Muscular dystrophies

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Search strategy and selection criteria

Data for this Review were identified by searches of MEDLINE, Current Contents, PubMed, and references from relevant articles using the search terms “muscular dystrophy”, “’ Duchenne”, “congenital”, “limb girdle”, “therapy”, “care” . Abstracts and reports from meetings were included only when they related directly to previously published work. Only articles published in English between 2013 and 2018 were discussed. Current trials were identified using the websites “www.clinicaltrials.gov” and “https://www.clinicaltrialsregister.eu/”

Summary

Muscular dystrophies are primary diseases of muscle due to mutations in more than 40 genes which result in dystrophic changes on muscle biopsy. Now that most of the genes responsible for these conditions have been identified, it is possible to accurately diagnose them, and implement subtype-specific anticipatory care, as complications such as cardiac and respiratory muscle involvement vary greatly. This, and advances in the field of supportive medicine have changed the standard of care, with an overall improvement in the clinical course, survival, and quality of life of affected individuals.

The improved understanding of the pathogenesis of these diseases is rapidly been exploited for the development of novel therapies. In the most common form, Duchenne muscular dystrophy, a few personalized therapies have recently achieved conditional approval and many more are at advanced stages of clinical development. In this Seminar, we concentrate on clinical manifestations, molecular pathogenesis, diagnostic strategy, and therapeutic developments for this group of conditions.

Introduction

Muscular dystrophies (MDs) are a group of genetically inherited degenerative disorders of muscle sharing clinical features of progressive muscle weakness and dystrophic pathological appearance on muscle biopsy[1]. In a *Seminar* published in 2013[2] we reported how a better understanding of the genetic basis and of the mechanisms underlying these disorders had allowed to appreciate a previously not anticipated genetic, mechanistic and clinical heterogeneity. From a clinical perspective the spectrum expanded to include conditions with associated involvement of other organs, for example the central nervous system; from a genetic perspective, a much larger number of individually rare conditions were described[3,4]. At the time, the development of novel therapeutic approaches had just begun their course[5-9]. In this review we will discuss how the field has evolved over the last five years. We will provide an update on the mechanisms that can result in MD and an indication on how experimental therapeutic approaches are rapidly advancing.

Classification and pathogenesis

Molecular genetic tools have allowed the identification of more than 50 genes involved in MDs[10,11], very rare MD variants continue to be identified. The classification recognizes conditions with early onset grouped under the congenital muscular dystrophies (CMDs), and later onset conditions (limb girdle muscular dystrophies, LGMDs). Allelic mutations in several genes can cause onset either before or after the acquisition of ambulation, providing a pragmatic boundary between CMDs and LGMDs. Additional common variants are Duchenne muscular dystrophy

(DMD), Becker muscular dystrophy (BMD); Emery-Dreifuss muscular dystrophy (EDMD) and facio-scapulo-humeral muscular dystrophy (FSHD), all recognised and clearly described long before most of the LGMDs and CMDs, due to their unique clinical features or high incidence, or both.

We favour a classification recognising the major group of conditions, and the genes responsible for their causation, together with function of the responsible proteins. The main classes of proteins involved are: **a.** extracellular matrix (ECM) and basement membrane; **b**. sarcolemma associated proteins; **c**. enzymes or proteins with putative enzymatic function; **d**; nuclear membrane; **e**. sarcomeric; **f.** endoplasmic reticulum; **g.** others.

CMDs are typically due to mutations in proteins located in the ECM, or external membrane proteins or enzymes involved in their posttranslational modification (table 1 and figure 1). Mutations in nuclear proteins typically result in EDMD while defects in sarcolemmal and sarcomeric proteins mostly cause LGMDs (table 2).

a. *Proteins of the extracellular matrix and basement membrane (table 1).*

Abnormalities in proteins at these locations result in the most common variants of CMDs: collagen VI, laminin 211 and the cellular receptor alpha-dystroglycan (ADG). (figure 1)[12]

Collagen VI deficiency can be caused both by autosomal recessive or dominant mutations in any of the 3 major collagen 6 alpha chain genes (*COL6A1-3*) (figure 2) [13-15]. The severity ranges from the CMD variant Ullrich CMD, to a milder form which resembles a LGMD (Bethlem myopathy).

Laminin α2 (encoded by the *LAMA2* gene) is the large chain in the laminin heterotrimer laminin 211, composed of the subunits α2, β1, γ1, which represents the main isoform in the basement membrane of muscle (figure 2). Recessive mutations in *LAMA2* commonly lead to complete loss of protein production, associated with severe congenital presentation[16]; rare milder variants can resemble LGMD[17].

b. Sarcolemma associated proteins.

The major subcomplex in this category is the dystrophin-associated glycoprotein complex (DAGC) which comprises dystrophin and sarcoglycans, in addition to dystroglycan. Proteins belonging to this group give rise to Duchenne MD (DMD) the most common form in childhood, and its milder allelic condition Becker MD (BMD), due to mutations in the X-linked *dystrophin* gene (figure 1 and 2); and to 4 autosomal recessive LGMDs known as sarcoglycanopathies, each secondary to mutations in one of four sarcoglycan genes (Table 2). The DAGC plays an important role in stabilising the muscle fibre against the mechanical forces of muscle contraction by providing a shock-absorbing connection between the cytoskeleton and the extracellular matrix.

