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REVIEW

Ongoing therapeutic trials and outcome measures for Duchenne muscular dystrophy

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Abstract Muscular dystrophy is a heterogeneous group of genetic disorders characterised by progressive muscle tissue degeneration. No effective treatment has been discovered for these diseases. Preclinical and clinical studies aimed at the development of new therapeutic approaches have been carried out, primarily in subjects affected with dystrophinopathies (Duchenne and Becker muscular dystrophy). In this review, we outline the current therapeutic approaches and past and ongoing clinical trials, highlighting both the advantages and limits of each one. The experimental designs of these trials were based on different rationales, including immunomodulation, readthrough strategies, exon skipping, gene therapy, and cell therapy. We also provide an overview of available outcome measures, focusing on their reliability in estimating meaningful clinical improvement in order to aid in the design of future trials. This perspective is extremely relevant to the field considering the recent development of novel therapeutic approaches that will result in an increasing number of clinical studies over the next few years.

Keywords Duchenne muscular dystrophy · Clinical trial · New therapeutic approaches · Exon skipping · Readthrough · Outcome measures

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Introduction

Duchenne muscular dystrophy (DMD) is the most frequent dystrophy in childhood, affecting 1 out of 3,500 newborns [1]. The disease is caused by mutations in the dystrophin gene (*DMD*), which has 79 exons and is located on the X chromosome. Dystrophin is a cytoskeletal protein that assembles other membrane-associated proteins, forming the oligomeric dystrophin–glycoprotein complex (DGC). The DGC includes dystrophin, dystroglycan subunits (alpha and beta), sarcoglycan subcomplex, syntrophins, and dystrobrevins, and functions as a bridge connecting extracellular matrix and cytoskeletal proteins, promoting the integrity and stabilisation of the sarcolemma during contraction. Because DGC proteins are tightly connected, abnormalities affecting one of the proteins lead to aberrant expression of the others and to impaired muscular function [2]. A consequence of the disruption of the DGC in DMD is also the dislocation and altered activity of a splice variant of the neuronal nitric oxide synthase (nNOS), an enzyme which plays a key role in myogenesis and muscle repair following acute and chronic muscle injuries [3].

More than 4,700 mutations have been described in *DMD* and are subdivided into deletions (65.8 %), duplications (13.6 %), and point mutations (micro-insertions, micro-deletions, and nonsense, missense, and splicing mutations; 20.6 %) [4]. Nonsense mutations and mutations that change the reading frame generally result in premature stop codons and a complete lack of dystrophin. Muscle fibres lacking dystrophin are easily damaged by mechanical insults and undergo cellular necrosis and fibro-adipose substitution. The lack of dystrophin is pathognomonic of the DMD phenotype. The detection of rare dystrophin-positive fibres, so-called "revertant fibres", as well as traces of dystrophin within the skeletal muscle, do not have diagnostic

implications in DMD patients [5]. DMD is characterised by onset before 3 years of age, independent ambulation loss between the ages of 10 and 14 years, and death between the ages of 20 and 30 years [6].

Mutations that do not disrupt the reading frame allow the production of a reduced amount of a qualitatively abnormal, but partially functional, protein. The related clinical phenotype is known as Becker muscular dystrophy (BMD) and exhibits greater heterogeneity [7]. Disease onset is variable from adolescence to adulthood, and death occurs after 30 years of age, generally as a consequence of cardiomyopathy or respiratory involvement, which leads to pulmonary insufficiency and heart failure.

Cardiac involvement represents one of the most important causes of death in DMD and BMD patients. An improvement in managing skeletal muscle and respiratory functions may overload the cardiac muscle, leading to early onset of cardiomyopathy [8]. Cardiac disease usually consists of dilated cardiomyopathy, which causes a progressive decline in the ejection fraction and fractional shortening, and generally evolves to heart failure, even with concomitant arrhythmia [9]. The age of onset varies considerably, but abnormalities in systolic function are present in more than 80 % of boys older than 18 years of age [8, 10, 11].

In BMD patients, the clinical manifestation of cardiomyopathy may also be the presenting symptom of the disease, and heart failure can be severe enough to require transplantation [7]. Patients with isolated dilated cardiomyopathy (X-linked cardiomyopathy) have been described [4]; in these subjects, dystrophin deficiency is restricted to the heart, and skeletal muscle strength and functioning are preserved.

Outcome measures and biomarkers

No effective treatment currently exists for dystrophinopathies and other muscular dystrophies. Patients are treated primarily using symptomatic approaches, such as cardiac medical therapy, pulmonary ventilation, nutrition management, orthesis, physiotherapy, psychosocial support, surgery for tendon retractions and scoliosis [12]. These strategies improve the patient's quality of life and have a significant impact on survival. Few data are available regarding the efficacy of other assistance interventions, but clinical evidence supports their importance [13]. Corticosteroid therapy slows the evolution of the disease [14]. Many studies and clinical trials assessing the effectiveness of molecules that modulate gene expression are ongoing. As a consequence of the development of these new potential therapies, the definition of the natural history and clinical outcome measures that can be used for disease follow-up has become essential. Clinical outcomes are directly related to a detailed knowledge of both the natural history of the disease and the mechanisms of action of each drug. Defining proper outcome measures is crucial for demonstrating drug efficacy in clinical trials; they should have high sensibility, sensitivity, and reproducibility. In many cases, available drugs are likely to result in only mild improvement, so outcome measures should be able to detect minimal changes in natural history, not just major changes.

Different outcome measures have been used in clinical trials, but none of them seem to be the gold standard [15].

Evaluation of motor function

One of the primary endpoints in clinical trials of boys with DMD is improved motor function. Measures of strength, such as myometry, timed function tests, and the 6-min walking test (6MWT), have been used in the majority of clinical trials. However, each of these methods has limitations. The quantification of muscular involvement is operator-dependent and can present inter-individual and intra-individual variability. Patient compliance, which may not always be optimal, influences the results, especially in children. The scientific community is still discussing the best criteria for patient evaluation, though some parameters have been chosen and validated for use in clinical trials. The strength measures should be obtained in a sensitive and reproducible way, but a definite standard has not yet been defined.

Manual muscle testing (MMT) has low reliability: the same parameters evaluated by different operators presented large variations and relatively long training is needed to obtain reproducibility. Quantitative muscle testing (using a manual dynamometer) and the timed functional tests (time to climb stairs, to walk 10 m, and to rise from the floor) record quantitative data and are more reproducible between different operators [16]. These data were collected as second outcome measures to evaluate patients who participated in trials with Ataluren and exon skipping molecules as detailed below. However, the clinical measures that are most frequently used in clinical trials are the 6MWT and North Star Ambulatory Assessment (NSAA); these assessments provide quantitative measures and can be subjected to statistical analysis [17].

The 6MWT is generally chosen as a primary outcome measure in the majority of trials; it is reproducible and documents disease-related limitations on ambulatory functioning [18]. The preservation of independent ambulation remains a major goal of dystrophy treatment. The results of the 6MWT correlate with age, anthropometric characteristics, stride length, and cadence. However, some confounding factors can be present. For example, this test does not consider the natural improvement in motor function in young children up to the age of 7 years, which is related

to an improvement in compliance and natural increase in stride length and cadence. In healthy children younger than 5 years of age, the rate of completed tests is considerably lower than in patients of at least 5 years of age (39 vs. 93 %), which is consistent with the subjects' developmental immaturity [18]. In subjects older than 7 years of age, the disease leads to progressive deterioration of the 6MWT distance. Results of this test significantly correlate with steroid therapy [18].

In clinical trials, a difference of 30 m among 1 year between drug and placebo is generally required for a clinically meaningful functional change. This difference represents 10–15 % of the distance generally crossed by DMD patients aged 4–12 years [18].

