Review

Biomarkers in Duchenne Muscular Dystrophy: Current Status and Future Directions

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Abstract. Duchenne muscular dystrophy is a severe, X-linked disease characterized by decreased muscle mass and function in children. Genetic and biochemical research over the years has led to the characterization of the cause and the pathophysiology of the disease. Moreover, the elucidation of genetic mechanisms underlining Duchenne muscular dystrophy has allowed for the design of innovative personalized therapies.

The identification of specific, accurate, and sensitive biomarkers is becoming crucial for evaluating muscle disease progression and response to therapies, disease monitoring, and the acceleration of drug development and related regulatory processes.

This review illustrated the up-to-date progress in the development of candidate biomarkers in DMD at the level of proteins, metabolites, micro-RNAs (miRNAs) and genetic modifiers also highlighting the complexity of translating research results to clinical practice.

We highlighted the challenges encountered in translating biomarkers into the clinical context and the existing bottlenecks hampering the adoption of biomarkers as surrogate endpoints. These challenges could be overcome by national and international collaborative efforts, multicenter data sharing, definition of public biobanks and patients' registries, and creation of large cohorts of patients. Novel statistical tools/ models suitable to analyze small patient numbers are also required.

Finally, collaborations with pharmaceutical companies would greatly benefit biomarker discovery and their translation in clinical trials.

Keywords: Duchenne muscular dystrophy, biomarkers, proteins, miRNAs, single nucleotide polymorphisms

INTRODUCTION

Duchenne Muscular Dystrophy (DMD) is a severe X-linked recessive disorder, affecting 1 out of 5000 males born worldwide, caused by mutations in *DMD* gene. Located on the short arm of the X chromosome (cytogenetic location: Xp21.2-p21.1), the *DMD* gene is one of the largest genes in the human genome containing 79 exons that encodes the giant dystrophin protein (427 kDa) [1].

Dystrophin is a component of the dystrophin-glycoprotein complex (DGC), a large multicomponent complex with an essential role not only in the maintenance of sarcolemma but also in mediating interactions between cytoskeleton, membrane, and extracellular matrix [2]. Absence of dystrophin and subsequent loss of DGC causes a cascade of cell dysfunctions resulting in loss of physical integrity of

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muscle cells and contraction-induced muscle degeneration [3].

Due to the enormous size of *DMD* gene, the mutation rate is relatively high with approximately 1/3 of mutations occurring *de novo* and 2/3 of mutations inherited from carrier mothers or arising from germline mosaicism [4]. This high mutation rate also underlines the complex mutation spectrum that has been identified for DMD patients. Mutations can be of large intragenic deletions (∼65%) and duplications (∼10%); the remaining cases are small mutations (∼25%), deep intronic mutations and complex rearrangements (less than ∼1%) [5].

Clinically, affected DMD boys display a progressive disease characterized by muscle-mass wasting and severe weakness starting in early childhood. Loss of independent ambulation (LoA) generally occurs around 12 years old, and death is mainly caused by cardiorespiratory failure [6].

The milder form of the condition, Becker muscular dystrophy (BMD), is like DMD in the distribution of muscle weakness and wasting, which is mainly proximal; however, the course is more benign and heterogeneous with a wide spectrum of clinical presentations ranging from delayed loss of independent ambulation to almost asymptomatic cases with only elevated activity of creatine kinase (CK). Dilated cardiomyopathy (DCM) is a common complication of both DMD and BMD, the severity of which may also depend on mutation type, deletion interval, and location [7].

In addition to progressive muscular degeneration, DMD is more often accompanied by cognitive dysfunction, neuropsychological problems (anxiety, depression, and emotional disturbance), and neurobehavioral abnormalities (autism spectrum, attention-deficit hyperactivity disorder, and obsessive-compulsive disorder) [8].

Due to the full capacity of identifying *DMD* pathogenic variations and thanks to next-generation sequence strategies [9] several innovative therapeutic approaches have been developed over last years and are currently under investigation or even already approved as orphan drugs. These can be categorized into two main groups: 1) therapies aiming at restoring dystrophin protein via inducing favorable exon skipping by antisense oligoribonucleotides (AONs), via ribosomal stop codon reversion (Translarna) or by gene therapy [10]; 2) therapies aiming at the mitigation of secondary downstream pathological mechanisms caused by the absence of dystrophin protein [11].

BIOMARKERS: WHY ARE THEY SO IMPORTANT

There is a general agreement on the importance of specific, accurate, and sensitive biomarkers to monitor disease severity, stratify disease subtypes, and accelerate drug development and related regulatory processes [12]. In the last decade, multiple studies have focused on discovery and validation of candidate biomarkers with the potential of being 'surrogate endpoints', defined as 'a biomarker intended to substitute for a clinical endpoint' [13].

Surrogate endpoints can predict a response to therapy and are tools that can be used to facilitate the regulatory approval of drugs, showing less variation than functional tests.

The association of a biomarker with a clinical endpoint is essential for the translation of candidate biomarkers in surrogate endpoints.

Given the numerous applications of biomarkers and their extensive applications, classification and standardization are crucial. Biomarkers are classified in: 1) diagnostic, 2) pharmacodynamic/response, 3) disease progression monitoring, 4) prognostic, 5) predictive, 6) safety, 7) susceptibility/risk biomarkers [14].

