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Review article

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Therapeutic approaches for Duchenne muscular dystrophy

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Abstract

Duchenne muscular dystrophy (DMD) is a monogenic muscle-wasting disorder and a priority candidate for molecular and cellular therapeutics. Although rare, it is the most common inherited myopathy affecting children and so has been the focus of intense research activity. It is caused by mutations that disrupt production of the dystrophin protein, and a plethora of drug development approaches are under way that aim to restore dystrophin function, including exon skipping, stop codon readthrough, gene replacement, cell therapy and gene editing. These efforts have led to the clinical approval of four exon skipping antisense oligonucleotides, one stop codon readthrough drug and one gene therapy product, with other approvals likely soon. Here, we discuss the latest therapeutic strategies that are under development and being deployed to treat DMD. Lessons from these drug development programmes are likely to have a major impact on the DMD field, but also on molecular and cellular medicine more generally. Thus, DMD is a pioneer disease at the forefront of future drug discovery efforts, with these experimental treatments paving the way for therapies using similar mechanisms of action being developed for other genetic diseases.

Sections

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Introduction

Duchenne muscular dystrophy (DMD) is a genetic muscle-wasting disease and the most common inherited paediatric myopathy, affecting 1 in 3,500–5,000 live male births[1]. It is characterized by progressive muscle weakness and loss of ambulation around age 10 years, and is ultimately fatal owing to cardiorespiratory failure around age 30 years[2–4]. In addition to muscle wasting, commonly observed clinical features of the disease include scoliosis, joint contractures and calf pseudohypertrophy[5]. DMD is caused by mutations that disrupt production of the dystrophin protein and therefore sensitize muscle to contraction-induced damage[6].

Standard of care for patients with DMD is corticosteroid therapy (for example, prednisone or deflazacort), which has demonstrated some limited efficacy in terms of prolonging ambulation and delaying disease progression[7,8] through an anti-inflammatory mechanism. Importantly, long-term steroid use is associated with undesirable side effects including Cushingoid symptoms, weight gain, growth delay, behavioural changes and osteoporosis[9]. The latter is particularly concerning because accidental fractures in patients with DMD often lead to a permanent loss of ambulation[10]. Efforts to develop corticosteroid treatments for DMD with improved side effect profiles (such as vamorolone)[11] have been discussed in detail elsewhere[9].

Newer DMD therapeutic approaches have focused on restoring dystrophin expression using multiple modalities. Drugs that restore the dystrophin reading frame via antisense oligonucleotide (ASO)-mediated exon skipping, stop codon readthrough and gene replacement have achieved regulatory approval for clinical use. Although these are major achievements for the DMD research and patient communities, the efficacy of these drugs is generally accepted to be very low. Various efforts are under way to enhance delivery of exon skipping drugs via novel chemical modifications and conjugation to delivery-assisting moieties. Conversely, multiple additional gene replacement therapies, comprised of viral vector-encoded compact dystrophin variants, are in late-stage clinical trials. Other therapeutic strategies, including cell therapy, gene editing, upregulation of compensatory genes such as utrophin and combination therapies, are also under investigation.

DMD is a key disease indication in the field of experimental therapeutics for several reasons: it constitutes an unmet clinical need with devastating disease progression; it has a relatively high incidence for a rare disease; a plethora of molecular and cellular medicines are being investigated for its treatment; and there have been multiple recent regulatory approvals. Advances in DMD therapeutics will undoubtedly have an impact on the development of therapeutics in other areas of medicine, as similar modalities can be applied to other disease indications. Here, we discuss the drug development landscape for DMD with a main focus on dystrophin restoration therapies, although other strategies are considered briefly. Recent drug approvals, progress in current clinical trials, improved delivery technologies, vector-associated safety issues, combination therapies and other novel approaches are discussed.

DMD genetics and pathophysiology

DMD is an X-linked recessive dystrophinopathy, caused by genetic absence of the dystrophin protein, which is encoded by the *DMD* gene at Xp21. Dystrophin is located at the intracellular surface of the sarcolemma, where it acts as an organizing centre for the dystrophin-associated protein complex (DAPC). Specifically, dystrophin binds to the transmembrane protein β-dystroglycan via its C-terminal cysteine-rich domain[12]. β-Dystroglycan binds to α-dystroglycan, which is exposed on the extracellular surface of the sarcolemma where it interacts with a complex of laminins. Dystrophin also binds to filamentous γ-actin, intermediate filaments and the microtubule network in the sarcoplasm via its N terminus. As such, it forms a mechanical link between the extracellular matrix and the actin cytoskeleton[13]. The primary function of dystrophin is to serve as a 'shock absorber' that protects muscle from contractile damage[6]. Additionally, it is involved in multiple signalling processes via DAPC interactions, including nitric oxide (NO) signalling via the activity of neuronal nitric oxide synthase (nNOS), the MAP kinase pathway[14] and MARK2 kinase signalling, which regulates muscle satellite (stem) cell polarity[15].

The loss of dystrophin results in disruption of the DAPC, and therefore many DAPC components become mislocalized from the sarcolemma[12] and are expressed at lower levels[16,17]. For example, the sarcoglycans are downregulated and mislocalized in dystrophin-deficient muscle[16,17]. Disruption of sarcoglycans is associated with various forms of limb-girdle muscular dystrophy[18], indicating that integrity of the DAPC is important for preventing muscle pathology. Dystrophin loss also leads to increased Ca^{2+} influx, oxidative stress and myonecrosis. Dystrophic muscle is characterized by foci of degeneration and regeneration and by persistent inflammation. During the early stages of disease, myofibre loss is balanced by compensatory regeneration driven primarily by satellite cells. In advanced disease, muscle quality declines as a consequence of extensive fibrosis and deposition of adipose tissue[19], which progressively replaces myofibres and generates a non-productive environment that is unable to support satellite cell-mediated regeneration (that is, functional exhaustion). Importantly, evidence suggests that the number of satellite cells is not reduced in dystrophic muscle and their regenerative potential is not diminished[20,21].

Aside from the structural and signalling functions described above, there is also evidence that dystrophin functions as a tumour suppressor in cancers that involve myogenic programmes (that is, rhabdomyosarcoma, gastrointestinal stromal tumour and leiomyosarcoma)[22] and in other cancers such as neuroblastoma[23]. Somatic mutations in the *DMD* gene are common in high-grade myogenic cancers[22]. Cell culture experiments demonstrated that dystrophin re-expression can reduce cell migration, invasion and anchorage independence in myogenic sarcomas, suggesting that loss of dystrophin expression promotes metastasis[22].

The genomic locus encoding the *DMD* gene is one of the largest in the human genome (~2.2 Mb) and exhibits a high rate of de novo mutation. Common types of DMD-causing mutation include whole exon deletions (68%), exon duplications (11%) and nonsense mutations (10%)[24–27]. Although mutations can occur throughout the genomic region, large deletions (and some duplications) are concentrated at two hot spots located at exons 3–19 and exons 45–55 (ref. 28). The *DMD* gene consists of 79 exons (Fig. 1a), many of which code for 24 spectrin-like repeat domains in the central rod domain of dystrophin. There is a degree of functional redundancy in these domains, meaning that in many cases they are dispensable for dystrophin function (Fig. 1b). Importantly, whole exon deletions that do not disrupt the translation reading frame lead to an internally deleted dystrophin protein, which retains partial functionality and is associated with Becker muscular dystrophy (BMD), a related dystrophinopathy[29–32]. Patients with BMD present with a wide range of disease severities, although disease onset is typically later and pathology is relatively mild compared with DMD. Life expectancy is longer than for DMD, with dilated cardiomyopathy typically appearing in the fourth decade of life[33,34]. However, in some