The NSAA is a 17-item rating scale. The items have a hierarchical order from easier (to stand, walk, get up, and stand on one leg) to more difficult (run, jump, and hop), and generally present similar trends; each one is quantified with a score from 0 to 2. The sum of each item gives a total score that is a reliable and valid measure of functional motor performance. This outcome measure also has some confounding factors: the presence of tendon retractions can negatively affect the total score, and this scale is not applicable to non-ambulant boys with DMD [15, 19].

Recent studies demonstrated a good correlation between the NSAA and 6MWT [20]. A 24-month observational study compared NSAA and 6MWT changes in a cohort of ambulant patients aged between 4.1 and 7 years with the objective of identifying potential early markers of a loss of ambulation. In particular, this study revealed that a distance of at least 330 m on the 6MWT or a NSAA score of 18 at baseline significantly reduces the risk of losing ambulation within 2 years. These data should be taken into account when selecting patients for clinical trials [21].

Even if trials are mainly designed for ambulant patients, outcome measures for non-ambulant subjects are necessary because some patients lose independent ambulation during trials. Furthermore future trials may also involve wheelchair-bound subjects. The Egen Classification (Egen Klassifikation, EK) scale is a validated scale consisting of 10 categories that test muscle strength, range of motion, and respiratory competence. In DMD patients, EK reflects the natural disease progression after years of wheelchair dependence [22], representing a valid instrument for evaluating the variation of clinical features during therapy.

The Motor Function Measure (MFM) is another scale adapted to all degrees of severity and applicable to both ambulant and non-ambulant patients. The MFM includes 32 items assessing the patient in lying, sitting, and standing positions. The scale is a good instrument for clinical trials because of its high reproducibility and reliability [23]. This scale is currently employed for the clinical analysis of patients affected with spinal muscular atrophy, but it could become a valid instrument for dystrophinopathic patients.

New studies are ongoing to evaluate upper extremity function in DMD [24].

Overall, these scales do not reflect all of the different levels of functional ability in daily living activities at different ages for DMD patients, and further studies are needed.

Another significant parameter that should be considered in patients with neuromuscular disease is the decline in pulmonary function, which is evaluated by forced vital capacity (FVC). The FVC provides an estimation of disease progression and its trend is predictive of survival time [25].

Other instruments have been used to quantify general muscular activity in children. For example, three different types of accelerometers have been tested. One of these accelerometers is the Step Watch Activity Monitor (SAM), which registers the frequency, duration, and intensity of movements. The Wear arm-band contains a two-axis accelerometer and is able to calculate data about the metabolic profile; it can also be used in non-ambulant patients and provides information about body position during the day. The last accelerometer is a wireless ambulatory system (ASUR) composed of three accelerometers and one gyroscope positioned in a t-shirt; this device is not yet commercially available [15].

Finally, motor function can be estimated through interviews or questionnaires (Pediatric Quality of Life, PedsQL) administered to the patient and/or the caregiver at different times to investigate the quality of life. The PedsQL is a reliable measure of health and, if integrated with other data, can contribute to an evaluation of potential improvement. It is currently used in every clinical trial [26].

Blood sample analysis

In clinical trials, blood samples are collected in order to study safety and efficacy. Serum creatine kinase (CK) levels are one of the parameters used most frequently, but it has never been chosen as an outcome measure because its interpretation can be misleading. CK levels at onset are typically high as an expression of muscle damage, but, in advanced stages, a decrease in CK is due to muscle substitution with adipose tissue. As a consequence, lower CK levels during a trial are not indicative of a potential therapeutic effect because they can be related to decreased cell death or the loss and substitution of muscle tissue.

Studies assessing the importance of cytokines and growth factors as markers of muscle degeneration and regeneration are currently ongoing. For example, in a recent work, TNF-α and β-FGF were found to be significantly increased, whereas VEGF was decreased in blood samples from DMD patients compared to controls [27].

These data can provide better insight into the pathogenesis of DMD.

Molecular biomarkers

Recent studies have evaluated the existence of potential new molecular biomarkers that can be used to gain insight into the cellular and biological mechanisms of the complete loss of dystrophin, and to obtain new parameters for assessing disease progression and the proper outcome of proposed therapeutic approaches. Although all DMD patients carry mutations that lead to a complete absence of dystrophin, the disease progression can differ considerably among subjects. The more likely explanation is the existence of genetic and environmental modifiers that are still not completely known.

Studies comparing gene expression patterns in dystrophic and wild-type cellular models have demonstrated a differential expression of 44 out of 21,920 analysed genes. Among these genes, 18 are related to processes involved in calcium homeostasis, and the others encode proteins taking part in antigen processing and presentation, muscle regeneration and differentiation, cytoskeleton activity, and extracellular matrix structure [28]. Recently, a correlation was demonstrated between the *SPP1*/*LTBP4* (human osteopontin protein/latent transforming growth factor β binding protein) genotypes and disease severity, including the loss of ambulation, and the response to steroid treatment. Both of these genes encode molecules involved in the TGF-pathways and likely influence fibrotic muscular substitution [29].

Other studies focused on modifications of the proteomic, metabolomic, and fluxomic profiles, which were explored using skeletal muscle and heart samples from *mdx* mice [30, 31]. Reduced expression of adenylate kinase 1 was the major proteomic alteration detected, and disrupted levels of muscle-specific chaperone proteins, such as cvHSP (muscle-specific heat shock protein), were demonstrated. At early stages, dystrophin deficiency leads to changes in the integrin-linked kinase pathway, actin cytoskeleton signalling, mitochondrial energy metabolism, molecules involved in skeletal muscle regeneration, and calcium homeostasis [32]. Fluxomic analysis allows alterations in cell dynamics that have not yet been related to structural changes to be highlighted, and the discovery of subclinical modifications.

The measurement and careful monitoring of serum markers of apoptosis (Fas/FasL and Bcl-2) and regeneration (TNF- α, β-FGF, and VEGF) has been proposed to monitor disease severity [27]. Chemokines are a large class of molecules implicated in inflammation, which is strongly activated during muscular degeneration and regeneration, promoting fibrogenesis as a secondary effect. These molecules are up-regulated in DMD patients [33], suggesting that their modulation can be targeted therapeutically. Unfortunately, the high number of these molecules, their different serum levels among patients, and the risk that, through immunomodulation, they may inhibit cells promoting skeletal muscle regeneration hamper their use in clinical practice.

In recent years, micro-RNAs (miRNAs) have been widely examined as potential biomarkers of DMD. They are small, non-coding RNAs involved in the regulation of gene expression and have been demonstrated to be deregulated in DMD patients. Increased serum levels of muscle-specific miRNAs, such as miR-1, miR-133, and miR-206 [34], were found in both animal models and human subjects affected with DMD, despite the reduction in muscle mass [35]. These data may be explained by the release of these molecules from damaged muscles into the blood. On the other hand, other miRNAs, such as miR-29 and miR-486, are down-regulated in tissues from *mdx* mice and human muscle, and they seem to be involved in muscle regeneration and differentiation [36]. Cacchiarelli et al. demonstrated a strong inverse correlation between miRNA levels and patient motor performance, suggesting their possible use instead of CK levels for studying disease severity and progression [37]. Interestingly, PMO administration normalises serum micro-RNA levels [35]. Thus, a potential future therapeutic approach could be the association of gene therapy with molecules modulating miRNA expression, as shown in *mdx* mice through intramuscular and intravenous administration [36]. Other studies should be implemented to gain better insight into the mechanisms leading to miRNA modification in order to identify reliable and more specific biomarkers, which will allow proper monitoring of the disease.