A separate but functionally related group of sarcolemmal proteins are dysferlin and anoctamin 5. Dysferlin plays a crucial role in the repair process of the sarcolemma[18-20], and anoctamin 5 also contributes to this process[21-24]. Dysferlin interacts at sarcolemma with caveolin 3, involved in a rare LGMD variant, while mutations in the Polymerase-I-and-transcript-release-factor gene (*PTRF*, also known as cavin), necessary for caveolar formation, result in a secondary deficiency of caveolin 3 and associated LGMD[25,26].

c. Enzymes or proteins with putative enzymatic function (Figure 1)

i. Defects in the glycosylation of ADG were originally identified in several CMD subtypes (table 1) but subsequently in LGMDs. These conditions are referred to as dystroglycanopathies[8,27]. ADG is highly post-translationally modified via numerous enzymatic steps [28]. Binding of ADG to its muscle extracellular matrix partners (of which laminin is a major one, figure 1) is dependent on its proper glycosylation, and mutations in 18 proteins with demonstrated or putative enzymatic function give rise to a dystroglycanopathy (Table 1). The clinical and pathological features range from LGMD phenotypes to CMD with structural brain involvement [29-31], underscoring a fundamental role of ADG glycosylation not only for muscle but also brain basement membrane maintenance and synaptic function[32].

ii. Enzymes not involved in ADG glycosylation (table 2). Calpain 3 is a Ca^{2+}- activated neutral protease. The precise pathophysiology of the corresponding dystrophy, LGMDA, is still incompletely understood.

d. Nuclear membrane proteins (Table 1 and figure 1)

The nuclear envelope is composed of 2 membranes, the outer and the inner nuclear membrane that interact with the underlying nuclear lamina. Mutations in proteins localized in the nuclear envelope, including lamin A/C, emerin, nesprin 1 and 2, TMEM43 (LUMA) have been implicated in conditions which share a progressive MD phenotype named Emery Dreifuss (EDMD). [33,34]. Mutations in another nuclear membrane protein, matrin 3, have been described in a distal myopathy with vocal cord paralysis[35]. There is a remarkable divergence of phenotypes due to allelic mutations in these genes, suggesting discrete roles of different protein domains[36]: The most striking example are mutations in lamin A/C which can give rise to **a**. EDMD; **b**. isolated cardiomyopathy with conduction system disease; **c**. LGMD + lipodystrophy; **d**. lipodystrophy with mandibuloacral dysplasia; **e**. CMD **f**. peripheral neuropathy; **g**. lethal restrictive dermopathy;. **h**. Hutchinson-Gilford progeria.

e*. Sarcomeric proteins (figure 1).*

While several of these disorders are subsumed under the non-dystrophic congenital myopathies, recent data also indicate their involvement in dystrophic muscle disorders. For the giant gene titin, encoded by *TTN,* the full range of phenotypes is only recently emerging, owing to the extremely large size of this gene for which next generation sequencing is necessary. At variance with most of CMDs and LGMDs, sarcomeric proteins associated conditions give rise to a prominent or even predominantly distal distribution of weakness[37,38]. In some of these conditions , and in particular *TTN* and *MYH7* [152] [39], severe cardiomyopathy, usually dilated, can co-exist, while allelic mutations in the same genes may lead to cardiomyopathy in isolation.

f*. Endoplasmic reticulum proteins.*

Mutations in TRAPPC11, a subunit of transport particle protein complex (TRAPPC) have been reported both in a form of LGMD (LGMD2S) [40] and in CMD with cerebellar involvement[41]. Mutations in another protein expressed both at the ER and the sarcolemma, BVES (or Popdci), result in a AR LGMD characterized by slowly progressive weakness and cardiac arrhythmias [42]

g. *Others.*

FSHD1 is one of the most common autosomal dominant adult MDs. Both cellular localisation and presumed function of the defective protein cannot be easily fit in our classification scheme. The causative gene defect was established following the finding of the inefficient repression of the transcriptional factor DUX4[43]. Patients with FSHD1 have deletion of integral copies of a tandemly repeated 3.2kb unit (D4Z4 repeat) at the subtelomeric region 4q35. This deletion loosens transcriptional repression of DUX4. In addition, for the disease to develop, the deleted 4q35 allele must be associated with a specific polymorphic variant in the final DUX4 containing unit, in which a single nucleotide polymorphisms leads to a canonical polyadenylation signal for transcripts derived from DUX4, which makes it stable and toxic for the cell. [44] In the clinically similar FSHD2 de-repression of DUX4 is based on hypomethylation of the same 4q35 locus, as a result of mutations in the chromosome modifier SMCHD1 located on chromosome 18p11.

Epidemiology

DMD represents the most common MD in children, with an annual incidence of ~ 1:5000 live males [45] and an estimated point prevalence in of 8.29/100.000 males[46]. BMD has a prevalence of 7.29/100.000 males. Regarding CMDs, recent studies based on nationwide referrals reported a point prevalence of 0.563: 100,000 in Italy [47]. This and other recent studies described a similar frequency of the 3 most common variants (dystroglycanopathies; laminin α 2 related, or CMD collagen-VI Ullrich CMD) [48] [49].

Regarding LGMDs, 2 large studies using next generation sequencing approaches identified a final diagnosis in in 59% [50], and 27% of cases respectively[51] In both studies autosomal recessive forms

accounted for the great majority of probands (>80%). In the former study LGMD2A (30% of probands) and LGMD2B (22.6%) were the most frequent forms, followed by the sarcoglycanopathies (21.3%) and LGMD2I (9.7%). In the latter study phenocopies of LGMD such as the glycogen storage disorder Pompe disease accounted for ~1% of the 4656 cases studied. This is important in view of the availability of enzyme replacement therapy for this metabolic disease. In the Northern Europe populations LGMD2I is one of the most common variants, [52] with a prevalence of 1/54,000 and a carrier frequency of 1/116 in Denmark [52] and the UK[46]. Regarding dominant disorders, FSHD1 is the most common form with an estimated prevalence of 1-15.00/20.000[53,54].

Clinical manifestations and diagnosis

The previous version of this Seminar[2] and other recent reviews provide in-depth information on onset and progression of clinical signs in the different forms of muscular dystrophies[55-57] .