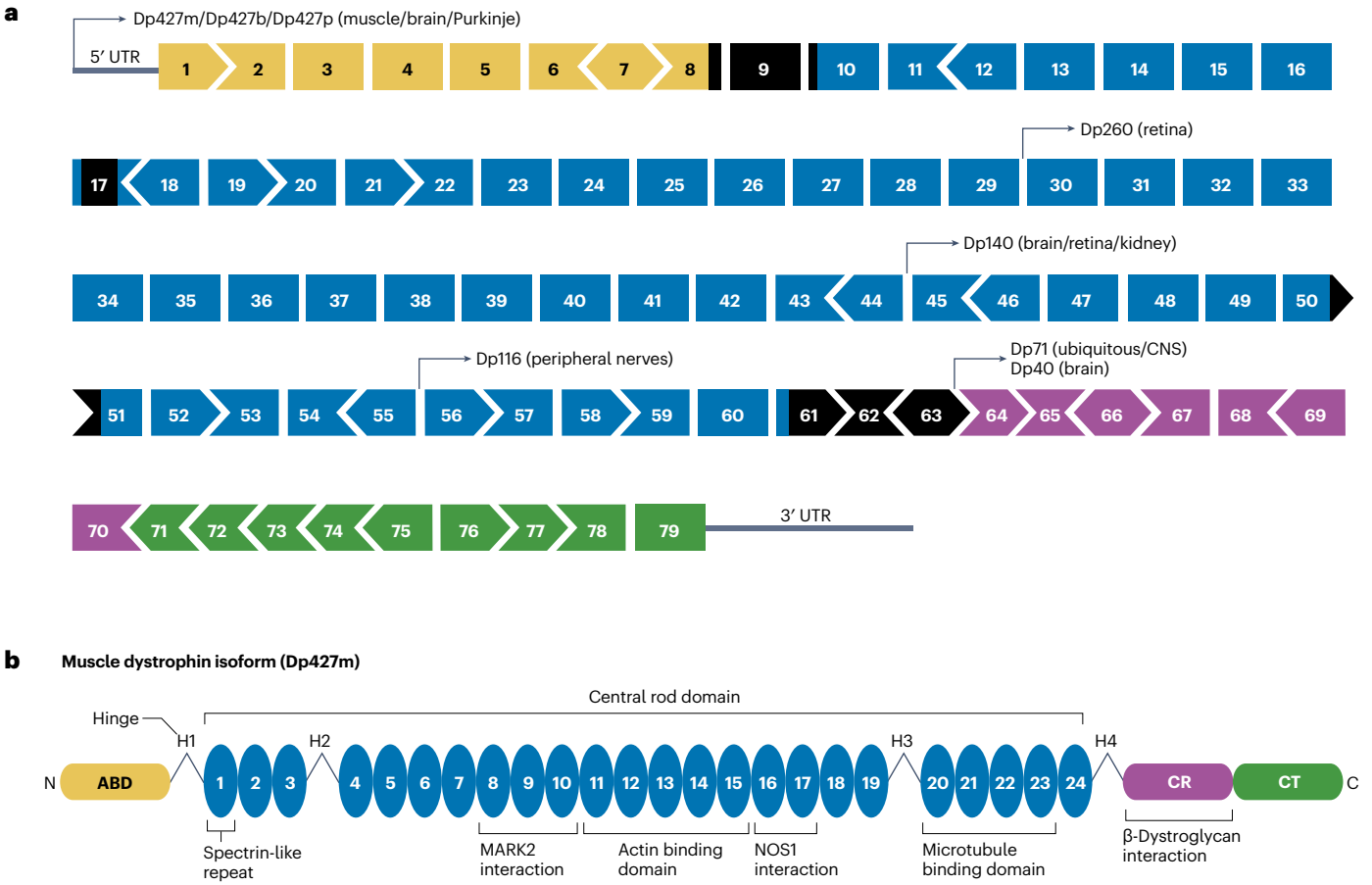

Fig. 1 | The dystrophin gene and protein. a, Schematic of the *DMD* gene. The colour of each exon represents the protein domain it encodes: the actin binding domain (ABD) in orange, the hinge regions in black, the central rod domain in blue, the cysteine-rich region (CR) in purple and the C-terminal domain (CT) in green. Exon shapes indicate how the triplet base code is distributed across the exons, such that they fit together to generate an in-frame mature *DMD* transcript. The locations of the transcription start sites for the various dystrophin protein isoforms are indicated by arrows. **b**, Structure of the full-length muscle isoform of dystrophin (Dp427m). Key interactions between dystrophin and other binding partners are indicated. CNS, central nervous system; UTR, untranslated region. Adapted from ref. 5, Springer Nature Limited.

cases dilated cardiomyopathy can be the initial presentation of BMD in younger patients[35]. Patients with BMD generate dystrophin protein at lower levels than in the case of DMD and/or produce a partially functional dystrophin on account of varying degrees of internal in-frame deletion[31]. In rare cases, some patients with BMD with large internal dystrophin deletions are effectively asymptomatic[29,30]. These observations motivated the development of therapeutic dystrophin restoration strategies that aim to convert the severe DMD phenotype into the milder BMD situation[36].

Dystrophin restoration strategies

Loss of dystrophin is the primary genetic cause of DMD, and so extensive research effort has been directed towards therapies that can restore dystrophin expression. A plethora of approaches have been tested, including splice correction (exon skipping) to restore the translation reading frame, stop codon readthrough for patients with nonsense mutations, gene replacement with internally deleted dystrophin transgenes, delivery of dystrophin-expressing myogenic cells and gene editing to repair the *DMD* locus at the DNA level.

Exon skipping

The leading dystrophin restoration strategy is currently exon skipping, whereby modulation of splicing is used to restore the translation reading frame and promote the generation of a partially functional, internally deleted pseudo-dystrophin protein (Fig. 2). Typically, this is achieved using steric block ASOs. These short (~20–30 nucleotide), single-stranded nucleic acid polymers interact with pre-mRNA transcripts via Watson–Crick base pairing and thereby influence splicing decisions by physically masking specific splicing signals, which include exon splicing enhancers and exon recognition sequences. Exon skipping ASOs must each be designed to target a single specific exon, and so an individual drug can only ever be capable of treating the limited number of patients for which a given exon skip will restore the translation reading frame. Importantly, many DMD-causing mutations cannot be treated with exon skipping approaches owing to certain regions of the dystrophin protein (and their encoding exons) being indispensable for function, or the absence of suitable mutation-adjacent exons that can be skipped in order to reframe the transcript. It has been estimated that exon skipping approaches might be applicable to 55% of DMD-causing

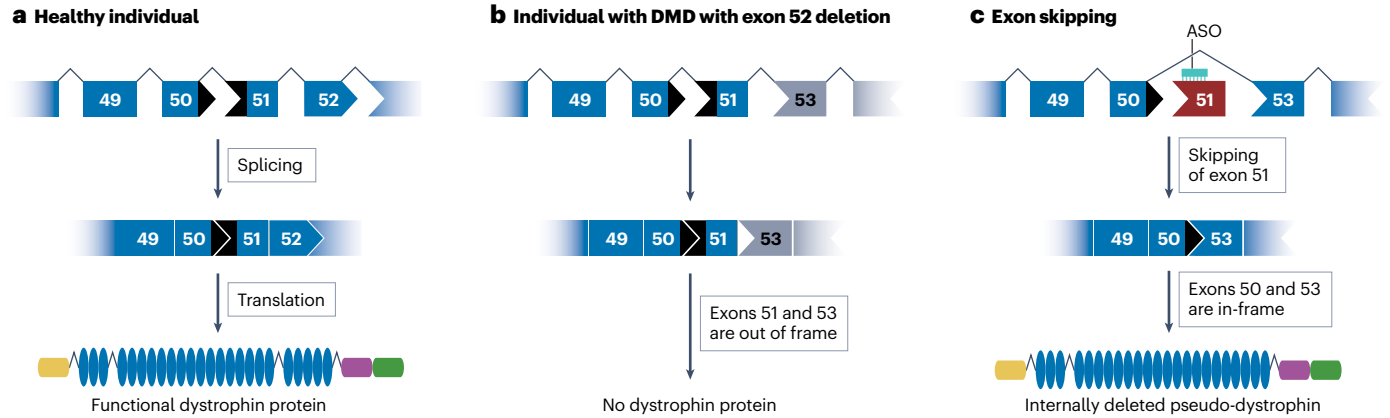

Fig. 2 | Restoration of dystrophin expression by exon skipping. a, In a healthy individual, the *DMD* gene undergoes splicing to excise intronic regions from the mature *DMD* mRNA transcript. A functional dystrophin protein is generated via the translation of this transcript. The schematic shows a region of the *DMD* gene covering exons 48–53, which encodes spectrin-like repeat domains (blue) and a hinge region (H3, in black). **b**, In individuals with DMD, mutations (often whole exon deletions) disrupt the translation reading frame of the *DMD* transcript. Here, a relatively common DMD-causing mutation is shown, in which exon 52 is deleted. As a result, exons 51 and 53 are out of frame, leading to a failure to generate dystrophin protein. Out-of-frame exons are shown in grey. **c**, Treatment with an antisense oligonucleotide (ASO) targeting an exon splicing enhancer motif in *DMD* exon 51 induces skipping of this exon by effectively hiding it from the spliceosome. As a result, exon 50 and exon 53 are spliced together, resulting in restoration of the dystrophin translation reading frame. Following translation, an internally truncated pseudo-dystrophin lacking the H3 domain is generated that retains a degree of functionality.

mutations and 80% of DMD-causing deletions[24]. These approaches have primarily been used for patients who carry whole exon deletions but can also be used for nonsense mutations, provided that these mutations occur within exons that can be skipped without disrupting the translation reading frame. For example, the commonly used *mdx* mouse model of DMD carries a premature termination codon in *Dmd* exon 23, and restoration of dystrophin expression can be achieved by skipping the mutation-containing exon. Exon skipping approaches can also be applied to some types of exon duplication, such as duplication of *DMD* exon 2, discussed below.

Initial efforts focused on skipping of exon 51, which would be applicable to ~13% of all patients with DMD[36] (such as those with whole exon deletions of *DMD* exon 50 or 52). The great promise of oligonucleotide therapeutics is that upon establishing platform chemistries and delivery strategies, novel drugs can be rapidly generated by the careful alteration of the constituent nucleotide sequence to target a different transcript. In the case of DMD, modification of ASO sequences can be deployed to target a wider range of DMD-causing mutations. So far, there are four US Food and Drug Administration (FDA)-approved ASO drugs designed to skip various DMD exons (Table 1 and Fig. 3a). These ASOs are all phosphorodiamidate morpholino oligonucleotides (PMOs) (Fig. 3b) developed by Sarepta Therapeutics (eteplirsen[37], golodirsen[38] and casimersen[39]) or NS Pharma (viltolarsen[40–42]). They target the exons that have the potential to treat the largest number of patients — that is, exons 45, 51 and 53. Both Sarepta and NS Pharma have pipelines with ASOs that target the skipping of additional exons (43, 44, 50, 52 and 55), although many of these programmes are still at the preclinical stage. Importantly, not all DMD-causing mutations are treatable with exon skipping approaches[36]. As the FDA has not approved exon skipping as a class of drug but has instead required separate trials for each ASO, the targeting of rare mutations is unlikely to be sufficiently commercially attractive for development by the pharmaceutical industry.

Notably, the European Medicines Agency has declined to approve any of the exon skipping compounds described above[43], based primarily on their low efficacy, marginal therapeutic benefit and small trial sample sizes. Indeed, the approval of eteplirsen by the FDA was particularly controversial, leading to accusations of 'railroading at the FDA' and the resignations of several FDA review team members[44–48]. Mean dystrophin protein expression after 180 weeks of eteplirsen treatment was determined to be less than 1% of healthy dystrophin levels[49]. The efficacies of viltolarsen, golodirsen and casimersen are similarly modest[39,40,50]. Nevertheless, PMOs have been remarkably safe in clinical trials[51,52], and doses of up to 3 g kg^{-1} are well tolerated in mice[53]. Despite the low levels of dystrophin protein restored by these compounds, clinical trial participants treated with eteplirsen (the most studied exon skipping drug for DMD) have maintained an attenuation in ambulatory decline over a treatment period of at least 4 years[54], which is not consistent with the established progression of the disease.