BIOMARKERS: DEFINITION

Molecular biomarkers are measurable properties or characteristics presenting as specific, accurate and sensitive indicators of a pathological state. In clinical context, molecular biomarkers, such as proteins, metabolites, miRNA, are increasingly explored as tools to monitor disease progression and response to therapy.

Single nucleotide polymorphisms (SNPs), acting as genetic modifiers, have also been extensively explored in their effect on functions in DMD patients, such as age of loss of ambulation (LoA), or on response to therapies, such as corticosteroids (CS) [15].

This review focuses on the most promising findings in development of DMD biomarkers and highlights the challenges encountered in translating biomarkers in a clinical setting.

PROTEIN BIOMARKERS

A plethora of high-throughput methods, including mass spectrometry, immunoassays and affinity-

based protein profiling approaches, is being increasingly applied for analysis of body fluids, aiming to identify many new potential biomarkers for diagnosis, prognosis or surveillance. In DMD context, several proteomics studies showed encouraging results regarding the use of proteins as potential biomarker candidates, including muscle-injury, extracellular matrix, muscle degeneration/regeneration-associated, energy metabolism, fibrosis and inflammatory, immune processes, and related proteins [12,16].

The most widely known and currently used biomarker is Creatine Kinase (CK), a muscle-specific protein that reflect sarcolemma damage and can be used as indicator of muscular dystrophy and inflammation. Serum CK activity has been extensively studied and CK levels remain the first element leading to the diagnosis of dystrophinopathies, as it was proven that specificity of CK is approximately 91% with a sensitivity of 100% in DMD [17].

CK is a dimeric enzyme, consisting of two subunits, M and B, and three isoenzymes, CK-BB, CK-MB, and CK-MM. Both MM and MB increase in cardiac muscle pathologies such as myocardial damage; indeed, the use of CK-MB was previously considered the gold standard for the detection of cardiac injury until a more specific marker, troponin T, became available.

Instead, the CK-MM isoform is found predominantly in skeletal muscle and results significantly increased in the serum of patients with skeletal muscle injury or inflammation [18].

Serum levels of CK-BB, isoform predominantly located in astrocytes, have been found elevated in various brain injury settings, including after cardiac arrest or subarachnoid hemorrhage [19]. However, a small amount of CK-BB is found also in the gastrointestinal tract, uterus, and vascular wall and seems to be one of the adenocarcinomas markers [20]. New applications (such as analysis of dried blood spots) for CK biomarker are being developed with the aim to detect asymptomatic individuals in the newborn period, to start early therapies and to prevent the diagnostic odyssey.

Since the 1970 s, there have been several pilot studies to detect DMD in newborns, many of which have used CK as a biomarker for early diagnosis of disease. These programs tested CK levels in dried blood spots for the first-tier screening, then tested positive boys using genetic tools, subsequently applying in positive infants clinical follow-up, and muscle biopsy [21, 22, 23, 24].

More recently, studies have piloted screening with quantitative detection of the CK-MM isoform as a specific biomarker of muscle damage using the FDA-approved GSP neonatal CK-MM kit (#3311-001U, PerkinElmer) [25, 26].

However, newborn screening (NBS) for DMD based on CK assay is still a controversial argument because of the rate of false positives and also due to lack of specificity of CK since its measurement could lead to the identification of other muscular dystrophies, for which treatment options are not available, therefore posing ethical issues about genetic equity.

Although CK is a good screening marker to early detect patients with suspected dystrophinopathies, it does not appear suitable to monitor disease progression and response to therapy [27]. Indeed, it peaks between one and 6 years and declines as disease progresses, reflecting the replacement of muscle tissue by fibrotic and adipose tissues. Moreover, CK is unspecific and elevated in other muscle diseases, either genetic (such as limb girdle muscle dystrophies) and acquired (such as inflammatory myopathies), and it is also highly influenced by environmental factors (such as age, metabolic changes, muscle trauma and exercise) [27].

Another pitfall in the use of CK levels as biomarker in DMD is that in female carriers, they showed a sensitivity of 33.3% and a diagnostic specificity of 50%, hence CK levels are not that useful for carrier detection [17].

In addition to CK, elevated levels of other muscle-specific proteins were detected by proteomic studies in DMD patients. The majority of the identified candidate biomarkers display a CK-like profile, declining over time as disease progresses and reflecting early loss of muscle mass [28]. Differently, other muscle injury biomarkers, such as troponin I (TNNI3), calcium/calmodulin-dependent protein kinase type II subunits alpha and beta (CAMK2A and CAMK2B), mitogen-activated protein kinase 12 (MAPK12), malate dehydrogenase I (MDH1), and glycoprotein I (GP1) tend to remain stable overtime. These muscle injury proteins could be useful exploratory biomarker to investigate the efficacy of dystrophin replacement therapies and other sarcolemma-stabilizing therapies in younger DMD patients [29].

Beside proteins that are ubiquitously expressed in skeletal muscle, muscle-injury proteins specifically expressed in other tissue (e.g. heart) could also be useful to monitor cardiac disease. For example, TNNI3 and Interleukin 1 Receptor-Like 1 Protein (ST2) are

potential biomarkers for cardiac injury, being associated with cardiac degeneration [30, 31].

