- or midi-dystrophins (μ/midiDys) that retain essential functional domains [46]. This strategy draws from Becker muscular dystrophy (BMD), where ~46% gene deletions still yield milder phenotypes [47], inspiring diverse μ/midiDys constructs (**Figure 2**). Systemic delivery of single AAV vectors expressing μDys has shown broad muscle transduction, dystrophic phenotype improvement, and functional benefits in preclinical DMD models [46,48,49]. These preclinical advances have paved the way for clinical evaluation, and several ongoing clinical trials are now reporting initial outcomes (**Table 1; Figure 2**).

Considerations on μDys gene therapy: safety issue and immunological response

Although preclinical models (including those developed by Sarepta, Pfizer, and Solid Biosciences) reported encouraging outcomes, the performance of μDys gene therapy across most clinical trials and publicly disclosed datasets has been more modest than anticipated, while also raising significant safety concerns in humans. Indeed, several serious adverse events (SAEs) have emerged in the context of AAV-mediated gene therapy for DMD. These events – including hepatotoxicity, myositis, and myocarditis – were observed in 6 of 18 clinical trials, notably the Sarepta trials (NCT03769116, NCT04626674, NCT05096221), the Solid Biosciences trial (NCT03368742), and the Pfizer trial (NCT03362502) (**Table 1**). Specifically, acute adverse effects, which arise within the first days to weeks following treatment and are driven by the innate immune response, may be triggered by CpG motifs present in the AAV genome [50] or by the capsid itself [51], which activates Toll-like receptor (TLR)–mediated immune signaling. Additionally, the AAV capsid can interact with the complement protein [52], leading to endothelial injury and thrombotic microangiopathy (TMA). TMA was observed in three independent clinical trials that shared no similarity in either the transgenes or capsid used; the only common denominator was the high AAV dose administered (> 1 x 10^{14} vg/kg) [53].

With respect to subacute and late SAEs, which manifest several months after gene therapy administration, these are thought to involve a T-cell response directed against the capsid and/or the μDys transgene. These subacute and late SAEs of μDys gene therapy clinical trials prompted a collaborative investigation

Table 1. List of clinical trials for µDys.

Company/ Sponsor	Clinical trial	Clinical trial Phase/type	Sample size	Criteria inclusion	Dose (vg/kg)	SAE	Status	Investigational product name (AAV serotype)	Notes	Reference
Sarepta	NCT03769116	Phase 1/2	41	Children from 4 to 7 years old	2,00E+14	Liver injury, hypertransalinasemia and rhabdomyolysis	Completed	SRP-9001(AAVrh74)		[89]
	NCT04626674 (ENDEAVOR)	Phase 1	55	Children from 4 to 8 years old	1,33E+14	Immune-mediated myositis, myocarditis, increased transaminases	Recruiting	SRP-9001(AAVrh74)		[90]
	NCT05096221 (EMBARK)	Phase 3	126	Children from 4 to 7 years old	1,33E+14	Myocarditis, left ventricular dysfunction, gamma-glutamyl transferase and transaminase increased, rhabdomyolysis	Completed	SRP-9001(AAVrh74)		[91]
	NCT05881408 (ENVISION)	Phase 3	148	Children from 4 to 7 years old	1,33E+14	NA	Active, not recruiting	SRP-9001(AAVrh74)		-
	NCT05967351 (EXPEDITION)	Phase 3	400	Children from 4 to 7 years old	NA	NA	Enrolling by invitation	SRP-9001(AAVrh74)		-
	NCT06128564	Phase 2	21	Children from 4 to 7 years old	1,33E+14	NA	Active, not recruiting	SRP-9001(AAVrh74)		[92]
	NCT06241950	Phase 1	6	Children from 4 to 9 years old	1,33E+14	NA	Completed	SRP-9001(AAVrh74)		-
	NCT06270719 (ENDURE)	Phase 4	500	Children from 4 years old Mutations Not within Exons 8-9	NA	NA	Enrolling by invitation	SRP-9001(AAVrh74)		-
	NCT06597656 (HORIZON)	Phase 1	16	Children from 4 to 8 years old Mutations Not within Exons 8-9	1,33E+14	NA	Active, not recruiting	SRP-9001(AAVrh74)		-
Solid Biosciences	NCT03368742 (IGNITE-DMD)	Phase 1/2	12	Children from 4 to 17 years old	5E+13 - 2E+14	Complement activation within first weeks following dosing (3 patients)	Active not recruiting	SGT-001 (AAV9)	Deprioritized	-
	NCT06138639 (INSPIRE DUCHENNE)	Phase 1/2	43	Children from 4 to 11 years old. Mutations Not within Exons 1-11, 42-45, 57-6	1,00E+14	NA	Recruiting	SGT-003 (AAV-SLB101)	First clinical trial using myotropic AAV vectors (AAV-SLB101)	-