Importantly, the four FDA approvals of PMO exon skipping drugs have not discouraged other companies from entering this space with enhanced chemistries and/or delivery technologies. The low efficacy of 'naked' PMO exon skipping drugs has motivated the development of improved delivery strategies, primarily based on bioconjugation[55]. The backbone linkages of PMOs are uncharged (unlike most oligonucleotide therapeutics), which permits facile covalent conjugation to cell-penetrating peptides (CPPs). These are typically arginine-containing short peptides that facilitate interaction with the outer surface of the plasma membrane and glycocalyx, and which might to some extent promote endosomal escape. The resulting peptide–PMO (PPMO) conjugates offer major increases in potency in preclinical DMD models compared with unconjugated, naked PMO[56,57]. PPMO conjugates are currently under investigation in two clinical programmes (Table 1). First, Sarepta is conducting a phase II trial (NCT04004065, MOMENTUM) of vesleteplirsen (SRP-5051), which consists of the exon 51-targeting eteplirsen PMO sequence conjugated to the arginine-rich R_6Gly peptide (Fig. 3c). Preliminary unpublished data reported by Sarepta suggest that vesleteplirsen exhibits greater

drug exposure and exon skipping activity than eteplirsen at equivalent doses (>10% mean exon skipping, >6% dystrophin expression after treatment with 30 mg kg^{-1} per month for 3 months)[58]. Secondly, PepGen has conducted a phase I clinical trial of PGN-EDO51 in healthy volunteers in Canada, based on novel PPMO technology developed by the Wood and Gait groups. These conjugates were designed to balance exon skipping activity with renal toxicity, which has been reported to be a potential limitation of PPMO technologies[59,60]. PepGen reported that PGN-EDO51 was safe and well-tolerated in healthy volunteers, with dystrophin exon skipping observed at low levels (2% mean exon skipping, assayed 28 days after injection), which was expected considering the relatively low dose (a single intravenous 15 mg kg^{-1} dose)[61]. Both companies reported cases of hypomagnesaemia after PPMO treatment, which required magnesium supplementation in some cases.

Entrada Therapeutics is developing PPMO exon skipping conjugates based on cyclic peptides using an enhanced endosomal escape vehicle (EEV) technology and is currently at the preclinical stage[62].

Other ASO conjugation approaches are also under investigation. Avidity Biosciences is exploring an antibody–oligonucleotide conjugate approach (Fig. 3d), with the leading compound AOC 1044, targeting *DMD* exon 44 skipping, being investigated in the EXPLORE44 phase I/II clinical trial in healthy volunteers[63]. AOC 1044 consists of PMO molecules conjugated to an antibody targeting the transferrin receptor (TFRC, TfR1), which is highly expressed in skeletal and cardiac muscle. Similarly, Dyne Therapeutics is undertaking a first-in-human phase I/II clinical trial of DYNE-251, targeting skipping of *DMD* exon 51 in amenable patients with DMD (NCT05524883). DYNE-251 consists of PMO molecules conjugated to a Fab fragment (Fig. 3e) also targeting TFRC. Dyne recently published preclinical exon skipping data in the *mdx* mouse using its FORCE platform[64].

The prospect of multi-exon skipping using a cocktail of ten octaguanidine dendrimer-conjugated PMOs (vivo-morpholinos) has been explored in a DMD mouse model lacking *Dmd* exon 52 (the *mdx52* mouse)[65]. Skipping of ten 'hot-spot' exons (*Dmd* exons 45–55) resulted in restoration of dystrophin expression and improvements in muscle function, although several skipped products were produced[65]. Importantly, skipping of these hot-spot exons would theoretically be applicable to ~63% of all patients with DMD[66]. Whether such an approach can be translated for use in human patients remains to be demonstrated, as overall such multi-exon skipping strategies have thus far proved only minimally successful.

Wave Life Sciences recently initiated testing of an ASO designed to skip *DMD* exon 53 (WVE-N531) in a phase Ib/II clinical trial in 15 boys with DMD (NCT04906460). According to news reports, preliminary results from this trial after 6 weeks of treatment indicated substantial RNA level exon skipping, although dystrophin protein was below the lower limit of quantification[67]. WVE-N531 is a chimeric stereopure steric block ASO that contains phosphorothioate (PS) and phosphoryl guanidine (PN) linkages, which reduce the overall charge of the oligonucleotide[68] (Fig. 3f–j). This drug also includes stereospecific linkages at one or more backbone linkages (both PS and PN linkages are chiral, unlike the analogous phosphodiester, PO, linkage) (Fig. 3i,j). Control of backbone linkage stereochemistry can influence a multitude of oligonucleotide properties, including hydrophobicity, nuclease resistance, target binding affinity and splice-switching activity[69]. The notion that control of stereochemistry could be used to optimize ASO development is appealing, as ASOs with chiral centres in their backbone (which is the vast majority) in reality constitute racemic mixtures of hundreds of thousands of different molecules. Among this population there might be hyperfunctional molecules that could be synthesized in a stereopure manner, thereby offering a substantial increase in potency. However, the importance of stereopure ASO backbone linkages has also met with scepticism from some others in the oligonucleotide field[70]. Beneficial outcomes that are obtained in certain properties (such as target binding) might be counteracted by detrimental changes in other properties (such as uptake efficiency). Notably, Wave Life Sciences has had several failures of its stereopure ASO technologies for both its DMD and its Huntington disease programmes[71,72]. Demonstration of efficacy for WVE-N531 will be important to support the continuation of stereopure ASO technology development.

Daiichi Sankyo is investigating renadirsen (DS-5141b) in a phase II trial in eight participants (NCT04433234). Renadirsen is a 'mixmer' ASO consisting of 2′-*O*-Methyl and ethylene-bridged nucleic acid (ENA) residues with PS linkages (Fig. 3g,k,l) designed to skip *DMD* exon 45 (ref. 73).

Table 1 | Exon skipping drugs approved and in clinical development

Name	Company	Chemistry	Target exon	Approval or clinical stage
Eteplirsen	Sarepta Therapeutics	PMO	51	FDA
Viltolarsen	NS Pharma	PMO	53	FDA, Japan
Golodirsen	Sarepta Therapeutics	PMO	53	FDA
Casimersen	Sarepta Therapeutics	PMO	45	FDA
Vesleteplirsen	Sarepta Therapeutics	PPMO (R_6Gly)	51	Phase II
WVE-N531	Wave Life Sciences	PS/PN stereoselective	53	Phase Ib/II
Renadirsen	Daiichi Sankyo	2'OMe/ENA mixmer	45	Phase II
AOC 1044	Avidity Biosciences	PMO–antibody conjugate	44	Phase I/II
DYNE-251	Dyne Therapeutics	PMO–Fab fragment conjugate	51	Phase I/II
ENTR-601-44	Entrada Therapeutics	PPMO (EEV)	44	Preclinical
PGN-EDO51	PepGen	PPMO (EDO)	51	Phase I
SQY51	SQY Therapeutics	Tricylco-DNA	51	Phase I/II (in 2023)

EDO, enhanced delivery oligonucleotide; EEV, enhanced endosomal escape vehicle; ENA, ethylene-bridged nucleic acid; PMO, phosphorodiamidate morpholino oligonucleotide; PPMO, peptide–PMO conjugate; PS/PN, phosphorothioate and phosphoryl guanidine linkages.

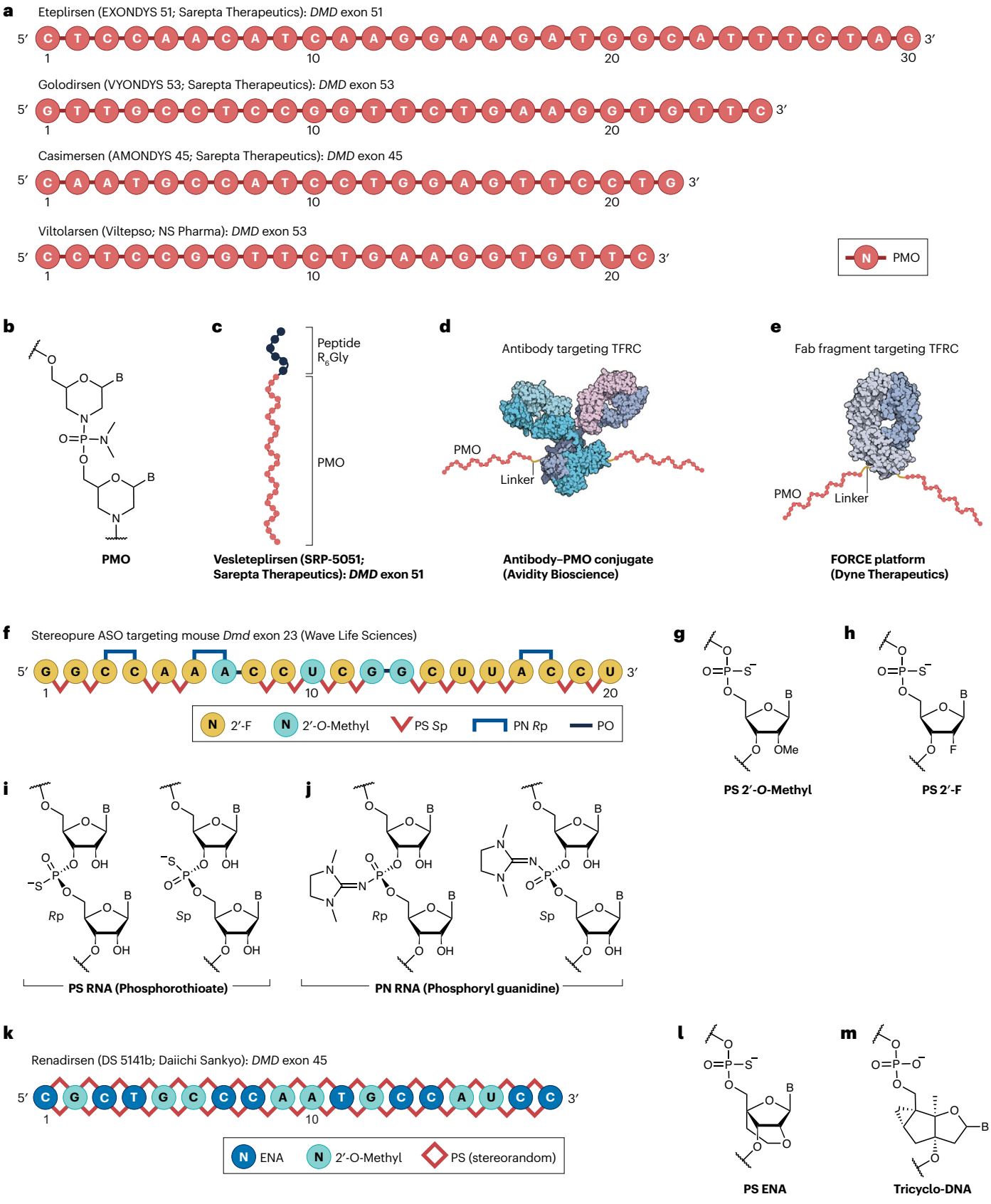
a
Eteplirsen (EXONDYS 51; Sarepta Therapeutics): DMD exon 51
5′ C T C C A A G A T C A A G G A A G A T G G C A T T T C T A G 3′
1 10 20 30
Golodirsen (VYONDYS 53; Sarepta Therapeutics): DMD exon 53
5′ G T T G C C T C C G G T T C T G A A G G T G T T C 3′
1 10 20
Casimersen (AMONDYS 45; Sarepta Therapeutics): DMD exon 45
5′ C A A T G C C A T C C T G G A G T T C C T G 3′
1 10 20
Viltolarsen (Viltepso; NS Pharma): DMD exon 53
5′ C C T C C G G T T C T G A A G G T G T T C 3′
1 10 20
N PMO
b
PMO
c
Peptide R8Gly
PMO
Vesleteplirsen (SRP-5051; Sarepta Therapeutics): DMD exon 51
d
Antibody targeting TFRC
PMO
Linker
Antibody–PMO conjugate (Avidity Bioscience)
e
Fab fragment targeting TFRC
PMO
Linker
FORCE platform (Dyne Therapeutics)
f
Stereopure ASO targeting mouse Dmd exon 23 (Wave Life Sciences)
5′ G G C C A A A C C U C G G C U U A C C U 3′
1 10 20
N 2′-F
N 2′-O-Methyl
PS Sp
PN Rp
PO
g
PS 2′-O-Methyl
h
PS 2′-F
i
Rp
Sp
PS RNA (Phosphorothioate)
j
Rp
Sp
PN RNA (Phosphoryl guanidine)
k
Renadirsen (DS 5141b; Daiichi Sankyo): DMD exon 45
5′ C G C T G C C C A A T G C C A U C C 3′
1 10
N ENA
N 2′-O-Methyl
PS (stereorandom)
l
PS ENA
m
Tricyclo-DNA