In order to facilitate the diagnostic pathways, an integrated approach combining clinical signs, age of onset, distribution of muscle weakness, cardiac involvement, with laboratory results (serum creatine kinase, CK, levels, typically markedly elevated in MDs), muscle biopsy (figure 2) and muscle imaging (figure 3) (and for some subtypes of CMDs also brain imaging) is useful. Diagnostic guidelines are available [50,58-60] and a comprehensive e-tool developed by one of the LGMD advocacy groups provides very helpful information on how to approach their diagnosis (https://www.jain-foundation.org/lgmd-subtyping-diagnosis-tool).

Congenital Muscular Dystrophies.

This term typically describes children symptomatic at birth (sometime also before birth, with reduced foetal movement, polyhydramnios,) or in the first year of life.

LAMA2-CMD is evident in the first few days or weeks of life with generalized hypotonia, paucity of antigravity movements, talipes, occasional hip and knee contractures, and marked elevation of serum CK. Arm and axial weakness predominates the early clinical course, but lower limb weakness and progressive contractures invariably develop. Affected children typically only acquire independent sitting, only rarely the ability to stand or walk. Swallowing difficulties are common and result in failure to thrive in the first few years of life. Respiratory muscle weakness determines frequent chest infections and respiratory insufficiency requiring nocturnal non-invasive ventilation occurs by middle teens. Scoliosis is common. Cognitive function is typically preserved, although brain imaging demonstrates white matter abnormalities resembling a leukodystrophy. Focal epilepsy occurs in ~ 20% of affected children, with a very small proportion have neuronal migration disorder affecting the occipital lobes. Life expectancy is highly dependent on optimal standards of care, and currently survival in early adult life can be expected in the majority of patients. Clinically overt cardiac involvement is exceptional. The diagnosis is suggested by the severely reduced or absent protein expression in muscle (figure 2) or in skin, and requires genetic confirmation [58].

Ullrich COL6-related CMD may also be present at birth, with typical skeletal deformities including kyphoscoliosis, contractures of the larger joints, a striking hyperlaxity of hands and feet, often associated with torticollis and hip dysplasia. Creatine kinase is normal or mildly elevated. Nearly 50% of the cases acquire independent ambulation which is however lost by the early teens as a result of progressive muscle weakness and of joints contractures with rigidity of the spine. Failure to thrive commonly develops requiring gastrostomy; respiratory insufficiency sets in early and is fully manifest by the early teens requiring nocturnal respiratory support [61 62]. Cognitive and cardiac function are spared. The diagnosis is made by demonstrating absence, or more frequently reduction in collagen VI expression at the basal lamina of muscle fibres or in dermal skin fibroblasts in addition to the severe pathological dystrophic changes [figure 2]. Muscle MRI demonstrates a distinctive pattern of muscle involvement highly suggestive of a collagen VI

involvement (figure 3). The genetic analysis of COL6A1; COL6A2 and COL6A3 completes the diagnostic pathway with the identification of either recessive mutations, or de-novo dominant mutations. Recently a deep intronic de-novo dominant mutation in COL6A1 was identified by transcriptomic analysis and represents one of the most common single mutations in this condition. [63,64]

Dystroglycanopathies. This is an extremely heterogeneous group of conditions. Clinically, the variable severity is depending on the severity of the effect of the individual recessive mutations on the level of dystroglycan glycosylation. The increasing depletion of dystroglycan glycosylation affects, in a hierarchical way, skeletal and cardiac muscles first; more severe deficiency of glycosylation leads to central nervous system involvement, ranging from intellectual disability in absence of structural involvement and thus suggestive of synaptic involvement, to severe cortical dysplasia of the cobblestone type, a form of polymicrogyria with neuronal overmigration. Structural eye defects are common in severe patients. The conditions with severe structural brain involvement are Muscle-Eye-Brain disease and Fukuyama congenital muscular dystrophy, often complicated by epilepsy, and Walker Warburg syndrome (the extreme end of the severe lissencephaly spectrum), all with structural eye involvement. The diagnosis of these conditions is suggested by the markedly elevated CK, the dystrophic muscle biopsy findings, the reduction of glycosylated alpha dystroglycan at the sarcolemma. The diagnosis needs to be confirmed by the analysis of the genes responsible for this group of conditions (table 1).

Duchenne muscular dystrophy

DMD affected boys are typically symptomatic after the first years of life, often after mildly delayed acquisition of ambulation. Early complaints are frequent falls, inability to run and to climb stairs and difficulty to get up from the floor, requiring the help of the hands to push on the knees and provide sufficient momentum to get upright (Gowers' manouvre). Delayed acquisition of speech is common (50%). In addition, intellectual disability, autistic spectrum disorder and

attention deficit disorder are co-morbidities present in ~ 30% of cases [65]. The MD remains relatively stable until the age of ~ 7 years, when more rapid progression becomes apparent, leading to loss of independent ambulation by the age of 12 (mean age of 9.5 years), followed by scoliosis, loss of upper limb function, respiratory insufficiency and cardiomyopathy. These complications led to a mean age of survival in the late teens. With the recently revised standards of respiratory and cardiac management, the implementation of multidisciplinary care and the use of corticosteroids[66-68] , ambulation is now maintained until a mean age of 13-14 years and the mean age of survival has increased to the late twenties[69]. Cardiac death due to progressive dilated cardiomyopathy is now a leading cause for mortality in the older patients[62,70]. Recent data suggest a beneficial effect of eplerenone, an antimineralocorticoid of the spirolactone group, in addition to beta blocker or angiotensin converting enzyme inhibitors. [71] There is still no consensus on whether pharmacological cardiac protection therapies should be started at a young age, below the age of 10 years, as a preventive measure; data from a limited cohort supports this view[72], while the outcome of larger randomised studies are awaited (EudraCT2007-005932-10).

Unlike the progressive skeletal and cardiac muscle degeneration brain involvement is static. While muscle only produces one isoform, in the brain there are multiple dystrophin isoforms. The site of the *DMD* gene mutation determines how many brain dystrophin isoforms are affected, providing an explanation for the variability in brain involvement observed [65,73].