Muscle biopsy

Muscle biopsy is a fundamental outcome measure as it reflects the effective penetration of the drug into muscle tissue and its biological effects in regards to the preservation of muscle fibres and protein restoration. In DMD patients, revertant fibres and traces of dystrophin in the immunohistochemical (IHC) analysis are typical findings [38]. The most important parameters evaluated in muscle biopsies are the expression of dystrophin, its localisation on the membrane surface, the restoration of dystrophin-associated glycoprotein (DAG) complex expression, and the reduction in inflammation and necrosis.

A widely discussed issue is the necessity to perform different biopsies within a short time in young children. Revertant fibres do not replicate during the patient's life and, as a consequence, the new dystrophin expression resulting from treatment could be distinguished from protein that was already present. These data do not support the necessity of collecting a biopsy before any new experimental treatment [39].

The majority of clinical trials use IHC to detect dystrophin on muscle fibres. However, IHC analysis does not allow quantitative evaluation of the protein. None of the clinical studies have used a quantitative method. Western blotting (WB) analysis is not a quantitative technique; however, careful control of total loaded protein as well as the use of another non-sarcolemmal muscle protein for internal normalisation purposes, may help to estimate relative dystrophin amount. This methodology should be considered in future clinical trials.

Furthermore, the capability of dystrophin in muscle tissue to restore a functional DAG complex should also be considered. In a recent study, 65 patients were evaluated based on a motor ability score, and no differences were found between patients with or without revertant fibres or dystrophin traces at muscle biopsy [5]. This result can be explained by the lack of the protein domains involved in sarcolemmal binding or the dystrophin-positive fibres being too few to preserve muscle structure and function. In the muscle biopsy, re-expression of the DGC as far as nNOS denotes the functionality of the restored dystrophin [39].

Another important issue is to determine what level of dystrophin is adequate for determining a clinical improvement. Muntoni et al. [40] studied a group of BMD patients to evaluate the correlation between dystrophin expression and clinical phenotype with the objective of identifying a rational target for trials in DMD patients. The patients were selected according to their genotype, choosing patients who carried deletions around exon 51, the target of exon skipping therapy; in particular, eight patients carried deletions ending with exon 51, four patients carried large deletions of exons 45–55, and five patients carried deletions ending with exon 53. The semi-quantitative muscle analysis performed with IHC and WB revealed that the mean dystrophin expression was, respectively, 65 and 80 % in the first group, 70 and 83 % in the second group, and 56 and 77 % in the last group. Lower dystrophin expression was associated with the worst phenotype. Asymptomatic or mildly affected patients had at least 40 % dystrophin compared to control, with partial preservation of beta-dystroglycan and nNOS expression. Overall, based on these data, the cut-off of 40 % for dystrophin re-expression should be considered the minimal target necessary to change the evolution of DMD [40].

Muscle MRI

Muscle magnetic resonance imaging (MRI) recently became part of the neuromuscular diagnostic work-up, though it must be implemented further. This technique allows muscle tissue to be distinguished from fibro-adipose tissue and shows good correlation with histopathological findings [41]. MRI can be a good tool for selecting and monitoring patients with regards to the degree of muscular involvement in trial assessment.

Therapeutic approaches and clinical trials

Therapeutic approaches to DMD, translated into current clinical trials, have been focused on modifying the primary pathogenic event, aimed at expressing a functional dystrophin protein either by small molecule modulation of gene transcription or by gene transfer. Alternatively, the modulation of downstream targets in the DMD pathogenesis was pursued by different compounds, the first of which was historically corticosteroids. Furthermore, a myogenic regenerative program was under investigation using different type of stem cells. Successful treatment of DMD will probably ultimately consists of a combination of these approaches. We will revise the most important clinical trials that have been developed in recent years. As selection criteria, we considered the phase of the study, the sample size, and the available clinical results, focusing primarily on trials which were at most advanced stage, were multicentre and enrolled a number of DMD subjects possibly adequate to test the trial hypothesis. We also primarily considered trials which evaluated as outcome measures changes in motor function.

Pharmacological approaches that target the primary dystrophin defect

Readthrough strategy

Approximately 10–15 % of DMD patients carry mutations that generate a premature termination codon (PTC) [4]. In the past few years, several studies have aimed to select molecules capable of promoting mRNA translation despite the presence of a PTC. The first molecule tested was gentamicin, an aminoglycoside antibiotic that interacts with the 40S ribosome subunit and promotes ribosomal readthrough of PTC in mRNA, leading to the production of a functional full-length protein. In vivo studies of *mdx* mice have reported an up to 20 % increase in dystrophin-positive fibres [42]. In humans, a 15 % increase in dystrophin-positive fibres was found in 3 out of 16 patients [43]. Gentamicin exhibited limited clinical benefit because, to be effective, it needs very high concentrations, which are associated with severe side effects.

Recently, in vitro studies defined new molecules that act via the same mechanism. Among these molecules, Ataluren, previously known as PTC124, interacts with the 60S ribosome subunit and determines the bypass of PTC in mRNA. This molecule is orally bioavailable in aqueous suspension. In preclinical studies, muscle cells from

humans and *mdx* mice (both carrying *DMD* point mutations) that were exposed to PTC124 exhibited a two-fold increase in sarcolemmal dystrophin in the IHC compared to control cells [44]. *Mdx* mice were treated with oral, intraperitoneal, or combined administration of Ataluren. Mice treated with both oral and intraperitoneal administration exhibited a 20–25 % increase in dystrophin according to WB and decreased levels of CK [44].

The safety and tolerability of Ataluren was confirmed in a phase IIa study, which recruited 38 ambulant and non-ambulant boys older than 5 years of age who were not on ventilation support. IHC of muscle biopsy samples taken after 28 days of treatment revealed a mean 11.1 % increase in dystrophin expression. A significant reduction in CK levels was also recorded. A phase IIb double-blind, placebo-controlled clinical trial with PTC124 evaluated the safety and efficacy of administering the drug for 48 weeks in DMD/BMD patients [Phase 2b Study of PTC124 in Duchenne/Becker muscular dystrophy (DMD/BMD), NCT00592553, http://www.clinicaltrials.gov]. A total of 174 patients older than 5 years of age who were able to walk unassisted for at least 75 m during the 6MWT and who were steroid-free or on stable corticosteroid therapy for at least 6 months were enrolled. The boys were randomised to receive either a low dose (10, 10, 20 mg/kg/day) or a high dose of Ataluren (20, 20, 40 mg/kg/day) or placebo. The primary outcome was improved ambulation as assessed by the 6MWT (the goal was a difference of 30 m in the final distance compared to placebo). Ataluren pharmacokinetics, proximal muscle function and strength, cardiac function, frequency of accidental falls during ambulation, activity in the community setting, quality of life, cognitive ability, treatment satisfaction, safety, and compliance with treatment were also evaluated. The patients underwent two muscle biopsies of the brachial biceps, one at the beginning of the trial and the second at the end of the trial. The study was followed by an extension phase during which all the boys took a high dose of Ataluren. The study was stopped in March 2010 because the primary outcome was apparently not reached. However, a more detailed analysis of the data revealed a different response between the groups taking low and high doses. Pivotal data presented in October 2010 at the World Muscle Society Congress suggested that Ataluren is effective at low doses. Patients receiving low-dose Ataluren exhibited better performances in the 6MWT than patients receiving placebo (29.7 m more than placebo at the end of the study period) and less of a decline in timed function tests [45]. Patients treated with high doses exhibited a decline similar to the decline in patients taking placebo, suggesting an inverse correlation between clinical efficacy and drug concentration [46].