Company/ Sponsor	Clinical trial	Clinical trial Phase/type	Sample size	Criteria inclusion	Dose (vg/kg)	SAE	Status	Investigational product name (AAV serotype)	Notes	Reference
Pfizer	NCT03362502	Phase 1B	19	Children from 4 years old	1E+14 - 3E+14	Dehydration, acute kidney injury, thrombocytopenia. One patient died of cardiogenic shock.	Terminated	PF-06939926 (AAV9)		[93,94]
	NCT04281485 (CIFFREO)	Phase 3	122	Children from 4 to 7 years old	NA	Myocarditis, liver injury	Active not recruiting	PF-06939926 (AAV9)		[95]
	NCT05429372 (DAYLIGHT)	Phase 2	10	Children from 2 to 3 years old	2,00E+14	One patient died over one year after infusion (recurrent rhabdomyolysis)	Terminated	PF-06939926 (AAV9)		-
Genethon	2023-505187-11-00 GNT-016 MDYF	Phase 1/2/3	5 (estimated 50)	Children from 6 to 10 years old Mutation not affecting exon 8 and/or 9	1E+13 - 3E+13	NA	Recruiting	GNT-0004 (AAV8)		[54]
Regenxbio	NCT05693142 (AFFINITY DUCHENNE)	Phase 1/2/3	65	Children from 1 to 11 years old Mutation in Exons 18 and above	1E+14 - 2E+14	NA	Recruiting	RGX-202 (AAV8)		[96]
	NCT06491927	Observational study	19	Children from 1 to 11 years old Mutation in Exons 18 and above	1E+14 - 2E+14	NA	Enrolling by invitation	RGX-202 (AAV8)		-
Insmed	NCT06817382 (ASCEND)	Phase 1	12	Children from 3 to 5 years old Mutation between Exons 18 and 58	NA	NA	Recruiting	INS1201 (AAV9)	First clinical trial with intratecal administration	-