Fig. 3 | Antisense oligonucleotide therapies for Duchenne muscular dystrophy. a, Chemical composition of the FDA-approved exon skipping phosphorodiamidate morpholino oligonucleotide (PMO) compounds eteplirsen, golodirsen, casimersen and viltolarsen. **b**, Chemical structure of PMO chemistry. **c**, The peptide–PMO (PPMO) conjugate vesleteplirsen. **d**, An antibody–PMO conjugate developed by Avidity Biosciences, with a PMO molecule conjugated to an antibody targeting the transferrin receptor TFRC. **e**, A Fab fragment–PMO conjugate developed by Dyne Therapeutics, with a PMO conjugated to a Fab targeting TFRC. **f**, Chemical composition of a stereopure antisense oligonucleotide (ASO) targeting mouse *Dmd* exon 23 (Wave Life Sciences). This compound also includes phosphodiester (PO) linkages and is chemically similar to WVE-N531 (exact composition not publicly disclosed). **g**, Phosphorothioate 2′-*O*-methyl RNA (PS 2′-*O*-Methyl). **h**, Phosphorothioate 2′-fluoro RNA (PS 2′-F). **i**, *R*p and *S*p stereoisomers of PS linkages. **j**, *R*p and *S*p stereoisomers of phosphoryl guanidine (PN) linkages. **k**, Chemical composition of renadirsen. **l**, Phosphorothioate 2′-*O*,4′-*C*-ethylene-bridged nucleic acid (PS ENA). **m**, Tricyclo-DNA. The IgG (1IGY) and Fab fragment (5FUZ) structures were downloaded from the Protein Data Bank.

Also, SQY Therapeutics is developing tricyclo-DNA (Fig. 3m), an ASO chemistry that exhibits limited activity for dystrophin restoration in the brain[74,75], and it has initiated a phase I/II clinical trial (Avance 1) that is expected to be completed in mid-2024.

An alternative strategy is to use expressed exon skipping triggers based on the U1 or U7 small nuclear RNAs (snRNAs), which can be delivered via adeno-associated virus (AAV) vectors to enable systemic delivery throughout the musculature[76,77]. Such an approach is currently under investigation in a phase I/II clinical trial (NCT04240314) sponsored by Nationwide Children's Hospital, USA, that aims to induce exon skipping of a duplicated *DMD* exon 2, the most commonly observed DMD-causing exon duplication[25]. The experimental therapeutic in this case (scAAV9.U7-ACCA) is a self-complementary AAV9 encoding four U7 snRNA exon skipping transgene cassettes, two targeting the exon 2 splice acceptor and two the splice donor[78]. This approach is notable because exon skipping results in two possible beneficial splicing outcomes. In the first outcome, skipping of one copy of *DMD* exon 2 will generate full-length dystrophin and be a significant advantage over other approaches that restore internally deleted pseudo-dystrophins. In the second outcome, skipping of both copies of *DMD* exon 2 will result in cap-independent translation driven by an internal ribosome entry site (IRES) sequence located in exon 5, which generates a highly functional N-terminally truncated dystrophin isoform[79]. Evidence from preclinical studies suggests that the second splicing outcome is dominant[78]. Preliminary unpublished findings from investigations in three patients with DMD are promising[80].

Stop codon readthrough

Nonsense mutations in the *DMD* gene result in the generation of truncated protein products and/or promote reductions in mRNA levels via the nonsense-mediated decay (NMD) pathway. Stop codon readthrough therapies have therefore been developed that aim to promote ribosome miscoding of premature termination codons (PTCs) such that an alternative amino acid is incorporated and translation of the mRNA continues past the PTC instead of translation termination occurring. Stop codon readthrough approaches in the context of DMD are therefore expected to generate full-length dystrophin protein, albeit with a single internal amino acid change (Fig. 4a,b). Importantly, such stop codon readthrough therapies are suitable for the treatment of patients with single nonsense mutations (but not for patients in which a frameshift mutation generates PTCs downstream of the mutation). Early work focused on the use of gentamicin (Fig. 4c), an aminoglycoside antibiotic that binds in the aminoacyl-tRNA acceptor (A) site of the ribosome and interferes with codon–anticodon recognition. Gentamicin treatment showed some promise in preclinical studies[81], although results in clinical trials were less impressive[82–84]. Notably, aminoglycosides are associated with renal and otic toxicities[85], and clinical development of gentamicin was ultimately terminated.

PTC Therapeutics has developed an improved stop codon readthrough compound, ataluren (PTC124; Translarna). Ataluren is an orally bioavailable small molecule (3,5-diaryl oxadiazole, Fig. 4d) that has no structural similarity to aminoglycoside antibiotics, exhibits no antibiotic activity, does not influence NMD target expression and promotes readthrough of PTCs while not affecting normal termination codons[86]. In July 2014 ataluren was granted conditional approval by the EMA[87] and is currently approved for use in patients with nonsense mutation DMD age 2 years and older at a dose of 40 mg kg^{-1} daily. This approval was initially based on promising findings from a randomized, double-blind, placebo-controlled clinical trial (NCT00592553)[88], and was conditional on the findings of a second phase III trial (NCT01826487, ACT DMD)[89]. Neither of these trials met their primary end point of a statistically significant improvement in 6 min walk distance (6MWD) by more than 30 m at week 48 post treatment (relative to placebo-treated individuals). However, analysing a subset of the data revealed significant improvements in patients in the ambulation transition phase (that is, those with baseline 6MWD of 300–400 m)[89]. Importantly, the FDA declined to approve ataluren based on the same data[90]. Subsequent studies have provided further evidence to support the efficacy of ataluren. A meta-analysis in which data from these similar trials was combined found that the improvement in 6MWD on ataluren did reach statistical significance (for both all intention-to-treat patients and the ambulation transition patient subset)[91]. Nevertheless, controversy surrounding the approval of ataluren persists and several clinical trials are ongoing (phase II, NCT04336826 and phase III, NCT02369731, NCT01247207, NCT03179631). The mechanism of action for ataluren is currently unknown, and it was shown that this drug could bind to and stabilize firefly luciferase, leading to an increase in its activity in reporter assays similar to those used to identify it as a stop codon readthrough candidate[92]. Others have reported conflicting results using ataluren, such as a failure to show readthrough activity using multiple reporter assays[93]. A convincing demonstration of dystrophin restoration in ataluren-treated patient muscle biopsy samples and elucidation of the drug mechanism of stop codon readthrough will help to assuage these concerns.