The advent of clinical trials has highlighted the need for validated outcome measures and longitudinal, long term natural history studies. The 6 minute walking test (6MWD) has been used as primary outcome measure in most clinical trials; disease specific functional measures, such as the North Star Ambulatory assessment or the Performance of Upper limb test or timed items have also been increasingly used. Natural history studies have shown that DMD progression is not linear and that there is a huge variability in the progression even within 12 months[74-76]. Combining functional assessments (e.g. 6MWD with timed function tests) and imaging assessment (magnetic resonance imaging, MRI) further enhances the

possibility to identify patients with different disease progression. A more accurate categorization of trajectories in DMD progression have recently been achieved using a statistical approach that allows to identify how multiple variables (age, timed function tests, height, weight, BMI) help in defining the trajectory of progression of affected boys (figure 4) [77,78].

Limb Girdle muscular dystrophies

The clinical and genetic heterogeneity of the LGMDs, and similar clinical features of conditions that are not typically considered within this pathological categorization complicates their diagnostic approach[79,80]. Common to all forms is an onset after the acquisition of walking (i.e. not congenital), a predominantly proximal pattern of muscle weakness at onset with sparing of facial and extraocular muscles, and dystrophic changes in the muscle biopsy. The assessment of a panel of relevant protein on muscle biopsy (Figure 2) and suggestive patterns on muscle imaging (Figure 3) can help guide the diagnostic process. There are important subtype specific difference as respiratory and cardiac involvement are concerned, highlighting the importance of a specific genetic diagnosis. The advent of next generation sequencing has been particularly impactful in this situation and these are now increasingly used as first line investigations.[49,81,82]. A recent ENMC workshop suggested a new classification for LGMDs (table 2) [83].

Emery Dreifuss Muscular Dystrophy

The classical phenotype of EDMD is that of a slowly progressive muscle weakness and wasting affecting predominantly the biceps and calves. Contractures affecting the elbows, the Achilles tendons and the neck and spine are common. The onset is typically in the early teens; an invariable complication is the associated atrial and cardiac system conduction disease and, in a proportion of cases, ventricular arrhythmias and dilated cardiomyopathy that often determine the long term survival. Standards of care recommend the implantation of a defibrillator, as pace-makers only have a modest impact on survival. Compared to the X-linked emerin-mutation related EDMD, dominantly acting *LMNA* mutation related EDMD overall has earlier onset, some children

resembling a form of congenital muscular dystrophy (LMNA-related CMD). These children may never acquire the ability to walk, or if they do, they lose this ability in the first decade of life; the severity of their muscle weakness is paralleled by a more severe respiratory and cardiac progression. Serum CK is typically moderately elevated and muscle MRI demonstrates a typical pattern of selective involvement. The clinical diagnosis requires genetic confirmation i.e. the analysis of the genes associated with this phenotype (table 1).

Facio Scapulo Humeral muscular dystrophy

The hallmark of FSHD is the progressive, frequently asymmetrical weakness involving the face, scapular muscles, proximal limb and peroneal muscles, often with foot drop. Marked, progressive winging and typical elevation of the scapulae associated with facial weakness is very common. Cardiorespiratory function is usually normal, and the progression of weakness is variable, with the majority of patients retaining the ability to walk in adult life. Rare patients have onset of symptoms in the first few years of life (infantile FSHD); this subgroup has more severe weakness with loss of independent ambulation by the early teens [84]. Additional features of FSHD which correlate with clinical severity are a retinal vasculopathy with telangiectasia and microaneurysms (Coates disease), and high-frequency sensorineural hearing loss. CK levels are normal or mildly elevated. The diagnosis requires the appropriate genetic analysis at the chromosome 4q35 locus (see above) or at the FSHD2 locus.

Therapeutic approaches in current clinical trials (table 3)

Duchenne muscular dystrophy.

The last decade has experienced an exponential increase in experimental therapeutic approaches for DMD, with 2 drugs having received conditional approval in 2016, one in the US and one in Europe. There are currently > 20 active clinical trials in DMD, ranging from phase I to more advanced studies, http://www.treat-nmd.eu/research/clinical-research/overview-current-trials-dmd/current-trials/.

The approaches focus on either improving structural integrity of the muscle fibres by restoring dystrophin production, or on the secondary consequences of dystrophin deficiency, i.e. the inflammatory process; fibrosis; muscle regeneration and muscle mass (table 3).

Restoring dystrophin expression at sarcolemma. The strategies used range from small molecules or antisense oligonucleotides, to adeno associated virus (AAV) gene therapy approaches.

Targeting nonsense mutations: ataluren, an orally bioavailable small molecule, induces read-through of nonsense mutations at the ribosome and partially restores dystrophin production. Two randomised, double-blind, placebo-controlled trials demonstrated a trend in therapeutic efficacy as measured by 6MWD changes, although both studies failed to achieve the 48 weeks primary endpoint [85]. The study design might have played a role on this outcome as both studies recruited a high proportion of DMD boys in the stable phase, blunting the possibility to appreciate a treatment effect. When considering only the boys in ambulatory decline (a pre-specified endpoint in the phase 3 study), a statistical difference between the treated children and the placebo group was detected[86]. Based on this and a favourable safety profile, EMA granted a conditional approval, while the company is running further trials.