The lack of improvement at high doses should be investigated further, but it can be explained by either a bell-shaped curve for drug efficacy or the activation of cell-mediated immunity, as described by Mendell [47]. A phase III open label study is currently ongoing in the United States: low doses of the drug are being administered to patients who received Ataluren in a prior PTC-sponsored study. This study will evaluate the long-term safety of Ataluren based on adverse events and laboratory abnormalities (Study of Ataluren for Previously Treated Patients With nmDBMD in the US, NCT01247207, http://www.clinicaltrials.gov). In addition, a new phase III study that will test both the safety and efficacy of the drug at low doses has started in other countries (Study of Ataluren for Previously Treated Patients With nmDBMD Nonsense Mutation in Europe, Israel, Australia, and Canada, NCT01557400, http://clinicaltrials.gov).

Overall, these studies play a relevant role in the search for an effective etiological therapy for this group of disorders. The premature cessation of the phase IIb trial due to an illusory failure in reaching the primary outcome measure underlines the importance of accurately designing the clinical protocol. In particular, higher dosages are not always better than lower dosages. The effective plasma concentrations and drug pharmacokinetic must always be considered when designing clinical trials because the long-term efficacy can be influenced by an unpredictable immune response.

Exon skipping strategy

Most mutations in the dystrophin gene are deletions that disrupt the open reading frame. The length and structural characteristics of dystrophin, which contains repetitive domains, suggest the possibility of excluding disruptive exons from mRNA during splicing, partially preserving protein function. Residual dystrophin levels of 29–57 % have been hypothesised to ensure the preservation of muscle strength [48]. Based on this evidence, clinical trials were designed to promote exon 51 skipping in DMD patients, expecting an improvement in their clinical phenotype to at least a BMD-like phenotype [43]. The choice of exon 51 was based on two considerations: in-frame deletions of this portion of the gene are generally associated with mild BMD phenotypes [49], and out-of-frame mutations that could benefit from exon 51 skipping account for at least 20 % of *DMD* mutations [4].

Modulation of splicing is achieved with antisense oligonucleotides (AONs), DNA molecules capable of binding intronic and exonic mRNA sites and modifying splicing events. In DMD patients, AONs are used to exclude some exons from the transcript, restoring the reading frame [50]. The AONs used in preclinical and clinical studies are

primarily a 2′-O-methyl-modified ribose molecule with a full-length phosphorothioate backbone (2OMePS) or phosphorodiamidate morpholino oligomers (PMO). In PMO, the sugar-ribose backbone is replaced by diamidate-linked morpholino moieties that are refractory to metabolic degradation in vivo. PMO has 25 or more nucleotides, whereas 2OMePS has up to 20 nucleotides. Differences in sequence length appear to influence both AON biodistribution and AON-mediated exon skipping for some target exons [51]. The non-ionic backbone of PMO facilitates in vivo delivery to the cell, in contrast to the ineffective transfection demonstrated in cell models. On the other hand, the charged 2OMePS seems to have greater difficulty reaching muscle tissues, probably because it interacts with cellular components. Nevertheless, both molecules appear to be safe, even at high doses (up to 3 g/kg in the mouse for 2OMePS) [52]. The results of current trials in humans based on systemic delivery suggest that PMO results in higher dystrophin levels, but differences in target sequences and dosing preclude a strict comparison [52, 53].

An important limit in clinical trial design is that different deletions require specific AON sequences and specific regulatory approval. Theoretically, sequence-dependent 'off target' effects cannot be reliably tested in non-human species and each sequence should be considered a different agent that requires a complete preclinical pharmacological work-up and passage through the various phases of human trials. For deletions, which are present in a small number of patients, obtaining a significant population will be impossible. Some studies are ongoing to estimate the application of multiple exon skipping in order to increase the number of patients that can be subjected to the exon-skipping approach [54]. The multiple exon skipping strategy was developed to evaluate the possibility of treating with the same compound patients carrying different deletions. Patients with genotypes suitable for applying this multiple skipping approach represent 63 % of DMD patients. Recent studies using different AONs targeting up to 10 exons demonstrated the feasibility of this strategy in both cellular and animal models [55–57]. However, in-depth analyses are required before translating this approach to clinical practice [57]. Generally, the fewer exons excluded, the more functional the dystrophin protein obtained, because the most important functional domains are preserved. On the other hand, the contemporary removal of a large number of exons allows more DMD-causing mutations to be covered and the treating of a greater number of patients with a single mixture of AONs. For example, Aoki et al. suggested skipping exons 45–55, considering that individuals with this deletion present with an exceptionally mild BMD phenotype [56]. The feasibility of skipping these exons was tested with a mixture of 10 AONs in *mdx52* models, which represent another kind of DMD model carrying a deletion of exon 52, obtaining good results in muscle, but only low dystrophin levels in the heart. This therapeutic approach should be improved to obtain higher levels of dystrophin in the heart, the impairment of which represents one of the major causes of death in DMD patients [56]. These observations require further studies to discern the best approach in clinical practice and to define the most suitable AON molecule for clinical trials. Even with appropriate caution, some regulatory flexibility will be needed [52].

2′-O-methyl-phosphorothioate

Dystrophin expression was measured in vitro using 2OMePS-treated muscle cells from DMD patients and in vivo in *mdx* mice treated with intramuscular, intravenous, or subcutaneous injections of 2OMePS [58]. 2OMePS was tested for the first time in four DMD patients in an exploratory, open-label, single-centre study [58]. In that study, patients received a single 0.8-mg dose of PRO051/GSK2402968 (a 2OMePS antisense oligoribonucleotide capable of inducing exon 51 skipping) injected into the tibialis anterior muscle. Muscle biopsy was performed after 28 days [59]. The primary outcome of this trial was to assess the presence of adverse events in the four subjects, and the secondary outcomes were specific exon 51 skipping and dystrophin expression. The injection was safe, causing only moderate adverse events, such as mild local pain at the injection site. In all patients, RT-PCR identified a novel, shorter dystrophin gene fragment lacking exon 51. Immunofluorescence analysis of muscle biopsies revealed the presence of dystrophin in the majority of fibres. The amount of dystrophin in total protein extracts ranged from 17 to 35 % compared to control specimens and laminin α2 expression. Lower levels of dystrophin were detected in a patient with advanced disease, suggesting the importance of performing clinical trials in early phases. The dystrophin-restoring effect of PRO051 was limited to the treated area, and no functional improvement was observed.

In an open label phase I/IIa study [60], the same drug was injected subcutaneously into 12 patients for 5 weeks at four different doses (0.5, 2.0, 4.0, and 6 mg/kg). Muscle biopsy was performed on the tibialis anterior muscle between 2 and 7 weeks after the last dose. After 6–15 months, all patients were treated for 12 weeks at a dose of 6 mg/kg. The primary outcome was a safety assessment, but pharmacokinetic, molecular, and clinical parameters were also estimated. Only mild side effects were noted, including skin reactions at the injection site, increased urinary α1-microglobulin, and reversible proteinuria. After 12 weeks of treatment, no improvement was observed in specific muscle strength, but an increase in the distance covered during the 6MWT was observed. Muscle biopsy revealed an increase in dystrophin expression (number of

fibres and signal intensity) proportional to the injected dose compared to baseline. In 10 out of 12 patients, new dystrophin expression was demonstrated in 60–100 % of fibres, with an intensity of 15.6 % compared to healthy muscle. WB analysis of patients who received higher doses (4.0 and 6.0 mg/kg) found 1.5- to 8.2-times greater dystrophin expression compared to baseline if normalised to muscle-fibre content (represented by dysferlin levels).

Interestingly, pharmacokinetic studies demonstrated that multiple administrations of 2OMePS increased basal levels of dystrophin, and no antibody production was shown. Unfortunately, a correlation between dystrophin levels and 6MWT results were not identified because no muscle biopsies were performed at the end of the 12-week phase. An extension phase of this study, in which the same patients are treated with a dose of 6 mg/kg weekly, is currently ongoing.