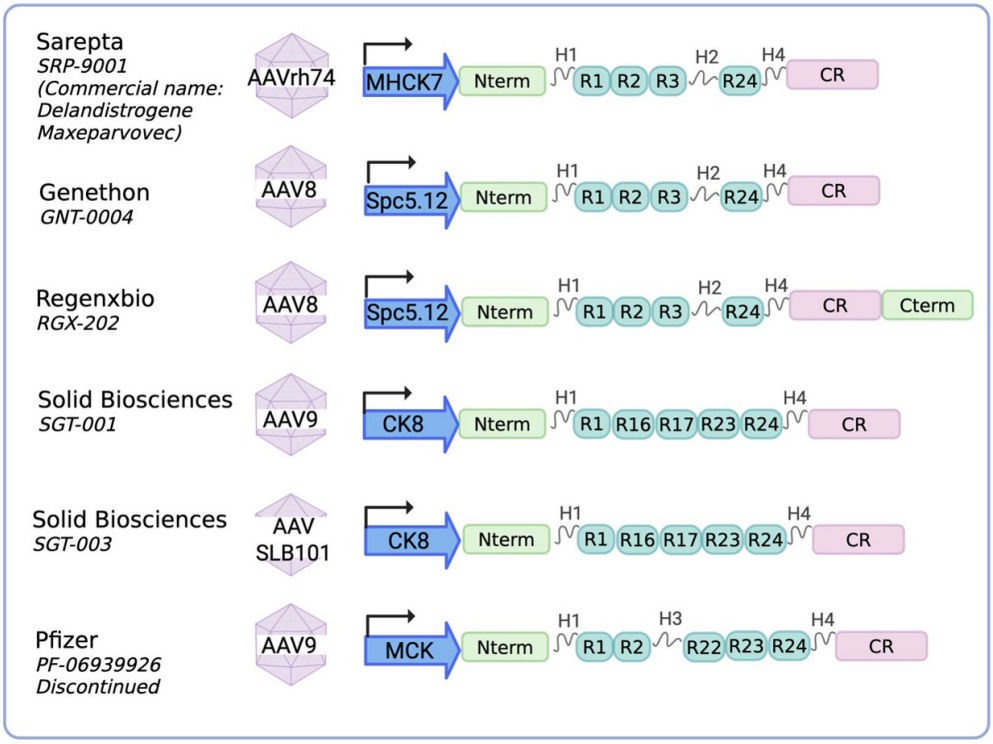

Figure 2. Micro-dystrophin (µDys) construct designs across clinical-stage gene therapy programs. All constructs are delivered via AAV vectors of different serotypes (AAVrh74, AAV8, AAV9, or AAV SLB101) and are driven by muscle-specific promoters (indicated in blue: MHCK7, Spc5.12, CK8, or MCK), selected to maximize muscle tropism and transgene expression. Despite sharing a common structural backbone (comprising an N-terminal domain (Nterm) and a cysteine-rich domain (CR), both essential for membrane anchoring and interaction with the dystrophin-associated protein complex (DAPC)) the constructs differ in their rod domain composition, particularly in the number and identity of spectrin-like repeats (R) and hinge regions (H) retained. These differences reflect distinct design strategies aimed at optimizing functional output while remaining within the AAV packaging size limit (~4.7 kb). The domain architecture of each µDys construct is shown for all gene therapy products currently in clinical development or approved by the FDA, including Delandistrogene Maxeparvovec (SRP-9001, Sarepta), GNT-0004 (Genethon), RGX-202 (Regenxbio), SGT-001 and SGT-003 (Solid Biosciences), and PF-06939926 (Pfizer, discontinued). Created in BioRender. Palmieri L. (2026) https://BioRender.com/ztj6ipq

among the various companies/sponsors developing µDys-based therapies to share and analyze safety data across clinical programs. This effort revealed that patients who developed subacute SAEs shared a common pathogenic variant involving exons 8 to 11 of the *DMD* gene, which encodes the highly immunogenic H1 region. Consequently, these patients were immunologically naïve to transgenes epitopes present in the gene therapy product. In consequence, clinical trial inclusion criteria changed to exclude patients carrying deletion in the region encompassing exons 8–11 [54].

Nevertheless, these adjustments in clinical trial criteria inclusion, four late-onset fatalities have been reported: three in the context of Elevidys clinical trials (Sarepta) and one in the PF-06939926 trial (Pfizer) [55,56]. The three children treated with Elevidys died 2–5 years post-dosing from causes that remain to be fully established. Nevertheless, elevated cardiac troponin-I concentrations in the blood of these patients may indicate a cardiac-related cause of death [57]. The patient who died 14 months after PF-06939926 administration exhibited a distinctive clinical course. This patient carried an in-frame deletion of exons 45–48, a mutation typically associated with the milder BMD phenotype yet presented a clinical course consistent with DMD [58], representing an apparent exception to this rule. The patient experienced recurrent rhabdomyolysis and ultimately died of hyperkalemia-induced cardiac arrest during the final episode. Notably, no immune response against the exogenous µDys transgene was detected, and the underlying mechanism remains uncertain.