Another class of biomarkers is represented by extracellular matrix proteins. A recent study reported significantly lower serum concentrations of proteins involved in cell adhesion, proteins regulating cell differentiation and growth and other extracellular proteins in CS-naïve DMD patients compared to healthy controls [29]. Several of these proteins such as osteomodulin (OMD), advanced glycosylation end product-specific receptor (AGER), cadherin-5, and contactin-4, were found decreased in DMD patients at baseline and further decreased following CS treatment [29].

Among proteins involved in DMD pathogenesis, matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1), were found increased in the blood of DMD patients, compared to controls. Interestingly, MMP-9 levels were significantly higher in older, non-ambulant patients compared to the younger, suggesting an increase in MMP-9 levels with disease progression [32, 33]. A similar study did not confirm these finding raising questions regarding the differences between the cohorts analyzed in terms of age, severity of skeletal muscle or cardiovascular disease, or differences in assays [34].

Biomarkers involved in myogenesis, and muscle development were also found to be different in concentration in DMD patients compared to controls, such as disintegrin and metalloproteinase domain-containing protein 12 (ADAM12), brother-of-CDON (cell adhesion molecule-related/downregulated by oncogenes-CDON) (BOC), cysteine and glycine-rich protein 3 (CSRP3) and growth differentiation regulating factors (GDF11 and GDF8) [29]. In particular, three myogenic biomarkers were found to be significantly elevated in their concentrations in DMD patients that have never been treated with CS, compared to controls (e.g. ADAM12, BOC and CSRP3). ADAM12 was the only marker in this category that responded to CS treatment in DMD patients showing a decrease after CS treatment [29]. Conversely, GDF11 and GDF8 were detected at lower concentrations in DMD patients compared to controls and slightly decreased with age in CS-naïve DMD patients [29].

By focusing on young CS-naïve DMD patients, *Hathout and colleagues* [29] were able to identify also a large set of pro-inflammatory biomarkers that were significantly elevated in untreated DMD patients compared to controls, such as fibrinogen gamma chain (FGG), interleukin 6 (IL6), C-X-C motif chemokine ligand 10 (CXCL10), C-C motif chemokines 2 and 18 (CCL2 and CCL18), angiopoietin-2 (ANGPT2), Tumor Necrosis Factor Receptor Superfamily Member 1A (TNFRSF1A), Collagen Type XII (COL12), and components of the complement complex (C5-b-C6). Moreover, several reports show that the degree of inflammation can be also investigated by plasma haptoglobin levels [35, 36]. All of these last reflect the known activation of inflammatory pathways in DMD resulting therefore rather unspecific.

Proteins involved in energy metabolism were also found to be altered in DMD/BMD patients. Myosin light chain 3 (MYL3), carbonic anhydrase III (CA3), mitochondrial malate dehydrogenase 2 (MDH2), and electron transfer flavoprotein subunit alpha (ETFA) presented different serum and/or plasma levels between DMD patients and both controls and female carriers [37]. Moreover, it has been recently suggested that the serum levels of MDH2 correlates with the stage of the disease and with response to treatment with CS. The same longitudinal study revealed that the serum levels of MDH2 are of particular interest, being associated with the risk of wheelchair dependency and pulmonary function. MDH2 was thus proposed as a potentially prognostic biomarker [38].

Biomarkers associated with muscle function, inflammation, and fibrosis may recapitulate disease progression, but additional biomarkers are required to monitor drug safety in new therapy developments.

Cystatin C (CST3), which is clinical biomarker for kidney injury and altered glomerular filtration rate, is increasingly used to monitor nephrotoxicity of AON therapies in DMD clinical trials. The applicability of this 'toxicity biomarker' potentially extends to other neuromuscular diseases, being independent of age, ambulatory capacity and type of steroid employed for treatment [39–41].

There is also a clear need to identify and qualify further sensitive and specific biomarkers to aid in the detection of drug-induced injury.

Preclinical evidence for a panel of biomarkers including MYL3, serum troponin I (sTn1), fatty acid binding protein 3 (FABP3), and creatine kinase measured by a mass assay (CKm) show that this muscle injury biomarker panel (MIP) outperformed and added value to the routine biomarkers, as CK and aspartate transaminase (AST). Inclusion of the MIP biomarkers in preclinical and clinical projects should help to define their utility to assess drug-induced

injury and the potential therapeutic effectiveness of treatments for inherited muscle diseases [42].

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are commonly used in clinical practice for routine safety monitoring and determination of risk of drug-induced liver injury (DILI). However, these serum enzymes may be significantly elevated in patients with underlying muscle disease in the absence of hepatocellular injury, making diagnosis of DILI challenging.

Recent evidence from a phase II clinical trial demonstrated that, compared with ALT and AST, GLDH may be a more specific biomarker to monitor for signs of liver injury in DMD patients. Consequently, GLDH should be considered as a safety biomarker capable of detecting DILI in clinical trials for patients with elevated serum transaminases due to muscle injury or degeneration [43]. Despite extensive efforts dedicated to the identification of muscle-related biomarkers, no surrogate biomarkers, which may anticipate clinical trial results, were discovered.