Recently, a phase III double-blind trial started with 180 DMD patients in order to assess drug efficacy and safety and collect pharmacokinetic, molecular, and clinical data. Patients will be treated with subcutaneous 6 mg/kg GSK2402968 versus placebo for 48 weeks, followed by a 2-year open label study (A Clinical Study to Assess the Efficacy and Safety of GSK2402968 in Subjects with Duchenne muscular dystrophy, NCT01254019, http://clinicaltrials.gov/). The primary outcome will be a difference of 30 m in the 6MWT between the two groups. The secondary outcomes will be functional tests, respiratory performance, and quality of life. The trial requires two muscle biopsies, which will be performed for each patient at different times, but never at baseline.

If the exon skipping approach is effective, it can be applied to other kinds of mutations. Previous studies have mainly targeted exons whose skipping can restore the reading frame in the greatest number of patients. Skipping exon 44 could restore the reading frame in approximately 5 % of patients [4] and is currently being investigated in a phase I/IIa trial with PRO044 [Phase I/II Study of PRO044 in Duchenne muscular dystrophy (DMD); NCT01037309, http://clinicaltrials.gov]. This trial recruited 18 boys aged between 5 and 16 years who have a life expectancy of at least 6 months and have been treated steadily with steroid therapy. The molecule was administered once a week for 5 weeks subcutaneously (0.5, 1.5, 5, 8, and 10 mg/kg) and endovenously (1.5 and 5 mg/kg). The results of intravenous administration could be interesting, as the majority of side effects of subcutaneous administration are skin reactions.

Morpholino phosphorodiamidate

PMO was tested in *mdx* mice to evaluate skipping exon 23 of *DMD*. The molecule was administered in different ways (intramuscularly, intravenously, intraperitoneally), but an increase in dystrophin synthesis was found predominantly in the skeletal muscle, and partially in the myocardium [61]. The primary endpoint of this treatment was the restoration of dystrophin expression, but other proteins are also deregulated in *mdx* mice [62]. Doran et al. demonstrated a restoration of secondary pathological changes after *PMO*-mediated treatment in *mdx* mice compared to untreated mice. In particular, the study reported restored expression of the β-dystroglycan subunit, an increase in metabolic enzyme density, and re-establishment of calcium-regulating proteins, such as calsequestrin [62]. These findings suggest that *PMO*-mediated exon skipping therapy may have multiple effects, restoring not only dystrophin expression but also modulating other pathological changes involved in the pathogenesis and progression of DMD. The drug was also administered intravenously in Golden Retrievers, resulting in increased dystrophin expression in 25 % of fibres in muscle biopsies [63].

Dystrophin restoration and improved muscle performance were also achieved with exon 51 skipping promoted by PMO in *mdx52* mice [64]. The skipping of exon 51 was tested for the first time in humans in a single-site, non-randomised, single-blind study [65] that recruited seven boys aged between 10 and 17 years. The inclusion criteria were fewer than 5 % revertant fibres at muscle biopsy, preservation of the extensor digitorum brevis muscle, and forced vital capacity of 25 % or more. The primary endpoint was the safety of morpholino oligomer AVI-4658 (Eteplirsen), and the secondary endpoint was biochemical efficacy.

AVI-4658 was injected into the extensor digitorum brevis at high doses (0.9 mg, $n = 5$) or low doses (0.09 mg, $n = 2$). The contralateral muscle was injected with saline solution. Muscle biopsy was performed on the treated side after 3–4 weeks. As a baseline biopsy, the original muscle biopsies used for diagnosis in each patient were re-evaluated. The patients did not present with adverse events, with the exception of a localised skin reaction at the injection site. AVI-4658 induced skipping of exon 51 at the mRNA level in all patients in a dose-dependent manner. In the low-dose group, RT-PCR and sequencing confirmed the skipping of exon 51, but no dystrophin was detected at the muscle membrane. IHC revealed that patients treated with high doses of AVI-4658 exhibited an increase in the number of dystrophin-positive fibres and increased dystrophin intensity in each fibre (42 % of the intensity in healthy muscle) with proper localisation of the protein at the sarcolemma. These results were confirmed by WB. Whether the expression of dystrophin resulted in improved muscle function was not studied. No anti-dystrophin antibodies were produced.

This study was followed by an open-label phase II study [53] that tested systemic administration of AVI-4658 with a dose-escalating model. AVI-4658 was

administered intravenously to 19 ambulant DMD patients aged between 5 and 15 years at different doses (0.5, 1, 2, 4, 10, or 20 mg/kg) for 12 weeks. Muscle biopsy was performed after 12 weeks and compared to a muscle sample obtained before treatment [Dose-Ranging Study of AVI-4658 to Induce Dystrophin Expression in Selected Duchenne muscular dystrophy (DMD) Patients, NCT00844597, http://clinicaltrial.gov]. The primary endpoint was to assess the safety and tolerability of AVI-4658, and the secondary objectives were the pharmacokinetic properties and ability of AVI-4658 to induce skipping of exon 51 and dystrophin restoration.

Systemic administration was safe and tolerable. Dystrophin expression was proportional to the administered dose. Patients receiving high doses exhibited an increase in dystrophin-positive fibres, mean fluorescence intensity in IHC, and protein in WB. The proportion of dystrophin-positive fibres in the three patients with the greatest responses was 21, 15, and 55 %.

Dystrophin-positive fibres exhibited a restoration of α-sarcoglycan and nNOS expression and reduced CD3 cell count. Antibodies against dystrophin were not detected. Considering that muscle perfusion, damage, and inflammation can influence the PMO distribution, a longer period of administration will be appropriate to achieve homogeneous dystrophin expression.

In this study, muscle performance remained stable during follow-up, but the total duration (12 weeks) of the experimental design was not sufficient to assess the significance of the data. A 48-week extension study is currently ongoing and a pivotal phase 3 trial will be planned.

Recently, Anthony et al. [66] developed a reliable and sensitive Taqman qRT-PCR assay for determining the levels of exon skipping in DMD patients treated with Eteplirsen, showing that the level of dystrophin protein restoration correlates with the RNA level. This assay could provide an important baseline for quantifying treatment efficacy and is essential for developing new therapies.

Optimisation of AON-mediated exon skipping

Overall, exon skipping using AONs is the approach closest to clinical application. However, previous clinical trials demonstrated that the production of dystrophin, even if detectable in the muscle biopsy, is inadequate for determining effective clinical improvement and varies widely among different muscles. Furthermore, neither PMO nor 2OMePS generate significant amounts of dystrophin in the heart. In general, these chemicals have poor cellular uptake and rapid renal clearance. Several strategies for improving the efficiency of this approach are currently being investigated, and various adjustments have been tested with promising results. The strategies are mainly directed at improving cell penetration (in both the muscle and heart) to reduce the turnover of the compound and enhance exon skipping. All these structural modifications should balance improvements and potential toxicity [52].

Cell penetration can be implemented through the conjugation of AONs with cell-penetrating peptides (PPMOs) or dendrimeric octaguanidine polymers (vivo-morpholinos). For example, positively charged arginine-rich peptides administered in adult *mdx* mice by systemic intravenous injection have been shown to function as cell-penetrating peptides, enhancing the cellular uptake of a variety of AONs and resulting in uniform systemic correction of dystrophin expression in muscle and cardiac tissue [67]. However, the potential toxicity of the peptide-conjugated AONs will require caution in clinical applications [68]. On the other hand, other small compounds capable of promoting exon skipping or improving AON-mediated exon skipping are currently being studied. For example, TG003, a kinase inhibitor specific for Cdc-like kinases, has been reported to induce exon 31 skipping in cells harbouring a point mutation in this exon [69]. This compound is specific for this mutation and its application is limited.