These recent cardiac-related events have stimulated an active and timely area of research aimed at elucidating the molecular mechanisms underlying AAV-µDys-associated cardiac toxicity. According to Sweeney lab, µDys-associated cardiac toxicity arises from the displacement of utrophin (normally upregulated in the DMD heart and partially

compensates for the absence of dystrophin). Because μDys is a truncated protein and less effective of utrophin in the heart, its replacement of utrophin is causing cardiac defects [59]. Nevertheless, the outcome of treating double knockout (dKO) mice (deficient in both utrophin and dystrophin) with AAV-mediated μDys gene transfer was likewise associated with cardiac toxicity [60]. Recent hypothesis identifies that accumulation of μDys at high concentrations may saturate the ubiquitin-proteosome system (UPS) and this proteostatic imbalance is causing cardiomyocytes toxicity [61,62]. In any case, further investigation is needed to elucidate the mechanisms underlying the cardiac abnormalities observed in the DMD context following μDys overexpression.

Considerations on μDys gene therapy: lack of functional primary endpoints

Although refined inclusion criteria (notably excluding patients with exons region 8–11) reduced SAEs incidence in children with DMD, the functional efficacy of μDys gene therapy products remain currently uncertain. Indeed, several clinical studies have failed to demonstrate statistically significant improvement over placebo in principle functional outcomes, such as North Star Ambulatory Assessment (NSAA). Among these, the Sarepta program is particularly noteworthy; in the phase III EMBARK trial, the primary endpoint changes from baseline in the NSAA score did not reach statistical significance between DMD patients after a single intravenous administration of delandistrogene moxeparvocec, the commercial μDys gene therapy product developed by Sarepta [63]. Nevertheless, in June 2024, the U.S. Food and Drug Administration granted full approval for Sarepta's therapeutic product for ambulatory patients with DMD aged 4 years and older, based primarily on post-treatment expression of μDys. Among published clinical studies, the only statistically significant improvement in NSAA score has been reported in the EUDRACT study conducted by Genethon, which observed a 4.7-point increase in NSAA score ($p<0.05$). However, this finding was derived from a very small patient cohort ($n = 5$), compared with the substantially larger EMBARK study population ($n = 126$).

The discrepancy between efficacy in preclinical models and the limited clinical benefit has raised a critical concern regarding the insufficient level of transgene expression achieved in skeletal muscle tissue. In response, two principal strategies have been pursued: (i) substantial escalation of the administered dose in humans, reportedly up to tenfold higher than those used in preclinical studies [46,49,64–66] and (ii) the rational engineering of muscle-tropic rAAV capsids. The latter approach has particularly gained momentum, as illustrated by Solid Biosciences' shift from its first-generation vector, SGT-001 (AAV9-based), to the more potent myotropic vector SGT-003, encapsidated with AAV-SLB101, a proprietary vector developed by Solid Biosciences. This next-generation platform has recently demonstrated encouraging early results. More broadly, advances in capsid bioengineering have led to the development of distinct classes of muscle-specific myotropic vectors known as AAVMYO, MyoAAV and LICA variants. These vectors share the common feature of displaying an integrin-binding RGD motif on the capsid surface, which enhances transduction efficiency in skeletal muscles of both mice and primates [67–69]. In addition, these engineered capsids exhibit liver-detargeting properties, thereby reducing hepatic exposure and the potential for liver-associated toxicities.