Proteomics profiling of urine revealed 32 differentially expressed proteins in DMD patients, with titin (TTN) presenting the highest fold change between DMD patients and dystrophin-deficient animal models (GRMD dogs and *mdx* mice) [44, 45].

Increased levels of TTN in *mdx*-4cv urine, as previously reported in dystrophic patients and *mdx-23* mouse, were clearly confirmed in a systematic mass spectromic survey of the urine proteome [46].

These findings corroborate the potential of urinary TTN as a non-invasive and translational biomarker for DMD.

It is known that dystrophinopathies are characterized by a highly complex process of pathophysiological effects due to dystrophin deficiency that are reflected by multi-systemic abnormalities and 'organ crosstalk' [47]. This body-wide aetiology requires the establishment of novel biomarkers that are suitable for the identification and monitoring dystrophinopathy-related abnormalities in different tissues and organ systems.

Abnormal protein expression has been documented in the heart, stomach, brain, liver, kidney, and spleen of the dystrophic phenotype [48–52].

In particular, because of a close relationship between nutritional uptake by the gastrointestinal tract, liver metabolism and skeletal muscle function, dystrophinopathy-associated changes that affect the inter-organ crosstalk of metabolic regulation have been extensively investigated.

Excellent indicators of disturbed fatty acid metabolism are the various isoforms of fatty acid binding protein (FABP). Several proteomic surveys have demonstrated that while skeletal muscles and the heart are associated with a reduction in FABP3, the liver and kidney show elevated levels of FABP5 and FABP1, respectively, and serum exhibits an increased concentration of FABP3 [53].

A recent proteomic profiling of the interface between the stomach wall and the pancreas in the *mdx-4cv* model of dystrophinopathy has confirmed the multi-systemic character of the dystrophic phenotype. In detail, the interface between the pancreas and the stomach of the *mdx-4cv* mouse model was characterised by a drastic reduction in dystrophin and concomitant reduction in sarcoglycan, dystroglycan, laminin, the sarcomeric protein titin and the actin-binding protein filamin [48].

Disease processes occurring in the kidney and bladder can be studied non-invasively by investigating alteration in biomarkers in urine samples. The most relevant proteomic changes in urine samples of DMD patients are associated to muscle-specific or body-wide alterations, as the previously described muscle TTN fragments [44] and high levels of ferritin [54]. An exception is represented by uromodulin, a protein exclusively produced in the ascending limb of the loop of Henle and in the distal tubular region of the nephron. This protein, which is a marker of chronic renal disease, was found to be significantly increased in urine of DMD patients [44].

Regarding the brain, a comparative proteomic profiling of wild type versus *mdx-4cv* brain extracts resulted in the biochemical identification of a large number of proteins with an altered concentration allowing to define a robust biomarker signature of brain DMD pathology [51].

Of special interest is the proteomic identification of the glial fibrillary acidic protein GFAP, an established biomarker of astrogliosis. Increased levels of the glial fibrillary acidic protein, an intermediate filament component that is uniquely associated with astrocytes in the central nervous system, imply neurodegeneration-associated astrogliosis in the *mdx-4cv* brain.

The up-regulation of annexin and vimentin probably represent compensatory mechanisms involved in membrane repair and cytoskeletal stabilization in the absence of brain dystrophin.

Moreover, alteration of neuronal proteins involved in Ca^{2+}-handling, metabolism and signalling in the central nervous system (as Ca^{2+}-binding protein

calretinin and the Ca^{2+}-pumping protein PMCA2) illustrate the complexity of the molecular pathogenesis in the dystrophic brain phenotype [51].

Overall, these new findings might be helpful to further develop a comprehensive biomarker signature of muscle and non-muscle-related abnormalities in DMD, which should improve I) our understanding of complex pathophysiological effects of dystrophin deficiency, II) the identification of novel therapeutic targets, and III) the design of differential diagnostic, prognostic and therapy-monitoring approaches.

Finally, easy dosability, low invasiveness for patients and repeatability over time, make proteins a class of molecular biomarkers very appealing.

Several assays have been considered for proteome screens biomarkers because of their potential for higher throughput and better sensitivity, which may help overcome the validation challenges of identified biomarkers. A study, using the SOMAscan assay, identified a large number of circulating serum biomarkers associated with DMD patients versus healthy controls from two independent cohorts with a 1% false-discovery rate [55].

However, the detection of an optimal and disease specific protein biomarker has so far been hampered by the high variability of the 'proteomic signature' in human fluids [56].

The advent of 'panels' of biomarkers, which can detect a particular disease 'signature' function-, time-, or drug-specific, will be an added value to precision medicine.

Moreover, although individual biomarker values are challenging to directly apply clinically, a recent study has demonstrated that trends of selected non-invasive biomarkers over time (as CK, serum creatinine, urine creatinine) may complement functional measures in the assessment of individuals with DMD [57].

Clinical trials should be encouraged to apply this approach in order to provide reliable and validated results in selected DMD patient cohorts. The use of multiple biomarkers panels should also empower the statistical analysis, making multivariate testing feasible, usually hindered by the small number of patients.

METABOLIC BIOMARKERS

Metabolites are small molecular mass components or intermediate products of metabolism that can be easily measurable in bio fluids and tissues using high throughput technologies; they are associated to various biological functions as regulating and main-

taining physiology homeostasis and can be influenced by genetics and environmental factors [58].