Recently, after screening a library of 2,000 compounds, 6-thioguanine (6TG), a guanine analogue with an anti-leukemic role, was suggested to enhance the exon skipping efficacy of PMO AONs in vitro and in vivo [70]. These data were not confirmed in vivo for either 2OMePS or PMO [71], and the known cytotoxic effects of 6TG after incorporation into DNA led the scientific community to reconsider its use as a chronic treatment.

The study by Kendall et al. [72] was more promising. Intramuscular or intravenous administration of dantrolene, which acts as a ryanodine receptor (RyR) antagonist and is currently used to treat malignant hyperthermia, potentiated AON-guided exon skipping in the skeletal muscles of *mdx* mice, increased muscle strength, and reduced serum CK levels. Ryanodine and Rycal S107, which function as RyR modulators similar to dantrolene, also promoted antisense-driven exon skipping, implying that RyR is a potential molecular target. Although the modulation of different RyR subtypes may play a role in the enhancement of exon skipping therapy [73], the exact mechanism through which these molecules act still needs to be clarified.

Finally, another limitation of AON-induced exon skipping is the need to re-administer very expensive molecules at regular intervals throughout life. One way to deal with this limitation is to embed the antisense sequences into modified U1 or U7 small nuclear RNA (snRNAs) genes from which they can be transcribed. These plasmids can be delivered efficiently by viral vectors (adeno-associated viruses, AAVs) to muscle fibres, where they do not integrate into the host DNA, but exhibit long-term expression and promote sustained AON-mediated exon skipping [52].

Experiments to validate this approach have been performed in dystrophic mice and dog models [74], reporting levels of efficiency comparable to AONs. These data are encouraging for the translation of this approach into a well-designed human clinical trial.

Gene therapy

The gene therapy approach is directed at restoring the contractile capacity of the skeletal muscle by introducing a functional copy of *DMD* in muscle fibres. The main challenge is the large size of dystrophin cDNA (13 kb of sequence). However, the presence of very mild cases of BMD characterised by a conserved reading frame and loss of a major portion of the central 'rod domain' led to the design of mini and micro-dystrophins that can fit in viral vectors (retroviruses and lentiviruses).

The lentiviral approach has been tested in adult and newborn mice. The best results were achieved in younger animals: muscle biopsy revealed increased trans-gene expression in 65 % of the fibres. In addition, the muscle was more resistant to contraction and able to generate greater strength [75]. Lentiviruses can also carry gene molecules capable of inducing exon skipping. Lentiviral vectors expressing AON were injected into the skeletal muscles of *scid/mdx* mice, a subtype of *mdx* mice carrying the severe combined immunodeficiency mutation, and, as expected, a month later, the muscle biopsy showed the re-expression of a shorter dystrophin protein lacking the sequence encoded by exons 49-51 [76].

AAVs have demonstrated more efficient delivery to skeletal muscle and reduced immunogenicity, but they manage to carry only small trans-genes. Thus, they can be employed to transport micro-dystrophins, in which the central region and C-terminal domain of the dystrophin gene are deleted but essential functional domains are maintained [77].

Which micro-dystrophins and mini-dystrophins will be the most appropriate for AAVs is uncertain. The use of "trans-splicing", which leads to the insertion of a full-length dystrophin by taking advantage of "trans-splicing" mechanisms that allow the assembly of multiple, single constructs encoding different functional domains during the splicing event, may be possible [52]. Different serotypes have been tested in various animals: *mdx* mice, dogs, and primates [78]. In mice and primates, the proportion of dystrophin-positive fibres is reported to be 80 %. However, this percentage decreased to 40 % in primates because they developed an immune response against the virus. Dogs presented with an important cytotoxic immune activation, but this event is unlikely in rodents.

A clinical trial was recently carried out in six dystrophic patients. Low and high doses of AAV serotype 2 carrying a mini-dystrophin were injected into the biceps muscle [47]. Muscle biopsies were performed 42 days after administration in four patients and after 90 days in two patients and compared to a sample of contralateral untreated muscle. All the samples contained the DNA vector, though in different amounts. Three patients exhibited mini-dystrophin-specific T cell activity, though with differences in the timing and duration of the immune response. Lymphocyte infiltration suggested an unpredictable T cell immune response against the viral vector, which should be considered when designing and monitoring experimental therapies. The major obstacles limiting further development of the AAV approach are: (1) defining an appropriate construct for delivery, (2) the technical production of a sufficient amount of infectious viral particles at a reasonable cost and at the best quality for human application, and (3) the possible immunogenic response, which is associated primarily with neutralising antibodies that block subsequent transductions with the same AAV serotype and a cell-mediated immune response due to the persistence of viral proteins. Immune system activation might be overcome with short-term immunosuppressive therapy at the time of AAV administration [43].

Recently, micro-RNAs were demonstrated to play a role in the pathogenesis of DMD. The down-regulation of key micro-RNAs in mice leads to difficult muscle regeneration and fibrogenesis as a result of the conversion of myoblasts into myofibroblasts. An interaction between cytokines and micro RNA has also been demonstrated, as high levels of inflammatory mediators (e.g. TGF-b) in dystrophic muscle determine the loss of micro-RNAs and promote fibrogenesis. These findings suggest a possible use of micro-RNAs for slowing the progression of DMD, but the reliability of these data has not been evaluated [79, 80].

Pharmacological approaches that target the secondary pathogenic effects downstream of dystrophin deficiency

An alternative strategy to the reintroduction of functional dystrophin protein and restoration of the DAG complex is the modulation of the downstream pathogenic effects of dystrophin deficiency, which ultimately lead to muscle loss.

These therapies will be applicable to all DMD patients, irrespective of their dystrophin mutation, and can also be combined in order to improve their efficacy.

Immunomodulation: steroid therapy and other treatments

The use of corticosteroids leads to an improvement in the muscle strength of patients affected with DMD [14]. Long-term therapy delays the loss of ambulation (by 2–5 years), reduces the need for vertebral surgery, improves

cardiopulmonary function, postpones non-invasive ventilation, and generally improves life expectancy and quality [81]. Starting treatment before the plateau in motor skills (4–6 years of age) is strongly recommended [12], whereas therapy is not indicated in patients younger than 2 years of age. Introducing treatment after the loss of ambulation appears to preserve upper limb strength, reduce the progression of scoliosis, and delay pulmonary and cardiac decline [12].

The key mechanisms of action of corticosteroids are still poorly defined but are probably related to the modulation of cellular events, including apoptosis, inflammation, regulation of Ca2 + concentration and myogenesis. In particular, benefits resulting from steroid treatment in DMD are likely related to the modulation of NF-kB, a cytoplasmatic transcription factor, whose activation leads to an increased expression of proinflammatory molecules including cytokines, chemokines, immunoreceptors, cell adhesion molecules and inflammatory enzymes. In dystrophic muscle, sarcolemmal instability and consequent altered calcium homeostasis promote cytokine-mediated NF-kB activation, which perpetuates the immune response involved in muscle wasting [82].

Patients should be monitored to manage side effects (e.g. hypertension, growth retardation, immune/adrenal suppression, glucose intolerance, weight gain, osteoporosis, and hyperactivity) [14]. Guidelines recommend daily or discontinuous prednisone (0.75 mg/kg/day) or deflazacort (0.9 mg/kg/day) [83], but no data yet demonstrate which choice is better.

A planned multi-centre, double-blind clinical trial (Duchenne muscular dystrophy: double-blind randomised trial to find optimum steroid regimen; NCT01603407; http://www.clinicaltrial.gov) will compare three different corticosteroid regimens widely used in DMD: daily prednisone (0.75 mg/kg/day), intermittent prednisone (0.75 mg/kg/day, 10 days on, 10 days off), and daily deflazacort (0.9 mg/kg/day).