Antisense oligonucleotides (AONs) for out-of-frame deletions. This strategy uses AON, which need to be administered systemically weekly, to manipulate the pre-mRNA splicing of patients with eligible out-of-frame deletions and generate an in-frame message by removing one exon adjacent to the deletion breakpoint (frame restoring exon skipping, see for example https://www.musculardystrophyuk.org/progress-in-research/background-information/what-is-exon-skipping-and-how-does-it-work/). This approach is mutation specific and facilitated by clustering of deletions in specific hot-spots: skipping exon 51 for example is applicable to ~ 13% of all the boys with deletions. Two different AON chemical modifications, the morpholino and the 2'OMe have been used in DMD trials. Both AONs induced frame-restored dystrophin production when administered following a single intramuscular injection to DMD boys. [87,88]. In 2 subsequent phase 2 studies, the 2'OMe AON drisapersen met the clinical endpoint [85]. However, a larger phase 3 study failed to demonstrate clinical

benefit. The inclusion criteria might have played a role; a recent post-hoc analysis suggested a significant improvement in 6MWD in favour of drisapersen in boys in moderate decline[89]. Nevertheless, the limited clinical efficacy combined with the unfavourable safety profile led Biomarin to abandon the development of the 2'OMe AON platform for DMD.

The morpholino AON designed to skip exon 51 (eteplirsen) also induced dystrophin restoration in a dose escalation i.v. phase II study. [90,91] Subsequent studies confirmed the production of dystrophin in most of the treated patients [92], and a divergence of the clinical course between the treated children and a concomitant natural history study based on the 6MWT [93]. These data led the FDA to conditionally approve eteplirsen in the US despite criticisms due both to the limited size of the treated patient population, the low amount of dystrophin produced [93] and the lack of a placebo arm. Recent additional data on the long term preservation of respiratory function in the DMD boys studied over the course of 5 years were published[94]. Currently confirmatory studies are targeting exon 51, but also exons 45 and 53 [95]. (table 3).

Next generation AON are also now in phase I studies: a peptide conjugated morpholino AON for improved muscle targeting; and a novel stereochemical modification of the 2'OMe backbone which is expected to improve efficacy, which should result in reduced dosage and adverse events[96], [97].

AAV gene therapy. The large size of the *DMD* gene precludes packaging of the full cDNA in AAV vectors. However considerably shortened "microdystrophin" versions, originally inspired by some patients with large intragenic deletions and yet a BMD phenotype, have been developed which can be accommodated in AAV vectors. As viral delivery to muscle is associated with an immune response to the AAV that precludes repeated administrations with the same serotype, it is essential to develop efficient strategies with a realistic perspective of producing a therapeutic benefit for the patients to minimize the need for a repeat treatment. Recent preclinical studies have shown the potential of AAV8 and AAV9 to target muscles, with histological and clinical improvements [98-104] Several academic groups and industrial partners have initiated phase I clinical trials in which escalating doses of AAV9 and AAVrh74 are being administered systemically to DMD boys.

Dealing with secondary consequences of dystrophin deficiency. (table 3).

Numerous clinical phase I-III trials are underway to assess safety and efficacy of: **i.** targeting the NF-kB inflammatory pathway (Catabasis); **ii**. a dissociative steroidal drug which aims to retain the corticosteroid efficacy but reduce their chronic side effect profile (ReveraGen Biopharma); **iii**. downregulation of the myostatin pathway, which in animal models induces improved regeneration and increases muscle mass (Pfizer; Roche); **iv**. compounds with an effect on fibrosis and muscle regeneration (Italfarmaco); **v**. or improved mitochondrial bioenergetics (Santhera).

Table 3 provides a list of the DMD ongoing clinical trials.

COL6-related CMD. In this condition myofibre degeneration is at least partially due to mitochondrial mediated muscle fibre apoptosis, driven by inappropriate opening of the mitochondrial permeability transition pore (PTP) [105,106]. Pharmacological interference with this mechanism can be achieved using cyclosporine A, exploiting its inhibition of cyclophilin D[107]. A small uncontrolled trial of cyclosporine A in patients with COL6-RD apparently improved mitochondrial PTP dysfunction on pathology, however the study was not designed to detect functional improvement [108]. Other studies have suggested that the muscle autophagic machinery is underperforming in COL6-related MD, causing backed up clearance of the abnormal mitochondria. Autophagy can be induced by protein restriction, leading to a small uncontrolled trial of protein restriction in patients with COL6-related MD. While it was possible to suggest an induction of autophagy in the patients, the study design did not allow the detection of clinical efficacy [109,110]. The antiapoptotic compound omigapil has recently been studied in a phase 1 PK study at the NIH in children with both COL6 and with LAMA2-related CMD (see below).

LAMA2-related CMD. Preclinical work had identified inappropriate premature apoptosis of myofibers as one potential disease driver. Omigapil is a GAPDH interactor and interferes with the GAPDH/Siah1 mediated apoptosis pathway. Preclinical studies in mouse models of the disease

have suggested disease modifying potential for this compound. A phase 1 PK study of omigapil in children with LAMA2 as well as COL6 related MD has been recently conducted. Pharmacological approaches to other potential mechanisms for this disease (overactive autophagy and underactive proteasome, TGFbeta mediated fibrosis [111], and inflammation) have been studied preclinically but not yet in clinical trials. Functional replacement of laminin-α2 (which is too large for AAV mediated delivery) has been achieved preclinically using two ingenious linker molecules in combination, mini-agrin and mini-nidogen[112], which could be delivered using AAV.

Limb Girdle muscular dystrophies. The translational research in these conditions is mostly focused on preclinical models. In the dystroglycanopathies, gene transfer approaches are being explored since these conditions are based on loss of function and most of the cDNAs are small enough to be packaged into AAV. Relevant animal models, most importantly the more common *FKRP,* are being studied. For the subtypes that also involved the central nervous system it will be important to target the brain as well, although the effect of postnatal correction of the primary defect in the brain remains to be explored. Another approach that has been explored pre-clinically includes upregulation of the glycosyltransferase LARGE, involved in adding an important glycoepitope that confers the binding activity to laminin. This approach may be able to compensate for a variety of defects more upstream[113]. However, there may be risks in upregulating this too much that still need to be explored.