So far, only a few studies have explored whether metabolites in biofluids could serve as biomarkers in DMD patients [59, 60] and DMD animal models [61, 62].

The ratio of two metabolites, creatine, and creatinine, was significantly associated to the progression of disease, increasing with age in DMD patients [59, 60]. Interestingly, creatinine and guanidinoacetic acid showed intermediate levels in BMD patients compared to DMD patients and controls, suggesting a possible 'metabolic signature' to discriminate the two types of muscular dystrophies [60].

Regarding animal models, *mdx* muscle exhibits lower levels of carnosine, taurine, glycine, methionine, and creatinine comparing to healthy muscle [63, 64]. On the contrary, glutamine, succinate, isoleucine, acetate, alanine, and glycerol were increased in *mdx* samples [63].

Besides serum, urine has also been analyzed as a potential source of metabolic biomarkers, within the context of DMD. In particular, the levels of 3-methylhistidine urine decreased with age in DMD patients, whereas appeared to remain stable in healthy controls [65]. Conversely, prostaglandin D2 metabolite was found elevated in the urine of DMD patients compared to controls; moreover, collected data revealed an increase of this metabolite above 8 years of age, proposing it as a potential biomarker candidate also for patients in the declining ambulatory phase [66].

In a recent study, the use of metabolomics for monitoring disease progression was also explored, revealing promising results. This 7-month longitudinal study, performed in plasma of *mdx* and wild-type mice, identified a signature of 31 metabolites able to discriminate between healthy and disease at various stages of the disease [67].

Although many metabolic biomarker candidates have been detected, the correlation of metabolic levels with clinical markers and disease progression is still challenging and needs to be elucidated.

microRNAs BIOMARKERS

microRNAs (miRNAs) are a class of short, noncoding RNAs that function post-transcriptionally to regulate gene expression, in a sequence-specific manner [68, 69]. The intricate functions of miRNAs make them key players in various mammalian processes

that are essential for development and survival [68].

In mammalian tissue miRNA activity is involved in cellular proliferation and differentiation. In particular, several miRNAs' families have shown an essential role in the control of cardiac and skeletal muscle development; not surprisingly, several miRNA families are dysregulated in various human neuromuscular diseases [70, 71].

miRNAs were originally studied in muscle tissue samples from patients affected by 10 different muscular diseases, revealing several trends of dysregulation of miRNAs expression. Interestingly, five miRNAs (miR-146b, miR-155, miR-214, miR-221 and miR-222) showed a dysregulated pattern of expression in each sample, consisting of up- or downregulation according to disease type. 'DystromiRs' was the definition attributed to these types of miRNAs involved in cellular response to muscle damage and potentially usable as highly specific biomarkers [72].

Significantly, amounts of miRNAs were also detected in extracellular body fluids, as blood serum and plasma, and thus identified as potential accurate and non-invasive biomarkers for various diseases [73]. Therefore, several studies are now focusing on their use as non-invasive biomarkers for diagnosis, prognosis, and efficiency of clinical trials.

Blood levels of muscle-specific miRNAs, such as myomiRs miR-1, miR-133, and miR-206, were found to be inversely related to the North Star Ambulatory Assessment (NSAA) score of DMD patients, increasing when the severity of muscle damage worsens [74].

Another study highlighted the utility of miRNAs also as a biomarker able to predict severity of the disease in an individual patient's case. Collected data revealed that miR-181 and miR-30c positively correlate with motor function in DMD patients, being significantly elevated in blood of DMD patients with a better motor performance. Interestingly, these results were consistent and not associated to patient's age or previous CS treatment [75].

Up or down-regulation of miRNA associated with heart and/or skeletal muscle pathologies, including cardiac hypertrophy (e.g., miR-22 and miR-26a), fibrosis (e.g., miR-26a, miR-222, and miR-378a-5p), muscle cell death (e.g., miR-342), and regulation of skeletal muscle mass (e.g., miR-378 and miR-29c) regulators have been detected in biofluids of DMD female carriers [76, 77].

In female DMD carriers, down-regulation of circulating miR-29c appeared associated to the presence of functional and/or structural cardiac abnormalities,

resulting a promising novel biomarker for an early diagnosis of cardiomyopathy [77].

Several lines of evidence suggested a potential use of miRNAs as a method to assess the efficacy of a treatment. Serum levels of myomiRs were analyzed in DMD patients who participated in two clinical trials testing exon skipping therapy mediated by a phosphoromediate morpholino oligomer (PMO), known as Eteplirsen. None of the four myomiRs (miR-1, miR-133a,b, miR-206 and miR-31) showed a significant statistically difference in pre-treated and Eteplirsen post-treated samples [78].