A total of 300 children between 4 and 7 years of age who have never taken steroids will be recruited. The primary outcome measures will be: time needed to rise from the floor, respiratory function evaluated by forced vital capacity (FVC), and parent/subject satisfaction with treatment (Treatment Satisfaction Questionnaire for Medicine, TSQM). Secondary outcomes will be tolerability, adverse events, North Star Ambulatory Assessment (NSAA), 6 min walking test (6MWT), range of motion (goniometry), quality of life, and cardiac function.

This study will be relevant not only for the identification of the more appropriate steroid protocols but also to define the natural history of DMD patients treated with steroids. The majority of new trials will likely combine new target molecules with standard of care.

A potential role of cyclosporine A as an immunomodulatory agent was also suggested following its use in stem cell therapies. Cyclosporine A, as glucocorticoids, modulates the chronic immune response in dystrophic muscle, but its mechanism is immunosuppressant, probably through a direct inhibition of T cell sub-populations and the release of proinflammatory cytokines. Other mechanisms of action involve the prevention of the formation of mitochondrial permeability pore through the blocking of cyclophilin D activity and, unfortunately, an impairment of muscle regeneration resulting from the inhibition of calcineurin [82, 84]. A double-blind clinical trial was performed with 153 DMD patients aged 5 years or older who were still ambulant. The drug was administered orally twice a day at a dose of 3.5–4 mg/kg for 3 months, followed by prednisone for 12 months. The primary outcome measure was muscle strength as estimated by the Medical Research Council (MRC) Scale. The results revealed no difference between patients who received cyclosporine A followed by steroids and patients who received only steroids, but assessed the safety of cyclosporine A in DMD population [84].

However, knowledge of the impact of immunosuppressant therapy on the natural history of the disease is important in regards to its use in cellular transplantation approaches and gene therapy trials.

Histone deacetylase inhibitors

Recently, givinostat, a histone deacetylase (HDAC) inhibitor used to treat juvenile arthritis, has been suggested as a potential therapeutic approach in DMD. Acting on muscle-resident stem cells, HDAC inhibitors increase skeletal myogenesis in vitro and in vivo, restore normal muscle morphology, and enhance the size and strength of myofibres in *mdx* mice, the most common animal model of DMD. *Mdx* mice carry a point nonsense mutation in exon 23 [85]. Long-term exposure to givinostat effectively increases the cross-sectional area of myofibres, decreases the cellular (inflammatory) infiltrate, and prevents the formation of fibrotic scars in this model.

HDAC inhibitors promote the transcription and the activity of the muscle-specific transcriptional activators MyoD and MEF2 proteins, that are, instead, inhibited by the epigenetic negative regulation of HDAC [86]. Further, they favour muscle myogenesis and regeneration inducing muscle-restricted expression of follistatin that inhibits myostatin activity and promotes myoblasts recruitment and increases their fusion index [87].

A two-part open label clinical study that will assess the efficacy of givinostat in ambulant DMD patients aged 7-11 years will begin soon (A 2-Part Study to Assess the Safety and Tolerability, pk, Effects on Histology and Some

Clinical Parameters of Givinostat in Ambulant Children With DMD, NCT01761292; http//:www.clinicaltrial.gov).

Muscle nitric-oxide dependent pathways

The dislocation and decreased function of nNOS, caused by dystrophin deficiency at the subsarcolemmal region, result in reduction of associated nitric oxide (NO).

This loss is not only detrimental to the role played by NO as a free radical scavenger but also to the consequent drop in NO-cyclic guanosine monophosphate (cGMP) signalling. This leads to impaired cardiac, smooth and skeletal muscle contraction and relaxation. NO has also been implicated in satellite cell activation and differentiation, key events of muscle repair [88].

Several other NO-related functions are likely impaired in DMD skeletal muscles, including the functional sympatholysis of the basal adrenergic vasoconstriction of muscle vessels which follows physical activity: in fact, muscle fibres of DMD patients exhibit an increased susceptibility to functional ischemia [89].

The importance of NO pathways has encouraged the use of its potentiation as therapeutic approach in DMD [3]. Indeed, the exposure to a combination of NO donors and anti-inflammatory drugs, for example non-steroidal anti-inflammatory agents (NSAIDs), was tested in *mdx* mice and demonstrated long-term therapeutic effects on disease progression and muscle function [90, 91]. Recently, the safety and tolerability of the co-administration of isosorbide dinitrate, a NO donor, and ibuprofen, a NSAID, in dystrophic patients were confirmed in an open-label, single-centre pilot study [92]. These data open novel perspective for the use of NO-based approaches in DMD and support the design of new clinical trial to evaluate their efficacy.

Recently, the regulation of phosphodiesterase type 5 (PDE5) has also been suggested as a potential therapy. In fact, this enzyme is essential to maintain the balance between synthesis and degradation of cGMP, a critical player downstream to the NO pathway. Tadalafil is a potent and selective PDE5 inhibitor, presently approved for the treatment of erectile dysfunction, pulmonary arterial hypertension and prostatic hyperplasia [93]. Following the amelioration of muscle damage demonstrated in *mdx* mice treated with PDE5 inhibitors, a randomised placebo-controlled clinical trial was performed with BMD patients confirming that tadalafil enables to restore a physiological muscle response to exercise [94, 95]. An open-label, single-dose study of tadalafil in DMD patients aimed at defining an efficient dose-titration for the design of a larger randomised multicentre trial is ongoing (functional muscle ischemia and PDE5 inhibition in Duchenne muscular dystrophy: acute dosing study; http//:www.clinicaltrial.gov).

Oxidative stress

Idebenone is a synthetic analogue of coenzyme Q10 with strong antioxidant properties and a protective role against mitochondrial damage. Long-term treatment with idebenone in *mdx* mice revealed its role in cardiac dysfunction prevention and improvement of motor performance [96]. Based on these data, a phase IIa randomised placebo-controlled clinical trial was designed to investigate effects on cardiac, respiratory and skeletal muscle function and to assess the safety and tolerability of the molecule (a phase IIa double-blind, randomised, placebo-controlled, single-centre study at the University of Leuven to assess the efficacy and tolerability of idebenone in 8- to 16-year-old males with cardiac dysfunction associated with duchenne muscular dystrophy; http//:www.clinicaltrial.gov). Compared to patients on placebo, patients treated with idebenone exhibited a significant increase of peak systolic radial strain in the left ventricle infero-lateral wall, an early marker of systolic dysfunction, and an improvement of respiratory parameters (peak respiratory flow and maximal inspiratory pressure). No differences in upper limb muscle strength were disclosed. The significance of these findings was limited by the small number of patients, the fixed dose of idebenone independently from the body weight and the disparity of age difference between the treatment and placebo groups [97]. A larger open-label extension study aimed to explore long-term safety and tolerability of idebenone and its effects on cardiac, respiratory and motor function has just been completed and the analysis of data is still ongoing (a phase II open-label extension study to obtain long-term safety, tolerability and efficacy data of idebenone in the treatment of Duchenne muscular dystrophy—Extension to Study SNT-II-001; http//:www.clinicaltrial.gov). A phase III study with a larger cohort of DMD patients is underway (a phase iii double-blind, randomised, placebo-controlled study of the efficacy, safety and tolerability of idebenone in 10- to 18-year-old patients with duchenne muscular dystrophy; http//:www.clinicaltrial.gov).

Insulin-like growth factor-1

Insulin-like growth factor-1 (IGF-1) is a hormone whose receptors are expressed in all cell types and tissues. Its interaction with its tyrosine kinase receptor promotes a cascade of events involving PI-3/AKT kinase pathways which, finally, lead to an increased protein synthesis and the inhibition of apoptosis. In skeletal muscle IGF-1, modifying protein phosphorilation status modulates the expression of myogenin, a protein involved in differentiation process, inhibits p27 KIP, a cell-cycle inhibitor, and promotes myoblast and satellite cell proliferation, and MyoD and Mef2 expression to control differentiation. [98].