Novel Strategies for Large Dystrophin Delivery

The limited and inconsistent functional benefit observed in μDys human clinical trials has prompted the development of next generation approaches based on the delivery of larger dystrophin constructs. These strategies are grounded on the rationale that additional dystrophin domains may be required to achieve a more robust therapeutic effect. To overcome the packaging constraints of AAV vectors, this challenge has been addressed through multi-vector delivery approaches, in which the dystrophin coding sequence is split across two or three AAV vectors. Upon co-transduction of the same myofiber, these truncated sequences can recombine or reconstitute a longer, functional dystrophin protein. This reconstitution can occur through several mechanisms, including DNA trans-splicing, homologous recombination (HR), RNA/mRNA trans-splicing, or protein trans-splicing. The optimal vector design and choice of reassembly mechanism depend on the specific features of the transgene sequence as well as the biological properties of the target tissue. In DNA trans-splicing, AAV inverted terminal repeats (ITRs) drive tail-to-head genome concatenation: the 5′ vector delivers promoter, 5′ coding sequence (CDS), and splice donor (SD), while the 3′ vector provides splice acceptor (SA), 3′ CDS, and polyA signal. Subsequent ITR-mediated splicing generates intact full-length mRNA, though efficiency is limited by concatenation orientation. The overlapping HR approach offers a simpler alternative, relying on recombination between homologous CDS overlaps: the 5′ vector carries promoter and 5′ CDS, paired with a 3′ vector bearing 3′ CDS and polyA. This approach eliminates the need for exogenous splicing elements and can be extended to triple-vector full-length dystrophin delivery, although reconstitution efficiencies remain dependent on the transgene design and the target cell type [70]. An alternative strategy that relies on mRNA trans-splicing to reconstitute large transcripts. In this approach, two engineered DNA constructs encode the 5′ and 3′ segments of a larger pre-mRNA. These fragments incorporate a shared intronic hybridization domain (HD) which promotes trans-splicing and enables the precise joining of the two partial transcripts into a full-length mRNA [71]. Despite promising experimental results, this approach has not achieved a sufficient level of pre-mRNA trans-splicing, to support its

implementation in dual-AAV strategies. Methods such as DNA trans-splicing AAV [65,72] and overlapping HR [73,74] have enabled the transfer of midiDys genes in dystrophin-null mice. However, these approaches remain limited by relatively low reconstitution efficiency, necessitating higher doses of AAVs, which in turn increase the risk of immune response and associated toxicities.

Another method for creating larger proteins from smaller transcription units involves the use of split-inteins to fuse two proteins. Split-inteins, protein orthologs of introns, are small polypeptides that undergo a distinct post-translational process called protein trans-splicing (PTS). During PTS, the N- and C-terminal residues (exteins) flanking the intein are naturally ligated, resulting in the formation of one functional protein while removing the reconstituted intein [75]. Work from Pinto and colleagues highlights GP41-1 as the most performant intein *in vivo* [76]. Intein-mediated PTS has been extensively investigated in the context of retinal and liver disease [77,78] as well as in DMD [79–81]. These studies have demonstrated significant amelioration of the dystrophic phenotype and physiological improvement in both young and old *mdx* mouse models. Remarkably, the inteins PTS technology has been successfully used for the delivery of full-length dystrophin transgene [81,82]. Tasfaout *et al.* demonstrated that full-length dystrophin improves cardiac function at later stage of disease in mdx, although evidence for long-term cardiac efficacy is lacking [82]. More notably, recent work from Tian *et al*. demonstrates that EV-mediated delivery of full-length DMD mRNA achieves higher therapeutic efficacy than AAV-µDys in mdx mice, although a fair comparison at equivalent dystrophin expression levels is lacking [83] Nevertheless, comparisons between full-length dystrophin and µDys have shown resulted broadly similar levels of functional improvement and histological rescue in skeletal muscle [81].

Rethinking Dystrophin Domain Inclusion: Is Larger Always Better?

Despite the remarkable progress achieved in delivering full-length dystrophin, several important considerations remain. First, the introduction of full-length dystrophin may increase immune reactivity due to the presence of novel epitopes not naturally expressed in patients. Second, the use of dual or triple AAV vector systems to reconstitute larger dystrophin constructs inherently requires higher vector doses, which can in turn increase immune responses against the viral capsids. Accordingly, the development of effective immune-modulating or tolerance-induction strategies will likely be essential to fully realize the therapeutic potential of full-length dystrophin in humans [50]. Third, the limited functional improvement observed in *mdx* mouse models compared with the current gold-standard µDys raises questions about the added therapeutic value of these approaches. However, this apparent equivalence may reflect the limitations of the mouse *mdx* model itself. The use of more representative preclinical models of DMD, such as rat models [84] or human organoids models [87], may better reveal potential differences and therapeutic benefits of full-length dystrophin constructs relative to µDys.