An even more minimally invasive method of early diagnosis was analyzed by another study, proposing to evaluate changes in miRNA levels in the urine of DMD patients. Findings of this study indicated that miR-29c-3p was significantly downregulated in the urine of DMD ambulatory patients whereas urine of non-ambulatory DMD patients showed downregulation of both miR-23b-3p and miR-21-5p, suggesting miRNA levels are a sensitive marker of the physical condition of patients [79].

miRNAs appear as promising non-invasive biomarkers to improve early diagnosis and to monitor disease progression and efficacy of treatments for DMD. However, there are several outstanding questions regarding the ability of miRNAs to act as biomarker. Firstly, it remains to be determined if there are individual or classes of miRNAs that can be consistently identified in serum of patients. Moreover, several comorbidities are associated with DMD: it is possible that these variable factors could independently influence miRNA expression at different rates between patients, making it necessary to consider groups of miRNAs together as a diagnostic tool [80].

Further studies need to be performed for validation of miRNAs as biomarkers and explore their specificity and sensitivity.

Table 1 summarizes molecular biomarkers identified and described in the text.

GENETIC MODIFIERS AS POTENTIAL BIOMARKERS

Despite genetic homogeneity, DMD subjects express a range of variable phenotypes. These include phenotype severity (age at LoA), cardiac involvement, intellectual disabilities, and neuropsychiatric comorbidities [81].

The etiopathogenic bases of this clinical variability are not fully elucidates and mutation types, DMD isoforms expression, or DMD-related proteins may

Table 1
Molecular biomarkers in serum, plasma, urine, muscle and organs of DMD patients and dystrophic animal models

Class of molecule	Biomarkers	Sample	Species	Type of biomarker	References
Proteins	Creatine kinase	Serum	Human	Diagnostic	[27]
	TNNI3, CAMK2A, CAMK2B, MAPK12, MDH1, GP1, ST2	Serum	Human	Pharmacodynamic, prognostic disease progression monitoring	[29][30][31]
	OMD, AGER, Cadherin-5 and Contactin-4	Serum	Human	Prognostic, disease progression monitoring	[29]
	MMP-9, TIMP-1	Serum	Human	Prognostic, predictive, disease progression monitoring	[32][33][34]
	ADAM12, BOC, CSRP3, GDF11,GDF8	Serum	Human	Prognostic, disease progression monitoring	[29]
	FGG, IL-6, CXCL10, CCL18, CCL2, ANGPT2, TNRSF1A, COL12, C5-b-C6 complex, haptoglobin	Serum	Human	Prognostic, disease progression monitoring	[29][35][36]
	MYL3, CA3, MDH2, ETFA	Serum, Plasma	Human	Prognostic, disease progression monitoring	[37][38]
	CST3	Serum	Human	Safety	[39][40][41]
	GLDH	Serum	Human	Safety	[43]
	TTN, Ferritin, Uromodulin	Urine	Human, GRMD dogs, mdx mice	Diagnostic, prognostic, disease progression monitoring	[44][45][46][54]
	FABP3	Muscle,Serum	Human, GRMD dogs, mdx mice	Prognostic, disease progression monitoring	[53]
	FABP5,FABP1	Liver, Kidney	mdx mice	Prognostic, disease progression monitoring	[53]
	GFAP, Annexin, Vimentin,Calretinin PMCA2	Brain	mdx mice	Prognostic, disease progression monitoring	[51]
Metabolites	Creatine/creatinine ratio	Serum	Human	Prognostic, disease progression monitoring	[59][60]
	Guanidinoacetic acid	Serum	Human	Prognostic, disease progression monitoring	[60]
	Carnosine, taurine, glycine, methionine, creatinine	Muscle,Plasma	mdx mice	Prognostic, disease progression monitoring	[63][64]
	Glutamine, succinate, isoleucine, acetate, alanine, glycerol	Muscle	mdx mice	Prognostic, disease progression monitoring	[63]
	3-methylhistidine	Urine	Human	Prognostic, disease progression monitoring	[65]
	Prostaglandin D2	Urine	Human	Prognostic, disease progression monitoring	[66]
miRNA	miR-146b, miR-155, miR-214, miR-221, miR-222	Muscle	Human	Prognostic, disease progression monitoring	[72]
	miR-1, miR-31, miR-133, miR-206	Serum	Human	Prognostic, disease progression monitoring	[74][78]
	miR-181, miR-30c	Serum	Human	Prognostic, disease progression monitoring	[75]
	miR-22, miR-26a, miR-378a-5p, miR-342, miR-378, miR-29c*	Plasma	Human	Diagnostic, prognostic, disease progression monitoring	[76][77]
	miR-29c-3p, miR-23b-3p, miR-21-5p	Urine	Human	Prognostic, disease progression monitoring	[79]

*female DMD carriers.

play a role in this wide clinical spectrum. Understanding factors underlying these differences has a great impact on disease prognosis evaluation, stratification of patients for clinical trials, therapeutic targets choices, and assessment of drug efficacy. Therefore, in recent years efforts have been directed at identifying genetic pathways that interact with *DMD* gene mutation.

Genetic modifiers are genetic variations, generally SNPs, occurring in genes that can positively or negatively modulate the phenotype via interactions between genes and their environment [82].

To date, variants in five loci have been associated with variability in human DMD sub-phenotypes and were validated in several study cohorts: *SPP1*, *LTBP4*, *CD40*, *ACTN3*, and *THBS1*. Four of these genes (*SPP1*, *LTBP4*, *CD40*, and *THBS1*) are implicated in several interconnected molecular pathways regulating inflammatory response to muscle damage, regeneration, and fibrosis [83].