Gene replacement therapy

Classical gene therapy for DMD aims to introduce DNA that encodes a functional dystrophin protein into patient muscles. Transgene DNA is typically delivered using viral vectors, with AAV being the vector of choice. Treatment with viral gene therapy usually results in 'immunization' of the treated individual against the vector, meaning that repeat administration of the therapy is precluded[94]. AAV is a single-stranded DNA parvovirus that is generally considered to be non-pathogenic in humans and has been widely used for gene therapy applications, with two AAV-based gene therapy products reaching marketing approval for non-DMD indications. These products are intrathecal injection of

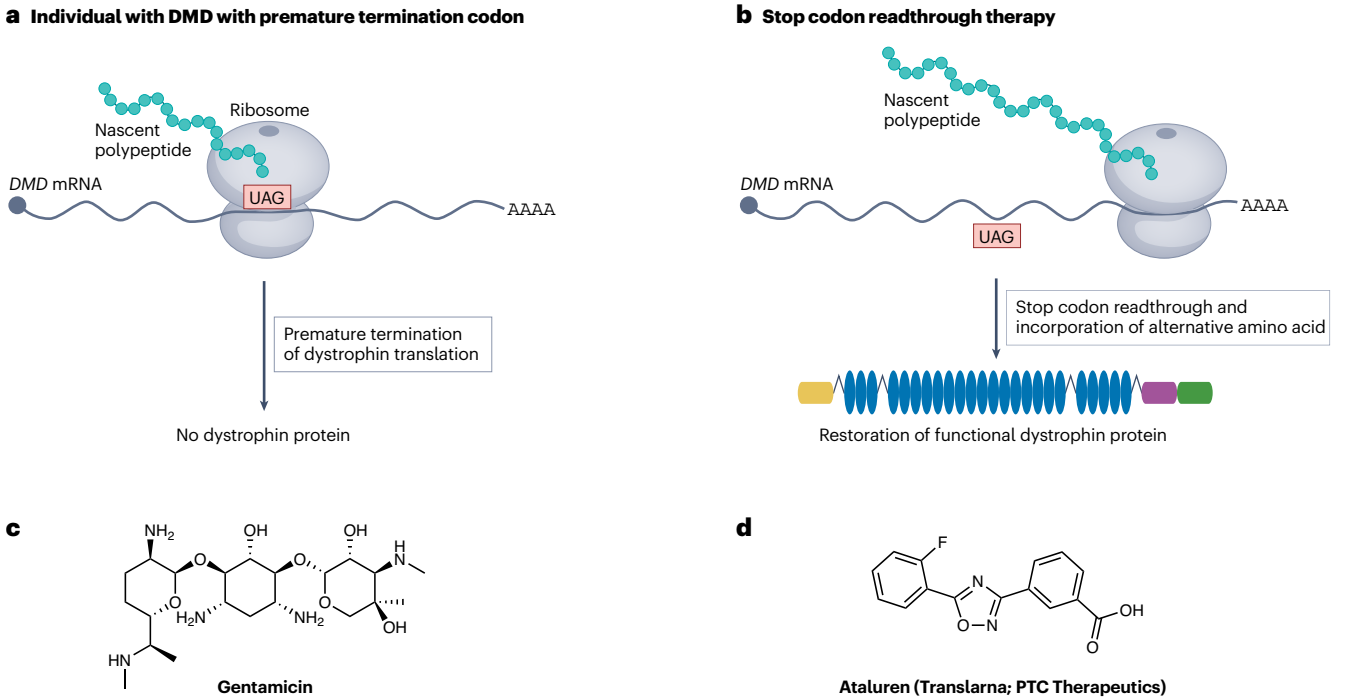

Fig. 4 | Restoration of dystrophin by stop codon readthrough therapy. **a**, Nonsense mutations that introduce premature stop codons in the *DMD* mRNA result in premature termination of translation by the ribosome and a failure to generate full-length, functional dystrophin protein. **b**, Treatment with a stop codon readthrough drug (such as ataluren) results in the incorporation of a random alternative amino acid at the premature termination codon site. The ribosome can therefore proceed through the premature stop codon and expression of full-length dystrophin protein is restored. **c**, Chemical structure of gentamicin. **d**, Chemical structure of ataluren.

zolgensma for the treatment of spinal muscular atrophy (SMA) and subretinal injection of luxturna for treating Leber congenital amaurosis. Importantly, multiple AAV serotypes exhibit tropism for skeletal and cardiac muscle[95–97]. However, the AAV genome has a maximum packaging capacity of ~4.8 kb and so cannot deliver the full-length *DMD* cDNA in a single vector given that the major muscle isoform is ~14 kb. Efforts have therefore focused on the generation of dystrophin minigenes in which non-essential internal domains are deleted, which are inspired by the example of a very mildly affected patient who expressed an internally truncated but functional pseudo-dystrophin protein[29] (Fig. 5). Expression of this minigene, with 46% of the normal dystrophin protein-coding region, prevented pathology in the *mdx* mouse, and the deletion was further extended to generate micro-dystrophin genes[98]. As such, gene replacement therapies for DMD have focused on the delivery of various micro-dystrophin transgenes (also named 'mini-dystrophin'). Micro-dystrophins typically lack large portions of the central rod domain — and therefore most of the spectrin-like repeat domains are also missing — but interactions between the DAPC and the cytoskeleton are maintained, thereby preserving the primary function of dystrophin. Most micro-dystrophin constructs lack the C-terminal domain, the inclusion of which confers little additional therapeutic benefit[99]. There are currently five micro-dystrophin drugs in clinical trials, sponsored by Sarepta, Pfizer, Solid Biosciences, Genethon (in partnership with Sarepta) and REGENXBIO. These constructs differ in terms of the micro-dystrophin structure, the choice of promoter and the AAV serotype used (Table 2). AAV-mediated delivery of micro-dystrophin can improve dystrophic pathology in various mouse and canine DMD models[100–103], suggesting that micro-dystrophin genes might be sufficient to convert the DMD phenotype into a milder Becker clinical course.

SRP-9001 (rAAVrh74.MHCK7.micro-dystrophin, ELEVIDYS, delandistrogene moxeparvovec; Sarepta) is the AAV–micro-dystrophin gene therapy that is at the most advanced stage and for which the most information is publicly available. It uses a codon-optimized human micro-dystrophin minigene driven by a synthetic MHCK7 promoter, consisting of the muscle creatine kinase (MCK) promoter fused with the MCK and α-myosin heavy chain complex (αMHC) enhancers to promote high expression levels specifically in skeletal and cardiac muscle[104]. This transgene cassette is delivered using an AAV variant derived from rhesus macaques (AAVrh74), which exhibits strong skeletal and cardiac muscle tropism[105], and for which seroprevalence of neutralizing antibodies is low in patients with DMD[106]. A phase I/IIa clinical trial (NCT03375164) in four patients with DMD treated with 2×10^{14} vector genomes (vg) kg^{-1} of SRP-9001 reported expression of dystrophin 12 weeks after injection and improved North Star Ambulatory Assessment (NSAA) scores and serum CK levels up to 1 year after treatment[107]. Three of these treated patients underwent further analysis by quantitative MRI and spectroscopy, which illustrated an improvement in muscle fat fraction and transverse relaxation time (qT_2, which is affected by inflammation and fat infiltration) values for patients treated with SRP-9001, compared with a natural history cohort[108]. (Comparison of experimental DMD therapies with natural history data has been commonly employed when the use of a placebo group is precluded by the relatively small number of available patients.) Multiple further

clinical trials of SRP-9001 are ongoing (phase I open-label extension, NCT03375164; phase II randomized placebo controlled, NCT03769116; phase I, NCT04626674; and phase III double-blind, randomized, placebo controlled, NCT05096221), with some preliminary data available through press releases and conference presentations[109–111]. On the basis of the promising findings from across its clinical programmes, Sarepta submitted a Biologic Licence Application for SRP-9001 to the FDA, which was granted Priority Review status[112]. In June 2023, SR-9001 was granted conditional approval for use in boys aged 4–5 years with DMD who do not have deletions in exon 8 and/or 9, making this the first approved gene therapy product for DMD[113].

Unfortunately, other micro-dystrophin gene therapy development programmes have not run so smoothly. In December 2021, Pfizer announced the death of a 16-year-old non-ambulatory trial participant with advanced disease treated with a high dose (2×10^{14} vg kg^{-1}) of PF-06939926 in an open-label phase Ib trial (NCT03362502), leading to a temporary FDA hold on the drug[114,115]. A randomized, double-blind, placebo-controlled phase III trial of PF-06939926 (NCT04281485, CIFFREO) is ongoing. No peer reviewed findings from these studies are currently available, but claims of relatively high levels of micro-dystrophin expression (24–50%, by anti-peptide antibody-enriched, immunoaffinity liquid chromatography tandem mass spectrometry assay) and significant functional improvement in the phase Ib trial have been reported by Pfizer[116]. However, several treatment-related serious adverse events have been reported, related to muscle weakness and myocarditis, leading to a protocol amendment to exclude patients with mutations that affect *DMD* exons 9–13, or deletions that affect both exons 29 and 30, and to include a 7-day hospitalization period after treatment administration[117,118]. A collaborative working group that combined the data and experience from Pfizer, Sarepta, Solid Biosciences and Genethon, together with experts from academia, was established to address the potential safety issues with micro-dystrophin gene therapy[119]. The observation that the worst serious adverse events occurred only in patients who carried deletions of dystrophin-encoding regions that are present in the micro-dystrophin transgene protein, suggested that a T cell-mediated immune response is responsible[119] and provides a scientific rationale for excluding such patients from clinical trials.

Similarly, a phase I/II trial (NCT03368742, IGNITE) of the micro-dystrophin therapeutic SGT-001 (Solid Biosciences) has been placed on hold by the FDA twice, as a consequence of serious adverse events in a patient in the high-dose (2×10^{14} vg kg^{-1}) cohort, including thrombocytopenia, complement activation, reduced red blood cell count, acute kidney injury and cardiopulmonary insufficiency[120]. Clearance to continue was given after several protocol amendments, including improvements to the AAV manufacturing process to remove the majority of empty viral capsids. Prophylactic measures to minimize immune reactions were also implemented (treatment with eculizumab and a C1 esterase inhibitor), together with an increase in corticosteroid dose in the first month after SGT-001 injection[121]. No peer reviewed data on the safety and efficacy of SGT-001 are currently available, but dystrophin levels of up to 17.5% (by western blot) of healthy levels and improvements in 6MWD, NSAA score and pulmonary function tests have been reported by Solid Biosciences[122]. An improved DMD micro-dystrophin gene therapy product (SGT-003) with better tropism for muscle and heart and reduced liver delivery is being developed by Solid Biosciences and is currently at the investigational new drug stage.

Notably, there have been several other deaths following treatment with high-dose AAV in clinical trials for X-linked myotubular myopathy (NCT03199469)[123], Sanfilippo syndrome (NCT03612869)[124] and in two patients treated with zolgensma (an FDA-approved gene therapy for SMA)[125]. High-dose AAV9 therapies (2×10^{14} vg kg^{-1}) have also been reported to induce severe hepatic and neurological toxicities in nonhuman primates and piglets[126]. However, even higher doses of AAV–micro-dystrophin gene therapies have been administered to patients with DMD (SGT-001 at 2×10^{14} vg kg^{-1} and PF-06939926 at 3×10^{14} vg kg^{-1})[116,127], suggesting that some patients are more susceptible to severe toxic effects than others. It is clear that a re-evaluation of the safety of high-dose AAV therapies is warranted.