Gene transfer strategies are also being explored for the sarcoglycanopathies as well as for calpainopathy and ANO5 in relevant preclinical model. Phase I studies of local (intramuscular) delivery of gamma, alpha and beta sarcoglycan have been performed in the corresponding LGMD variants. The next stage of systemic delivery of some of these transgenes has very recently been initiated with the delivery of beta sarcoglycan using the same AAVrh74 used for dystrophin (Clinical trial identifier: NCT03652259).

Pharmacological approaches aimed at intervening in secondary disease driving processes are also being pursued preclinically in dysferlinopathy and in calpainopathy. These include the upregulation or even the systemic administration of proteins that collaborate with dysferlin in the resealing process, such as mitsugumin 53[114-116]; and the identification of the targets responsible for progressive muscle damage in calpainopathy.

Genetic therapeutic approaches to the *EDMD disorders* are only at the preclinical stage. Gene replacement is conceivable in emerin deficiency since the gene product can be packaged into AAV. However the same therapeutic approach would not be appropriate for *LMNA* mutations, where the mutations act in a dominant negative way, but haploinsufficiency also leads to disease (cardiomyopathy), making even a mutation specific silencing approach a challenge.

Conclusions

In the last few years the genetic diversity of muscular dystrophies has been further elucidated, mainly due to the powerful genetic diagnostic tools which now, along with excellent clinical sorting and improvement on muscle pathology and imaging, achieves individual genetic diagnosis in the majority of patients.

Therapeutic developments have also seen a dramatic expansion of efforts, initially with mutation and gene directed approaches, which led to drugs targeting specific DMD mutations being commercially available. The second generation drugs of some of these approaches are now in early clinical trials. At the same time, approaches that are potentially applicable to patients irrespective of the mutations are also being developed. In the last few years increasing confidence in AAV gene therapy is leading to multi-partner efforts to take these viral vectors to clinical trials in DMD. These efforts, if effective, will pave the way for other forms of dystrophies in which this approach is feasible.

While mechanisms and pathways may be disease specific, in some instances they may also be valid across different diseases – with opportunities for use across entities. It is also likely that in the future we will see the development of combinatorial therapies for a number of these approaches.

While there is tangible excitement in this field for these efforts, it is nevertheless essential not to lose sight of the fact that the ultimate cure for muscular dystrophies will be very challenging, these being degenerative disorder of muscle. Significant obstacles to overcome are, in CMDs, the timing of onset of pathology; in DMD and LGMDs the abundance of the muscle tissue to target, together with the fact that symptomatic patients already have lost a considerable amount of muscle tissue which is not replaceable with the current technology.

Therefore implementation of optimal medical management based on standards of care remains as important as ever as management cans still be improved, remains often inconsistent between centers and countries, and needs to be optimal as a baseline on which to test additional experimental approaches.

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Disclosures

F. Muntoni reports grants and personal fees from Sarepta, grants from EU FP7 (SKIP-nmd grant), grants and personal fees from Pfizer, grants from Wave, grants and personal fees from PTC, personal fees from Roche, during the conduct of the study; .

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Legends to figures.

Figure 1

A schematic representation of the major class of proteins involved in MDs, and their subcellular localisation.