The use of IGF-1 in preclinical studies in *mdx* mice determined an increased body mass, fibre size and resistance to the fatigue and a reduction of inflammation [99]. Recently, a randomised clinical trial aimed at investigating IGF-1 effects on muscle functioning has started. The primary outcome consists in the evaluation of differences in 6MWT performance after 6 months of treatment; secondary outcomes are the growth rate, global motor performance and pulmonary and cardiac involvement (IGF-1 Therapy and Muscle Function in Duchenne muscular dystrophy; http//:www.clinicaltrial.gov).

Cell therapy

Studies and trials aimed at evaluating the applicability of stem cell therapy are currently ongoing. Transplanted stem cells can fuse with endogenous myofibres, providing regenerative potential or a wild-type copy of the defective gene. Unfortunately, previous trials of myoblast transplantation in humans were disappointing for several reasons, including a low migratory and engraftment ability of the cells [52].

Other cell populations characterised by more favourable engraftment properties have been tested, including mesangioblasts, pericytes, CD133+ stem cells derived from muscle and blood, and other primitive myogenic cells derived from muscle [43]. One of the most interesting and important aspects is the ability to migrate through vessels; therefore, they can be administered with a systemic injection, such as intra-arterially [100].

A clinical trial to evaluate mesangioblast allografts in patients affected with DMD has been designed. Another development may be the use of autologous cells corrected ex vivo instead of heterologous wild-type cells. The possibility to obtain mesoangioblasts from dystrophic *mdx* mice with an HAC vector holding the entire (2.4 Mb) human dystrophin genetic locus was recently described [101].

Another promising stem cell source is represented by the CD133+ cell population. The CD133 antigen is a member of a novel family of stem cell surface glycoproteins. Initially, expression of the CD133 antigen was seen only in hematopoietic-derived CD34+ stem cells. CD133 expression was demonstrated in undifferentiated cells, including myogenic and neural stem cell populations. Thus, CD133 may represent a novel marker of various stem cells and progenitor cell populations [102]. CD133+ stem cells isolated from human blood and muscle have been shown to be capable of promoting muscle regeneration after intramuscular or intra-arterial injection in *SCID* mice [103]. In addition, the safety of intramuscular transplantation of muscle-derived CD133+ cells in DMD patients has been tested [104]. To use autologous cells, the mutation in dystrophic cells must be fixed before transplantation. A possible strategy is the correction of CD133+ myogenic cells through permanent exon skipping, for example using U7 viral constructs, and then the delivery of these cells back to the dystrophic muscle. Human genetically engineered DMD blood- and muscle-derived CD133+ cells contribute to the expression of dystrophin in the muscles of *scid/mdx* mice [76]. A clinical trial is currently ongoing in order to investigate the safety and efficacy of autologous ex vivo-corrected stem cells in DMD patients. Cell therapy would be more advantageous in the early stages of the disease course, when tissue regeneration facilitates successful treatment.

Perspectives and conclusions

No effective therapy currently exists for dystrophinopathies. Efforts have been focused on discovering new therapeutic approaches, from drugs that mitigate the clinical presentation, acting on, for example, inflammation and remodelling, to gene and cell therapies. In this work, we have summarised the most important clinical research, including the trials that are still ongoing and those that are about to begin. The most promising approaches, which have reached more advanced clinical phases (IIb and III), are the readthrough strategies and the use of AONs. Gene therapy with AAVs and cellular therapies with mesangioblasts, pericytes, and CD133+ stem cells, are very promising, but they are currently in either a pre-clinical stage or a safety stage of development. Readthrough with Ataluren, studied in a phase 2b trial, is safe and well tolerated, with mild improvement in patients who receive lower doses; the ongoing open label trials in the USA and Europe will corroborate the long-term safety and contribute data on Ataluren's efficacy.

AONs are also safe and tolerable [48, 50, 31], determining an increase in dystrophin expression and improved 6MWT results. However, the target specificity of these drugs restricts the number of treatable patients with each oligonucleotide. Furthermore, the degree of muscle tissue uptake and drug efficacy have been inadequate for determining the effective clinical improvement, and additional studies are needed to identify new molecules with increased cell penetration capability, better tissue distribution, and higher exon skipping levels. However, every structural modification and drug association must balance improvement and potential toxicity [52].

None of these approaches, if taken alone, have been shown to restore adequate dystrophin levels and improve the clinical phenotype. Only the combined use of multiple approaches acting on both the molecular and symptomatic levels will likely achieve real efficacy. For example,

the combined use of AONs and molecules which enhance splicing, such as dantrolene, is currently being studied [72].

Generally, in recent years, the number of drugs that can be used in DMD clinical trials has significantly increased. The most accurate and reliable outcome measures to be used in clinical trials to evaluate patients over time, prove drug efficacy, and detect minimal changes and improvements are currently being debated [104]. Clinical parameters must be as uniform and quantifiable as possible in order to reduce operator- and patient age-dependent variables.

Because the patients are generally young children, tests must take into consideration problems regarding compliance and developmental phases, as natural growth may lead to a temporary improvement in muscular and pulmonary performance [1]. These aspects often limit the number of patients and the age range of the cohort. Outcome measures must be chosen that reflect the evolution of the disease and expected therapeutic effects as much as possible, and they can be defined only through proven experience in clinical trials and an accurate knowledge of the natural history of the disease. During the first phase of the disease (up to approximately 7 years of age), the children present with improvement due to growth and learning, but in the period that follows, they experience a progressive decline that ends with loss of ambulation. The time when therapy is started influences the success of the treatment.

We do not yet have a definitive description of the disease course using novel quantitative outcome measures, as patients evaluated according to these parameters are in different stages of their lives and the disease. The 6MWT and NSAA approaches appear to be the better approaches and to have a good correlation with disease progression [19]. However, quantitative measures that define upper limb strength in patients who have lost independent ambulation also have to be included and are under consideration [22]. Outcome measures should not only be reliable and reproducible but should also provide quantitative results that can easily be analysed and undergo statistical analysis.

A lot of outcome measures were used in the first trials, but the experience demonstrated that “fewer is better”. For example, whether the data obtained with the accelerometer and the myometer in the Ataluren phase 2b trial were useful is unclear, as they were abandoned in subsequent studies. Previous experience has taught researchers to pay great attention to muscle biopsy programming and analysis. Even if the final outcome is the amelioration of clinical aspects, muscle tissue is an important indicator of drug distribution and cell penetration. In contrast, improvements of muscle biopsies cannot be considered a unique marker because they do not necessary reflect clinical efficacy.

Quantitative analysis of dystrophin and DGC should be fully developed. Though the first trials scheduled two muscle biopsies at the beginning and the end of the study, recent work has demonstrated that revertant fibres do not replicate during the patient’s life [40]; therefore, muscle biopsy should be performed only during the treatment period.

The increasing number of clinical studies points to the importance of reaching a diagnosis using only molecular analysis, sparing a muscle biopsy, which will probably be performed when the patient is enrolled in the trials. Previous experience underlines the importance of a precise definition of pharmacokinetic parameters, plasma concentrations, and the immune response. Overall, clinical trials in the DMD population are demanding and require a design that takes into account both the natural history and the mechanism of action. Further studies are needed to clearly define the most suitable outcome measures and to develop drugs characterised by a strong efficacy in vitro and in vivo. We hope that the strong improvement in and increasing number of clinical trials in recent years will soon lead to the development of novel and more effective strategies for DMD patients.

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