Aside from safety issues associated with high-dose AAV, gene replacement therapy for DMD faces several additional challenges. Indeed, as many as ~40% of humans are already positive for anti-AAV antibodies as a consequence of natural exposure[128], which creates a key challenge for the application of AAV-derived vectors in patients with DMD. The presence of these antibodies is typically an exclusion criterion in gene therapy trials. The expression of micro-dystrophin in the muscle of patients with DMD has the potential to generate non-self antigens, leading to an anti-transgene immune response. The relative failure of the first clinical trial of AAV–micro-dystrophin was attributed to such an anti-dystrophin T cell response[129]. Evidence from preclinical studies suggests that the anti-dystrophin antibody response can be avoided by co-treating with immunomodulatory drugs such as rituximab and VBP6 (ref. 130). The success of AAV–micro-dystrophin therapy is predicated on long-term expression of the therapeutic

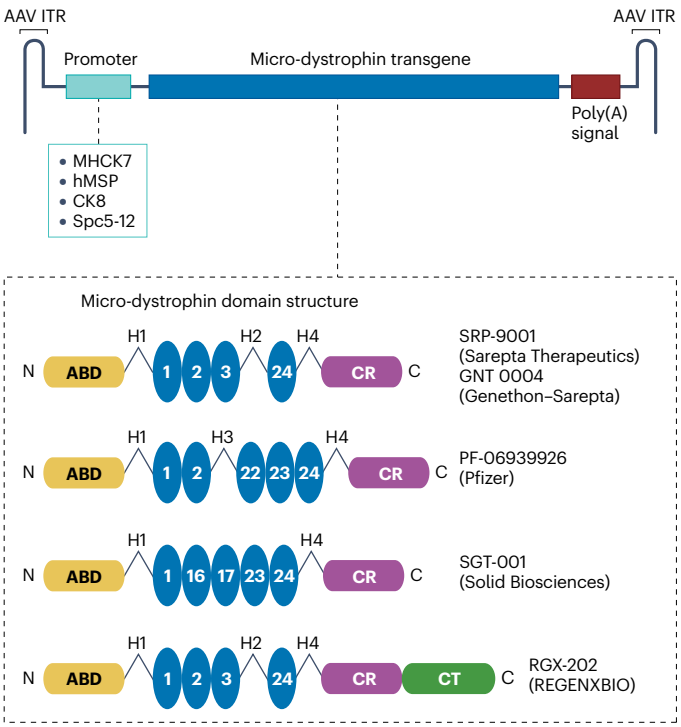

Fig. 5 | Micro-dystrophin gene replacement therapy. Micro-dystrophin transgenes expressed in adeno-associated virus (AAV) genomes. The constructs contain flanking inverted terminal repeat (ITR) regions, a muscle-specific promoter (one of the four variants shown), the micro-dystrophin transgene and a poly(A) transcription termination signal. Micro-dystrophin domain structures are indicated for all the micro-dystrophin gene therapy products currently in clinical development or FDA approved. ABD, actin binding domain; CR, cysteine-rich domain; CT, C-terminal domain.

Table 2 | Micro-dystrophin gene replacement therapies in clinical development

Name	Company	Micro-dystrophin	AAV serotype	Promoter	Approval or clinical stage
SRP-9001	Sarepta Therapeutics	ΔR4-23/ΔCT	AAVrh74	MHCK7	FDA
PF-06939926	Pfizer	ΔR3-19/20-21/ΔCT	AAV9	hMSP	Phase III
SGT-001	Solid Biosciences	ΔR2-15/R18-22/ΔCT	AAV9	CK8	Phase I/II
GNT 0004	Genethon–Sarepta	ΔR4-23/ΔCT	AAV8	Spc5-12	Phase I/II/III
RGX-202	REGENXBIO	ΔR4-23 (Includes CT)	AAV8	Spc5-12	Phase I/II

AAV, adeno-associated virus.

transgene. AAV vector genomes do not integrate into the host DNA, but are instead maintained as episomal chromatin with the potential for long-term persistent transgene expression[131,132]. However, in practice, AAV genomes are progressively lost from treated dystrophic muscle so that micro-dystrophin expression would be expected to progressively diminish, leading to a recurrence of dystrophic pathology[133]. Epigenetic silencing of the micro-dystrophin transgene cassette might also contribute to a loss of expression over time[134]. Intriguingly, Mollard et al.[135] recently reported a reduction in AAV transgene expression in post-regeneration mouse muscle. In this study, muscle necrosis and regeneration was induced by injection of cardiotoxin, and AAV1 particles were subsequently injected at time points when regeneration was completed and muscle morphology restored (3 weeks and 42 weeks after injury). Transgene expression was reduced in these animals relative to uninjured control animals, whereas there was no difference in vector genome numbers or subcellular localization. A similar effect was observed in *mdx* mouse muscle (which undergoes asynchronous necrosis and regeneration), suggesting that dystrophic pathology itself might be a limitation to the effectiveness of therapies that require AAV for delivery[135].

Cell therapy

Cell therapies for DMD aim to treat the disease via the transplantation of dystrophin-expressing pro-myogenic cells into patient muscle. Such therapies can involve cells derived from healthy, histocompatible donors (allogenic) or via patient-derived cells that are genetically corrected to express dystrophin ex vivo (autologous). Healthy cells are administered either via intramuscular injection (which can involve multiple injections per muscle) or via systemic administration. In both cases, the transplanted cell population expands, undergoes myogenic differentiation and fuses to generate new myotubes and/or integrate with existing or regenerating myofibres. Initial results in the *mdx* mouse demonstrated that implanted healthy neonatal muscle progenitor cells (myoblasts) can fuse with pre-existing *mdx* myofibres and render them dystrophin positive[136,137]. Subsequently, multiple other cell sources have been explored for cell transplantation, including satellite cells[138], bone marrow-derived myogenic cells[139], side population cells[140], mesoangioblasts[141], pericytes[142], $CD133^+$ cells[143] and induced pluripotent stem cells (iPSCs)[144]. Despite promising results in preclinical studies, results in human patients using these approaches have been relatively disappointing. For example, patients with DMD treated with a series of high-density injections of normal myoblast allotransplants (under tacrolimus immunosuppression) exhibited dystrophin expression that was mostly restricted to the area surrounding the injection sites and ranged from the presence of a single dystrophin-positive myotube to positivity in 26% of myofibres[145]. In contrast, $CD133^+$ cells introduced via intramuscular injection failed to fuse with the myofibres in muscle from patients with DMD[143]. Similarly, intra-arterial injection of mesoangioblasts derived from HLA-matched healthy donors resulted in the detection of dystrophin expression, but no functional improvement[141].

Cell therapies for DMD face several important challenges[146]. In many cases, obtaining sufficient numbers of cells to treat all muscles is difficult, especially for cell types such as satellite cells. Transplanted cells can face immune rejection, and typically large numbers of the donor cells die shortly after injection[147]. Delivery also constitutes a major challenge, as cells can exhibit reduced potential for migration or aggregate inside blood vessels following a failure to extravasate, which can potentially lead to pulmonary embolism or accumulation in filter organs[142]. Delivery to disease-critical muscles such as the diaphragm is also particularly challenging.

A novel alternative cell therapy approach using cardiosphere-derived cells (CDCs) has been pioneered by Capricor Therapeutics. Allogenic CDCs derived from healthy donors have been administered to patients with DMD via intracoronary[148] and intravenous[149] routes. Although these have the potential to express wild-type dystrophin protein, this is not the goal of the therapy per se. Instead, CDCs are hypothesized to release extracellular vesicles containing cargo molecules that exert anti-inflammatory and anti-fibrotic effects. In a recent double-blind, placebo-controlled, phase II clinical trial (NCT03406780, HOPE-2) patients with DMD were intravenously injected every 3 months with 1.5×10^8 CDCs (CAP-1002) for a total of four administrations[149]. CAP-1002-treated patients exhibited a slowing of the loss of upper limb function, together with improvements in cardiac structure and function[149]. An open-label extension study is ongoing (NCT04428476), and a phase III trial (NCT05126758, HOPE-3) is currently recruiting.

ENCell is conducting a phase I clinical trial (NCT05338099) in patients with DMD for its stem cell therapy EN001 based on the transfer of Wharton's jelly (umbilical cord)-derived mesenchymal stem cells.

Gene editing

With the repurposing of the CRISPR–Cas9 system for gene editing in mammalian cells, there has been intense interest in deploying this technology for the treatment of DMD[150]. In its simplest configuration, the CRISPR–Cas9 system consists of the Cas9 endonuclease, which induces double-strand DNA breaks (DSBs), and a single guide RNA (sgRNA) that acts to programme the Cas9 such that it cuts at a specific DNA sequence. Notably, the use of multiple guides enables the possibility of multiplex gene editing[151]. The host cell DNA damage repair machinery is of key importance for the success of CRISPR–Cas9 therapies, with the non-homologous end joining (NHEJ) pathway being the most relevant for the purposes of this Review.

Multiple CRISPR–Cas9-based strategies have been proposed for the treatment of DMD. The first demonstrations of CRISPR–Cas9-mediated correction in the context of DMD used an exon excision

approach[152–154]. This strategy is conceptually similar to exon skipping, whereby two DSBs are induced in intronic sequences flanking a target exon using a pair of sgRNAs and was initially demonstrated for *Dmd* exon 23 in the *mdx* mouse. The resulting lesions are joined via the NHEJ pathway and the intervening DNA removed. Such a corrected locus would now be 'permanently exon skipped' as the preceding exon (exon 22) will be spliced onto the following exon (exon 24).

Myofibres that re-express dystrophin are believed to have a selection advantage, meaning that they are progressively enriched over time[155]. Furthermore, early treatment in dystrophic mice is more effective than treatment at the adult stage, possibly as the former approach leads to tolerization towards dystrophin-associated antigens. The editing of satellite cells is desirable, as the resulting corrected stem cells will continue to add myonuclei with the potential to express dystrophin during growth and regeneration throughout the life of the treated individual. However, there have been conflicting reports regarding the potential of AAV vectors to transduce satellite cells[152,156–158].

Excitingly, multiplex gene editing enables the simultaneous deletion of multiple exons (analogous to multi-exon skipping described above), with the potential for a single therapy that could be applied to a large proportion of patients[144,159]. An alternative approach is the single cut strategy, in which the targeted introduction of an indel is used to either disrupt a splicing motif, such as an exon splicing enhancer or splice site, or reframe a transcript[155].