Another key consideration in addressing DMD is understanding the functional role of individual dystrophin domains and how different domains combinations influence therapeutic outcomes. The delivery of full-length dystrophin remains technically challenging, requiring multiple viral vectors, higher doses, and potentially introducing novel epitopes, raising the possibility that complete restoration of the protein may not be necessary. Instead, intermediate-length constructs may provide sufficient therapeutic benefit. This concept is supported by observations in patients carrying in-frame mutations in the *DMD* gene, who produce partially functional dystrophin variants associated with BMD. These cases highlight that certain domains are more critical than others, and their presence or absence in gene therapy constructs can markedly influence functional recovery. Notably, mutation hotspots in BMD patients are frequently observed within the ABD1 domain and in the spectrin-like repeat regions R17–R19 and R19–R23 [86]. One possible explanation is that some domains, particularly those involved in actin or microtubule binding, may exhibit a degree of functional redundancy. Alternatively, the relative contribution of dystrophin's interactions with distinct cellular components may differ between domains. Given that dystrophin's primary role is to link the contractile apparatus of myofibers to the sarcolemma and extracellular matrix, it is plausible that optimal combination of cytoskeletal-binding domains (for actin, microtubules, and intermediate filaments) and membrane-binding domains (via lipids or the DAPC) is sufficient to ensure functional integrity. For example, in the original design rationale of µDys constructs, the connection to intermediate filaments (mediated by R11–R15), an essential link to costameres and a key anchoring point for lateral force transmission in myofibers, was not originally incorporated [22]. Moreover, the relationship between specific dystrophin mutations and patient phenotypes provides key insights into the regions critical for dystrophin function. Notably, asymptomatic individuals often retain the DMD 3′ region (exons 56–79) as well as hinges 1 and 2, underscoring their importance in maintaining muscle function [86]. Although hinge regions have not been clearly associated with specific binding partner, they may nonetheless play a critical role in conferring elasticity and structural stability of the dystrophin protein [87]. Collectively, these observations suggest that preserving key structural domains may be sufficient to preserve dystrophin function, providing a strong rationale for the development of optimized, streamlined midi-dystrophin constructs.

Conclusion and Final Perspectives

Altogether, these findings underscore the importance of defining the minimal structural requirements for a functional dystrophin molecule. This body of evidence supports the development of optimized midi-dystrophin constructs that prioritize key functional domains, enabling the design of more efficient therapeutic transgenes. Looking ahead, the next phase in dystrophin gene therapy will likely rely on the rational design of constructs that balance functional sufficiency with efficient delivery. In this context, advances in structural prediction, including those enabled by AlphaFold-based approaches, offer a powerful framework to refine midi-dystrophin architecture *in silico* prior to experimental validation. Such strategies may help identify the minimal combinations of domains required to ensure mechanical stability, signaling capacity, and proper folding, while avoiding the complexity associated with multi-vector delivery systems. Beyond design considerations, the clinical translation of these constructs will depend on addressing several practical challenges. Among these is the precise titration required to co-deliver the dual- or triple-vector systems in the exact stoichiometry needed, so as to avoid expression of individual vectors in isolation. The removal of inteins derived from protein trans-splicing, which may trigger unwanted immune responses, should also be addressed, an approach already explored in the context of retinal genetic disorders [88]. Long-term safety must also be carefully evaluated, including the durability of transgene expression, the potential for immune responses against the vector or the transgene product, and the consequences of genomic persistence over time. Ultimately, integrating structural modeling with iterative functional testing should accelerate the development of more effective and clinically translatable dystrophin replacement therapies.

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