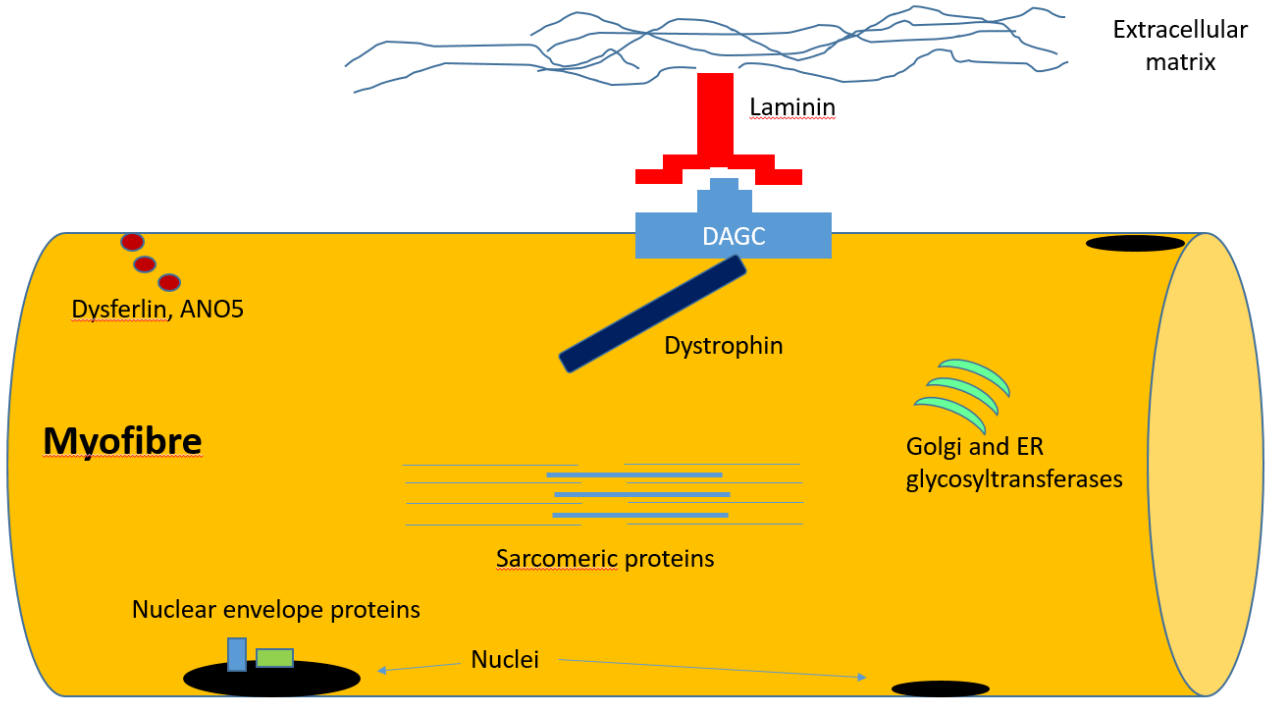
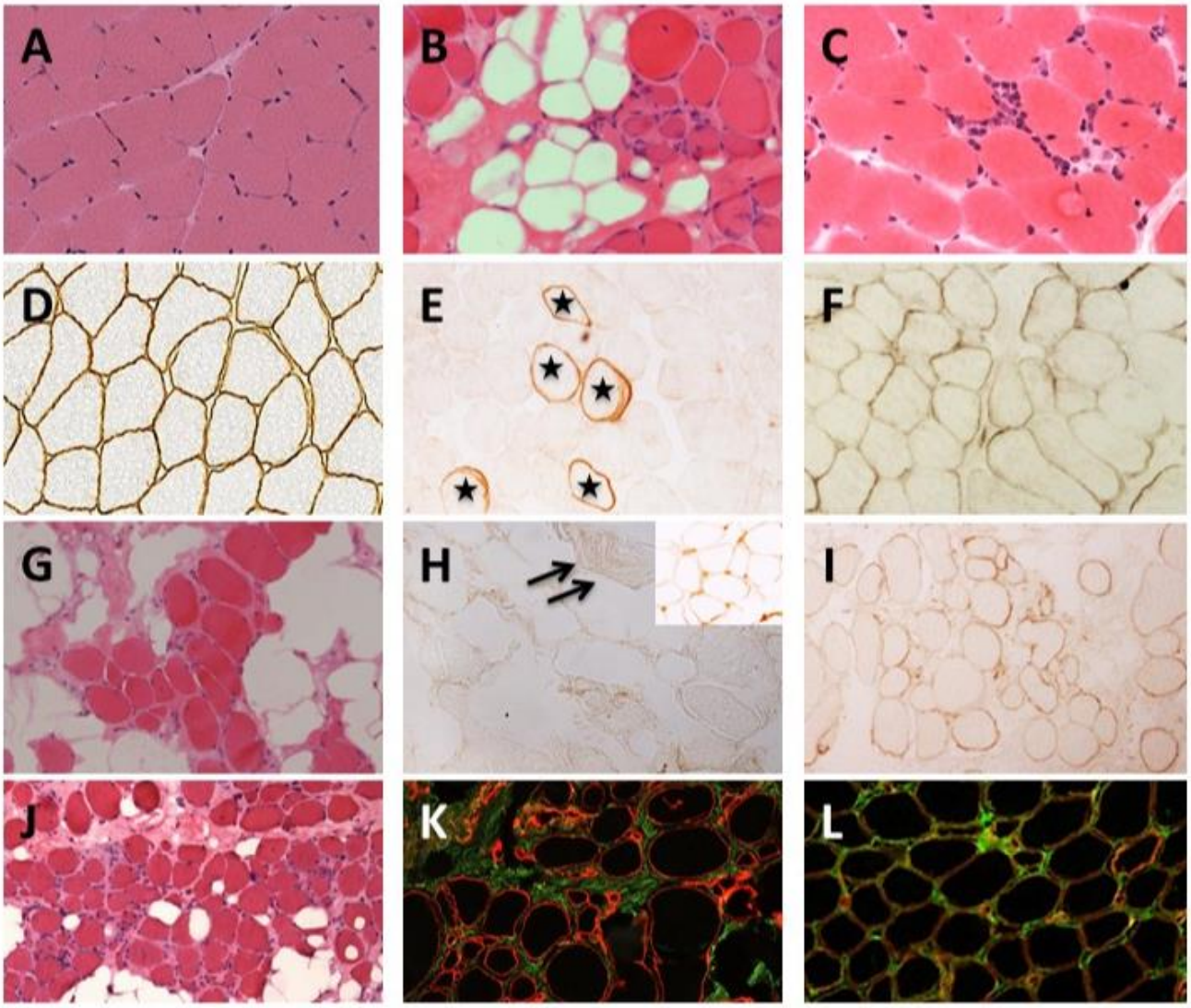


Figure2.



Haematoxylin and eosin stained sections (A-C). A histologically normal muscle biopsy (A) shows a compact fascicular pattern. The fascicles are segregated by thin slivers of perimysial connective tissue and comprise polygonal-shaped myofibres in cross-section with peripherally placed myonuclei. Biopsy from a patient with Duchenne muscular dystrophy (B) shows marked variation in fibre size, increased rounding and hypercontraction of fibres, and clusters of basophilic regenerating fibres with internal nuclei with accompanying fibrosis and fatty infiltration in the perimysium and endomysium. These dystrophic changes are milder in the biopsy of a patient with Becker muscular dystrophy (C). Immunostaining with an antibody to an epitope within the rod domain of dystrophin (Dys1) shows uniform sarcolemmal labeling in the unaffected control (D). There is a complete absence of sarcolemmal dystrophin labeling in Duchenne muscular dystrophy (E), except for a few strongly labeled 'revertant' fibres (stars, E). In Becker muscular dystrophy, there is patchy, mild-to-moderate reduction in sarcolemmal dystrophin labeling (F). Biopsy from a patient with complete laminin alpha 2 –deficient muscular dystrophy (G) shows marked dystrophic features with striking fatty infiltration. Immunolabeling for laminin alpha 2 (300 kDa antibody) shows complete absence at the myofibre basal lamina (H), as well as in intramuscular nerves

(arrows, H). There is secondary upregulation of laminin alpha 5 at the myofibre basal lamina (inset, H). In partially laminin alpha 2 deficient muscular dystrophy, there is variable, patchy, mild-to-moderate reduction of laminin alpha 2 labeling at the basal lamina of myofibres (I). Biopsy from a patient with Ullrich muscular dystrophy shows marked dystrophic changes with striking fatty infiltration (J). Immunofluorescent double-labeling with an antibody to an epitope in the heterotrimeric domain of collagen VI (green) and perlecan, a myofibre basal lamina marker (red) shows near-total absence of collagen VI at the basal lamina accompanied by increased deposition in the interstitium (K). In Becker muscular dystrophy, there is variable, patchy, partial reduction of collagen VI at the basal lamina (courtesy of Prof Caroline A. Sewry and Dr Rahul Phadke).