Vertex Pharmaceuticals and Sarepta are developing CRISPR–Cas9-based therapies for DMD, although both are at the discovery or preclinical phase. The most clinically advanced DMD CRISPR therapy is CRD-TMH-001 developed by a non-profit organization, Cure Rare Disease, in collaboration with the University of Massachusetts. Few details are publicly available about CRD-TMH-001 other than it is designed to activate dystrophin expression using a CRISPR activation (CRISPRa) approach. CRISPRa uses a catalytically inactive Cas9 variant (dCas9) fused to an effector domain (such as VP64) that promotes transcriptional activation without the introduction of DNA DSBs[160,161]. An $n = 1$ clinical trial (NCT05514249) with CRD-TMH-001 was initiated, but Cure Rare Disease announced that the single 27-year-old trial participant had died[162]. Within 1 week of treatment the patient suffered acute respiratory distress and cardiac arrest, leading to death at day 8 after injection[163]. Cure Rare Disease has multiple other personalized CRISPR-based therapies in preclinical development[164].

Most CRISPR–Cas9 therapeutics for DMD rely on the use of AAV vectors for delivery of the gene editing apparatus and, as such, are subject to all of the limitations of these delivery vectors described above (especially considering the high doses of vector that will be required). In addition, there are multiple additional CRISPR–Cas9-specific limitations that must be addressed before such strategies can be translated into new therapies. First, deleterious off-target editing events must be carefully considered. Non-productive on-target editing also has the potential to corrupt myonuclei, leading to a patchy pattern of dystrophin in treated myofibres[165]. Pre-existing anti-Cas9 antibodies and T cells are relatively common in the general population[166–168], which may further limit the applicability of these therapies.

Multiple other CRISPR-based technologies are under investigation for the treatment of DMD and are considered here briefly. A detailed discussion of these approaches is beyond the scope of this article, but has been presented elsewhere[150].

Precise gene editing can be achieved by leveraging the homology-directed repair (HDR) DNA repair pathway. HDR-based approaches require the introduction of a single DSB near to the target edit site and the provision of a single-stranded oligodeoxynucleotide (ssODN) repair template. Importantly, HDR is only active in cycling cells, meaning that its in vivo utility in postmitotic tissues such as muscle is limited. However, HDR-based approaches might be used for ex vivo correction of cells from patients with DMD[169]. Furthermore, an HDR strategy was applied at the zygote stage to restore dystrophin expression in the *mdx* mouse[170].

Base editing is a CRISPR strategy that can induce targeted single nucleotide variants into target DNA[171,172]. Base editing typically uses a 'nickase' Cas9 (nCas9) enzyme, which is capable of cleaving only one strand, fused to a base editing enzyme such as the adenosine base editor, ABE, which is derived from the *Escherichia coli* TadA protein[171]. For the nCas9–ABE system, a single-strand break (SSB) is generated on one DNA strand, while the ABE effector module catalyses the transition of an adenosine base to an inosine on the opposite strand. Inosine either functions as a guanosine base or is converted into guanosine by the base excision repair pathway[173]. Base editing using the nCas9–ABE system has been used to correct the nonsense mutation in the *mdx* mouse[174] and to achieve genomic DNA-level exon skipping via splice site disruption in dystrophic ΔEx51 mice[175]. Base editing has the advantage of minimizing the possibility of deleterious editing events such as indel formation and chromosomal rearrangements as no DSBs are formed. However, the nCas9–ABE system is large, and so strategies such as split vector (trans-splicing AAV) approaches have been used for in vivo delivery[174,175].

Prime editing is an alternative gene editing system that can directly install specific edits including transitions, transversions, insertions and deletions into target genomes[176]. The prime editing system consists of a catalytically impaired Cas9 (nCas9) endonuclease fused to a reverse transcriptase (RT) enzyme and a specialized prime editing guide RNA (pegRNA) that directs the Cas9–RT fusion protein to the complementary target site and also encodes the intended edit. Following nicking of the target sequence by nCas9, the pegRNA binds to the exposed strand, which serves as a primer for the RT reaction. New complementary DNA specifying the desired edit encoded by the pegRNA is subsequently generated, and the resulting lesion is resolved by ligation and mismatch repair[176]. Prime editing has been used to achieve reframing of the dystrophin open reading frame in iPSCs[175]. Although prime editing enables the installation of almost any desired edit, delivery of this system to dystrophic muscle in vivo remains to be demonstrated.

Utrophin upregulation

Utrophin (*UTRN*) is an autosomal (6q24) paralogue of *DMD* that is expressed during fetal development, at the neuromuscular and myotendinous junctions, and during muscle regeneration[177–179]. The 395 kDa utrophin isoform is upregulated in the muscles of dystrophin-deficient mouse models (such as *mdx* and *mdx52*)[16,17] and in patients with DMD[180], where it relocates to the sarcolemma and can bind to DAPC components[181]. These observations suggest that utrophin can substitute for dystrophin to some extent, and that its upregulation in dystrophic muscle might be a compensatory and protective mechanism. Accordingly, dystrophic pathology is severe in the dystrophin and utrophin double knockout (dKO) mouse[182,183], and genetic overexpression of full-length utrophin by 3- to 4-fold on an *mdx* background was sufficient to prevent the development of dystrophic pathological features[184,185]. Upregulation of utrophin is a particularly attractive therapeutic strategy as a single approach could be used to treat all patients regardless of mutation type. Furthermore, the *UTRN* gene is invariably unaffected in patients with DMD, and its expression is ubiquitous[186], meaning that

patients are already tolerized to UTRN-associated antigens[187]. Ubiquitous overexpression of a utrophin transgene in mice was also found to be non-toxic[186].

The leading strategy for utrophin upregulation is the use of small-molecule modulators. The drug ezutromid (SMT C1100; Summit Therapeutics), a 2-arylbenzoxazole, was identified in a screen of small molecules that promoted transcriptional activation of the utrophin A promoter[188]. Daily oral administration of this compound improved muscle pathology in the *mdx* mouse[189]. Investigation of ezutromid in an open-label phase II clinical trial (NCT02858362) showed evidence of utrophin upregulation and reduced muscle turnover after 24 weeks of treatment[190], but this effect was not present at 48 weeks[191]. Development of ezutromid was discontinued on the basis of these disappointing findings. Subsequent investigation revealed that ezutromid binds to, and acts as an antagonist of, the aryl hydrocarbon receptor (AHR) and that other AHR antagonists similarly promoted utrophin expression[191], suggesting that this could be used for future drug development. It has been proposed that the lack of sustained efficacy of ezutromid is a consequence of its cellular metabolism leading to reduced drug exposure[191], and that such metabolism of future utrophin modulator drugs could be avoided through medicinal chemistry optimization (A. Russell, personal communication). Similarly, second-generation utrophin upregulator compounds that are derivatives of ezutromid with improved activity, such as SMT022357, have been reported[192].

The sequence of utrophin is highly similar to that of dystrophin[177,178]. As such, internally truncated utrophin minigene variants have been generated, analogous to the micro-dystrophin approach described above[193,194]. To this end, transgenic micro-utrophin expression resulted in improvements in histopathology and reduced serum CK levels in *mdx* mice[193] and various canine models[187]. Similarly, AAV-delivered micro-utrophin constructs improved muscle function and increased lifespan in the severely affected dKO mouse[195].

Several other approaches have been explored for utrophin upregulation in preclinical models including CRISPRa[160], deletion of miRNA target sites in the *UTRN* 5′ UTR[196] and artificial transcription factors[197].

Notably, there are important differences in the functionality of utrophin and dystrophin, which suggest that utrophin might be insufficient to fully compensate for the absence of dystrophin. Specifically, utrophin is not capable of anchoring nNOS at the sarcolemma[198] nor of rescuing the disordered pattern of the microtubule network that is observed in dystrophic myofibres[199]. Furthermore, there is also evidence that the functional benefit of utrophin minigenes is substantially less than that of full-length dystrophin[184].

Other therapeutic approaches

A multitude of other disease-modifying approaches for DMD have been investigated that target the various pathological features of DMD. These include anti-inflammatory, vasodilating NO donor, modulation of Ca^{2+} handling, anti-fibrotic, antioxidant and myostatin pathway blockade strategies. A detailed discussion of these strategies is beyond the scope of this Review, and they have been reviewed elsewhere[200,201]. However, two promising strategies are of note, given their interesting and distinct mechanisms of action. Givinostat is a small-molecule histone deacetylase inhibitor (HDACi) developed by Italfarmaco as an epigenetic therapy for DMD. A recently completed randomized, double-blind, placebo-controlled phase III trial of givinostat (NCT02851797, EPIDYS) reported slowed disease progression in ambulant boys with DMD in the treatment arm. Eighteen months of givinostat treatment was reported to result in improved performance (that is, a reduced decline) in timed function tests, muscle strength analysis and fat infiltration in the vastus lateralis muscle as measured by magnetic resonance spectroscopy, according to Italfarmaco[202]. HDAC activity is elevated in dystrophic muscle as a consequence of impaired NO signalling[203], resulting in widespread alterations in gene expression that contribute to DMD pathology. Previously, givinostat-mediated HDAC inhibition was shown to improve muscle histopathology in the *mdx* mouse[19] and in a phase I/II clinical trial (NCT01761292) in boys with DMD[204].

Edgewise Therapeutics is developing EDG-5506, an orally bioavailable, small-molecule inhibitor of myosin that is specific to type II (fast twitch) fibres but not active against type I (slow twitch) fibres. A phase II clinical trial of EDG-5506 in patients with DMD (NCT05540860, LYNX) is currently recruiting. Fast twitch myofibres are more susceptible to contraction-induced damage in DMD[205,206], and individuals with inactivating variants in the *MYH2* gene encoding fast myosin exhibit mild proximal muscle weakness and typically lose ambulation[207]. As such, EDG-5506 is designed to protect dystrophic muscle by inhibiting the contraction of fast twitch myofibres and thereby paradoxically reducing muscle strength. Edgewise is pursuing clinical trials in both patients with DMD and those with BMD[208].