Among genes belonging to TGF-β pathway, *SPP1* and *LTBP4* haplotypes have been surveyed in several cohorts.

SPP1 gene (MIM *166490), encodes Osteopontin (also known as secreted phosphoprotein 1), a secreted glycoprotein that has roles in bone-remodeling, immune function, and muscle repair [84].

A polymorphism in the promoter region of the *SPP1* gene (-66T/G), annotated as rs28357094, has been found to be significantly correlated with a more rapid progression of disease, earlier LoA and reduction of grip strength in a cohort of DMD patients [85]. This association, however, was not confirmed by another study performed on a European cohort [86].

A further study indicated that the less common G allele was associated with more rapid disease progression, especially in patients treated with CS, implying that this variant may act as a pharmacodynamic biomarker of CS response [15].

More recently, SPP1 was found overexpressed in DMD myotubes carrying the G allele because of CS treatment [87].

Latent TGFβ Binding Protein 4 (*LTBP4*) has also been identified as a genetic modifier [88]; the two-allele polymorphism forms a haplotype in human *LTBP4* (MIM *604710) containing four nonsynonymous SNPs that are in linkage disequilibrium and compose two major haplotypes, IAAM and VTTT.

Two cohorts demonstrated that the IAAM haplotype significantly correlated with prolonged ambulation. In a multi-ethnic cohort of DMD subjects, IAAM homozygotes were ambulant around 2 years longer than heterozygotes or homozygotes for the VTTT haplotype [15]. *LTBP4* IAAM haplotype was also found to be significantly associated with prolonged ambulation in patients under CS treatment in a cohort of 265 DMD patients from many European Centers [86].

A SNP (rs1883832, *C* > T, minor allele T) in the 5' untranslated region of *CD40* gene (MIM *109535) was identified to be associated with earlier LoA in multiple independent DMD cohorts. Reduced CD40-mediated cell-cell signaling in carriers of the minor rs1883832 allele might precipitate failure of regeneration and fibrosis in DMD skeletal muscle [89].

Another marker with effect on the DMD phenotype is a common nonsense polymorphism (R577X, rs1815739) in the *ACTN3* gene (MIM *102574). This polymorphism appeared to be associated with significantly reduced muscle strength and poorer performance at the 10 m walk test in young, ambulant DMD patients [90].

A large genome-wide association study (GWAS) conducted by the United Dystrophinopathy Project (UPD) in their severe DMD cohort, revealed that the minor allele rs2725797 in *THBS1* gene (MIM *188060) appeared to be protective against DMD progression and may allow prolonged ambulation in DMD patients [91].

Altogether the above data suggest a relevant role of these gene modifiers on DMD disease severity and, quite importantly, on CS response. Regarding this aspect, a recent study aimed at identifying SNPs possibly linked to CS response in DMD boys.

Based on prioritization of SNPs in candidate genes by targeted resequencing, this study identified the *TNFRSF10A* C/T haplotype as associated to a better response to CS [92]. In detail, data collected suggested that the identified *TNFRSF10A* C/T haplotype confers a better response to CS since it reduces cytokines release and increases the beneficial effects of CS by decreasing their pro-apoptotic effect. Given these findings, the authors proposed a dual screening for *TNFRSF10A* and *LTBP4* SNPs in DMD patients to investigate the CS response [92].

Interestingly, and further supporting the *TNFRS10A* modifier role, the pro-inflammatory marker TNFRSF1A, which belongs to the same receptor superfamily, was found to be elevated in the serum of DMD patients' serum [29]. Table 2 summarizes DMD genetic modifiers described in the text.

Table 2
DMD genetic modifiers

Gene	SNP	Type of biomarker	Phenotype	Cohort	References
SPP1	rs28357094 (*T*>G, minor allele G)	Predictive, prognostic, disease progression monitoring, pharmacodynamic	More rapid progression of disease, earlier LoA, and reduction of grip strength	Two DMD cohorts: a Padova longitudinal cohort and the Cooperative International Neuromuscular Research Group (CINRG)	[85]
			No significant association between the SNP and the age of LoA	Five European neuromuscular centres	[86]
			More rapid disease progression, especially in DMD patients treated with CS	Cooperative International Neuromuscular Research Group Duchenne Natural History Study (CINRG-DNHS)	[15]
LTBP4	IAAM haplotype	Predictive, prognostic, disease progression monitoring	Delay in LoA in patients with dystrophinopathy	United Dystrophinopathy Project (UPD) cohort	[88]
			Prolonged ambulation in DMD CS-treated patients	Five European neuromuscular centres	[86]
			Prolonged ambulation in DMD patients	Cooperative International Neuromuscular Research Group Duchenne Natural History Study (CINRG-DNHS)	[15]
CD40	rs1883832 (*C*>T, minor allele T)	Prognostic, disease progression monitoring	Earlier loss of ambulation and possibility to precipitate failure of regeneration and fibrosis in DMD skeletal muscles	Multiple independent DMD cohorts	[89]
ACTN3	rs1815739 R577X	Prognostic, disease progression monitoring	Reduced muscle strength and poorer performance in the 10 m walk test in young, ambulant patients with DMD	Cooperative International Neuromuscular Research Group Duchenne Natural History Study (CINRG-DNHS)	[90]
THBS1	rs2725797	Prognostic, disease progression monitoring	Prolongation of deambulation; protective against DMD progression	United Dystrophinopathy Project (UPD) cohort	[91]
TNFRSF10A	C/T haplotype	Predictive	Better response to CS treatment in DMD patients	217 DMD patients	[92]

FUTURE PERSPECTIVES

During recent years, extensive research efforts and advances in the high-throughput omics technologies, has allowed discovery of clinical blood-based biomarkers for neuromuscular diseases, with a strong emphasis on DMD.