Figure 3

Muscle magnetic resonance imaging (T1 images) of the thigh muscles (upper panel) and calf muscles (lower panel) demonstrates distinct pattern of selective muscle involvement in the different conditions

(LGMDA: calpain related LGMD; LGMD2B/MM: dysferlin related LGMD / Myoshy Myopathy; FSHD: Facio-Scapulo Humeral muscular dystrophy)

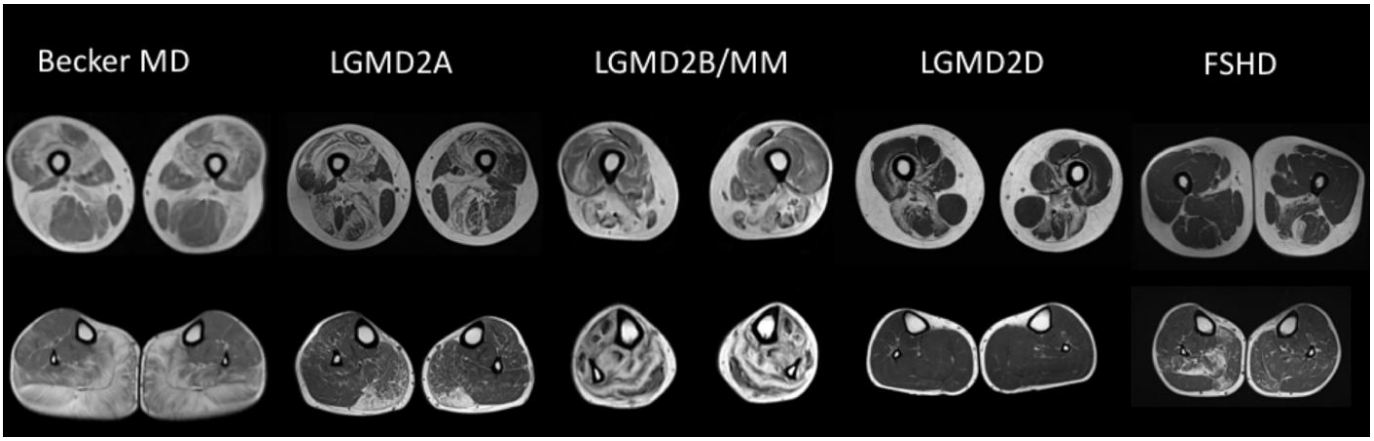
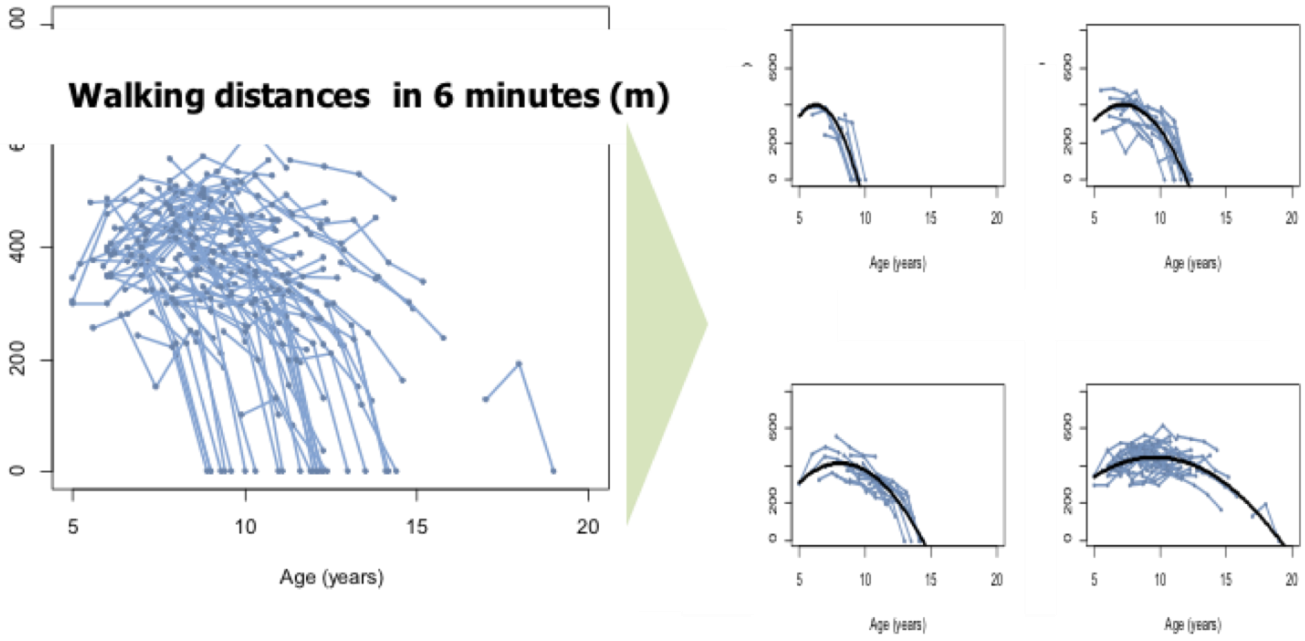


Figure 4.

On the left hand side the longitudinal 6 minute walk time (6MWT) distance walked by a group of DMD boys demonstrates major heterogeneity. On the right hand side different disease trajectories can be identified taking into account variables other than age and help to reduce variability



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