Combination therapy

It is increasingly apparent that there will likely be no 'one size fits all' therapy for DMD. The diversity of DMD-causing genetic insults means that a degree of personalization will be required to address specific mutation types. In addition, dystrophin restoration alone might be insufficient to correct the disease in patients with established pathology. For example, the progressive decline in muscle quality that results from chronic inflammation and fibro/fatty degeneration means that there might be relatively few fibres left in which to restore dystrophin by the time treatment is administered. Therefore, combination therapies capable of simultaneously restoring dystrophin expression and addressing the downstream molecular and cellular pathologies that occur in dystrophic muscle are desirable[209]. Notably, most novel DMD treatments are technically combination therapies as most patients are subject to chronic steroid regimens. However, there is a paucity of preclinical studies on the combination of experimental therapies with clinically relevant glucocorticoid cotreatment[210].

Although restoration of dystrophin protein expression by exon skipping has now been reported in numerous preclinical studies, achieving in human patients the kind of protein levels observed in dystrophic animals constitutes a significant challenge. Combining various dystrophin restoration strategies might lead to a synergistic benefit. For example, the combination of ASO-mediated and AAV-U7-snRNA-mediated exon skipping strategies led to reduced AAV vector loss and prolonged dystrophin expression[211]. AAV is a single-stranded DNA virus, and so second strand synthesis is required before a therapeutic transgene can be expressed. This results in a time delay between injection of the virus and therapeutic dystrophin rescue, during which time the muscle turnover associated with dystrophic pathology results in the loss of AAV vector genomes from treated muscle, and therefore a reduction in therapeutic efficacy[133]. Pre-treatment of *mdx* mice with ASOs (PPMO conjugates) resulted in a transient restoration of high levels of dystrophin and concomitant stabilization of muscle turnover, and then therapeutic AAV vectors were injected 2 weeks later[211]. This pre-treatment strategy resulted in a tenfold increase in dystrophin protein expression after 6 months, compared with mice treated with AAV alone[211]. Importantly, PPMO pre-treatment also enhanced AAV-mediated micro-dystrophin therapy[211], meaning that

this combination strategy is likely to be beneficial for enhancing any therapy that relies on AAV transduction. It will be interesting to determine whether such an approach can be used to improve the efficacy of CRISPR–Cas9-mediated dystrophin recovery.

Conventional pharmacological means have also been used to enhance the efficacy of exon skipping, including cotreatment with so-called skipping enhancer drugs such as dantrolene[212] and with various HDAC inhibitors[213].

Similarly, the possibility of combining dystrophin re-expression and utrophin upregulation has also been investigated in the *mdx* mouse[214]. Genetic overexpression of utrophin combined with PPMO treatment resulted in a complete restoration of muscle function to wild-type levels, which was not observed for either approach in isolation, as measured by force drop measurements in isolated extensor digitorum longus muscles[214]. Both dystrophin and utrophin can coexist at the sarcolemma, suggesting that this approach could be used in patients with DMD. However, very high levels of utrophin transgene expression in a wild-type mouse resulted in a decrease in dystrophin expression, suggesting that these two proteins compete for a finite number of occupancy sites at the sarcolemma[214]. On balance, this is not likely to be an issue for therapy as such high levels of utrophin are very unlikely to be achieved, and the two proteins co-localize at the sarcolemma when expressed at levels that prevent pathology in the mouse.

Cell therapy has also been combined with AAV–micro-dystrophin therapy in the dystrophic $CXMD_J$ dog model[215]. Cotreatment of AAV with bone marrow-derived mesenchymal stromal cells (MSCs) resulted in an improvement in the dystrophic phenotype in the single dog tested, which was attributed to the immunomodulatory properties of the MSCs[215].

Dystrophin restoration therapies might be augmented by combining them with a microRNA (miRNA) inhibition strategy. miRNAs are small RNA molecules that typically regulate gene expression by binding to partially complementary mRNAs and repressing translation and/or inducing target transcript degradation[216]. For example, miR-31 is highly upregulated in dystrophic muscle[16,217,218] and has a target site in the 3′ untranslated region (UTR) of the dystrophin mRNA[219]. This miRNA–target interaction is not expected to have any effect in the dystrophic condition, as dystrophin protein is not expressed. However, when dystrophin expression is restored via exon skipping, the high levels of miR-31 limit the degree of protein recovery[219]. Inhibition of miR-31 using expressed miRNA sponges or anti-miRNA oligonucleotides resulted in enhanced dystrophin rescue after exon skipping using the U1 snRNA system in the *mdx* mouse[219]. Similarly, miRNA regulation of dystrophin expression was shown to account for differences in dystrophin protein levels between patients with BMD with varying levels of disease severity[220], suggesting that miRNA inhibition might be further exploited to maximize dystrophin rescue in a therapeutic context. Notably, miRNAs that regulate utrophin expression have also been identified[221], and masking of a site for the let-7c miRNA on the utrophin 3′ UTR resulted in functional improvement in the *mdx* mouse by inducing utrophin protein upregulation[222]. Another miRNA, miR-29, was shown to suppress the expression of pro-fibrotic factors in dystrophic muscle, such that synthetic mimics of this miRNA could be used for therapeutic purposes. However, the combination of such an approach with a dystrophin restoration therapy has not been tested yet.

Many other approaches have combined dystrophin restoration with strategies to improve muscle quality. For example, co-delivery of two AAV vectors, one encoding micro-dystrophin and the other encoding the muscle isoform of insulin-like growth factor 1 (IGF1), resulted in synergistic benefits that were not observed for either vector in isolation[223]. Specifically, stabilization of myofibre turnover and protection against contraction-induced injury (attributed to expression of micro-dystrophin) was accompanied by an increase in muscle mass and strength attributed to the *Igf1* transgene[223]. The combination of exon skipping and myostatin blockade (to increase muscle mass) has also been explored in several studies[224–226]. However, myostatin blockade strategies have so far proved ineffective in DMD clinical trials, which is likely due to the already low circulating levels of myostatin in patients with DMD[227].

Conclusions and perspectives

The genetic insult that underlies DMD is relatively simple, and yet the goal of restoring gene expression has proved to be a substantial challenge. Although there are multiple drugs that have achieved marketing authorization in various jurisdictions, these are applicable to only small subsets of patients, and expert opinion on whether these offer therapeutic benefit is mixed. It is clear that better therapies are still needed. Improved ASO delivery technologies based on peptide, antibody and Fab fragment ASO-bioconjugation strategies, together with novel nucleic acid chemistries, have the potential to overcome the low efficacy of naked PMO-based ASOs, although renal toxicity must be considered carefully. By contrast, therapies based on gene replacement, utrophin upregulation, and disease-modifying approaches are in theory 'mutation agnostic' with widespread applicability. However, this notion has been challenged recently, as patients with certain mutation types might be susceptible to anti-transgene T cell immune toxicity. Furthermore, the high diversity of DMD-causing mutations means that personalized medicine approaches will likely be needed to treat all patients. In many cases, there may be insufficient patients to undertake conventional clinical trials. Alternative approaches include the use of Bayesian statistics to predict the pathological trajectory within a single patient or clinical trials with one patient.

The success of dystrophin restoration therapies is likely to be dependent on three key factors. First, the total amount of dystrophin restored. Evidence from preclinical models suggests that >15% of wild-type levels are required for functional correction[228], and levels >10–20% were associated with less severe pathology in patients with BMD[229,230]. Second, the quality of dystrophin produced. Therapies such as exon skipping, micro-dystrophin gene therapy and CRISPR exon deletion/skipping result in generation of internally truncated pseudo-dystrophins that are expected to have reduced functionality relative to full-length wild-type dystrophin. The degree of internal truncation differs between therapeutic strategies and so should be considered carefully. Third, the correct localization of dystrophin at the sarcolemma. We have recently demonstrated the importance of uniform sarcolemmal dystrophin for stabilizing turnover in dystrophic muscle[231]. This is important because the various dystrophin restoration strategies can lead to distinct patterns of sarcolemmal coverage[165,232].

Although it is often assumed that re-expression of functional dystrophin protein in the muscles of patients with DMD will correct the disease, combination therapies might be required to both correct the initial genetic insult and address the myriad molecular pathologies that occur in dystrophic muscle. This is especially important in the case of patients with low overall muscle quality and as a consequence of established pathologies and chronic disease. However, performing clinical trials for combination therapies is likely to be highly complex, especially if the individual therapies to be combined are only minimally

efficacious in isolation. It is therefore likely that such combinations will necessarily consist of already-approved single therapies. Facilitating such a therapy might require cooperation between pharmaceutical companies with distinct intellectual property portfolios.

The recent approval of the micro-dystrophin gene therapy SRP-9001 developed by Sarepta is an enormous step forwards for the DMD field and would follow on from the highly successful drug zolgensma for the treatment of SMA. Whereas zolgensma is a life-changing treatment, there has not been an equivalent breakthrough for DMD. Importantly, early therapeutic intervention in SMA is much more effective than treatment later in life[233]. Whether such early intervention, before the onset of pathology, would be beneficial in the case of DMD is unknown. Identifying applicable patients with DMD for early treatment and trial participation would necessitate the implementation of newborn screening programmes. Notably, there is delay in diagnosis of 2.2 years for DMD, which has largely remained unchanged over the past three decades[234].

In conclusion, there is a plethora of molecular medicine approaches that are under investigation, or approved for use, in patients with DMD. However, there is still a need for improved therapies with higher efficacy and that are applicable to a wider group of patients. In particular, treatments that can correct cardiac pathology are needed for maximum benefit to patients. Additionally, treatments that are effective for the whole lifetime of a patient, or that can at least be repeat administered, are also desirable. Knowledge gained from current drug development programmes, and especially from clinical data, will be crucial for the ongoing development of therapies for DMD, but will also be highly useful in the development of treatments for other diseases.

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Author contributions

The manuscript was conceived by K.E.D. and T.C.R. The first draft was written by T.C.R. All authors researched data for the article. All authors contributed substantially to discussion of the content and edited the manuscript before submission.

Competing interests

K.E.D. is a member of the scientific advisory board of Sarepta Therapeutics. M.J.A.W. is an adviser and shareholder in PepGen Ltd and Evox Therapeutics. T.C.R. declares no financial competing interests.

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