The identified biomarkers can serve two important roles in clinical practice. Firstly, disease-specific biomarkers related to pathogenic mechanisms of the condition, can be useful to identify the response to disease-modifying therapies. Alternatively, non-specific disease biomarkers, linked to specific aspects of the disease, could provide information about the progression of the disease and/or serve as pharmacodynamics biomarkers to evaluate the improvement of a particular symptom/sign.

Biomarkers can thus expand our knowledge about the mechanisms underlying the disease and consequently, the identification of potential new therapeutic key targets.

Moreover, advancements in therapies for DMD have provided identification of biomarker for diagnosis within the newborn period a high priority.

The context of biomarkers' application greatly varies and defines the burden of proof required to promote their translation into clinical practice [93]. For example, in a screening setting (as NBS), higher false-positive rates are tolerable, and the focus of concern may be on false-negative rates; on the contrary, a biomarker intended to measure the efficacy and/or safety of therapies must have a very low false-positive rate.

However, the translation of biomarkers into clinical practice of a rare disease (RD) is adjoined by many challenges such as the technical validation of biomarkers, the limited sample resources, the high cost of technology, the management of high-throughput data and the education of healthcare professionals for interpretation of omics data.

Firstly, although many multiplexing proteomics methods was proven useful for large-scale biomarker discovery, their translation to clinical use remains challenging due to the lack of substantial evidence regarding their reliability as quantifiable indicators of disease state or outcome.

Comparison of results from different laboratories on biomarker discovery, indicate that biomarker candidates to discriminate between DMD patients and healthy individuals are not all reproduced even when the same analytical assay and technology are used [28, 29].

Errors related to poor analytical accuracy (variability related to the detection assay) and/or poor clinical accuracy (variability related to biological factors) can explain this discrepancy.

To overcome these challenges, a recent work used a sensitive and specific Parallel Reaction Monitoring Mass Spectrometry assay (PRM-MS) for biomarker confirmation to avoid errors/variability introduced by antibody-based proteomic methods; in addition, a novel orthogonal analytical validation was developed and used to confirm already identified serum biomarkers for DMD [94].

Furthermore, most strategies used for the identification and validation of biomarkers requires a large number of patients.

Within RDs, the number of samples mirrors the low prevalence of the disorders with a consequent difficulty in differentiate patients in phenotype sub-classes. The mathematical power of standard statistics is thus very poor and study reproducibility is hampered by confounding factors such as ethnicity and genome heterogeneity.

Furthermore, the statistical models normally used for polygenic traits do not appear suitable for DMD or other RDs. Indeed, novel tools/models suitable for analyzing small patient numbers should be adopted.

Collection of samples and multicenter data sharing has been standardized and procedures harmonized through several national and international collaborative efforts, e.g. EuroBioBank [95], RD-Connect [96], Orphanet [97], in order to overcome these difficulties.

The importance of sample collection and access to these resources is widely agreed and platforms are designed for selection and sharing of biological samples as well as integration of omics results with phenotypic data.

A strong support in this context derived also from the creation of large cohorts of patients.

Currently there are two large cohorts available for modifier identification: the CINRG cohort and the Bio-NMD cohort, linked by a reciprocal agreement for which modifiers identified in one cohort are validated in the other.

Another critical point is the integration of many datasets available for biomarkers discovery and validation.

One possible way to facilitate sample and data sharing could be the development of public repositories of biological samples and biobanks for biomarker validation studies as well as the use of bioin-

formatics tools to allow comparison of different datasets.

Many initiatives are ongoing in Europe, such as the Joint Research Programmes, which are funding novel '*ad hoc*' registries of diseases, the creation of which may greatly facilitate biomarker validation studies [98].

Another challenging aspect is the heterogeneity of the DMD and the limited knowledge of the pathophysiology of sub-phenotypes. For example, the patient variability in muscle function obscures correlation between biomarkers and functional tests. Moreover, the molecular bases of different clinical aspects, not only in skeletal muscle, but also in the heart and central nervous system, still need to be elucidated.

In the light of these challenges, studies are required to increase our understanding of disease pathology and different sub-phenotypes on both cellular and tissue level.

While disease progression biomarkers are useful for clinical management of the disease, treatment monitoring biomarkers can aid in accelerating discovery, validation, regulation, and commercialization of new drugs.

Although progresses have been made towards identifying and implementing translational safety biomarkers for kidney and liver, significant biomarker gaps still exist to monitor toxicities for other organ/tissue toxicities.

Several precompetitive consortia [e.g., Predictive Safety Testing Consortia (PSTC), Innovative Medicines Initiative (IMI)] are currently working with industry, academia, government, patient advocacy groups and foundations to qualify safety biomarkers to be used in preclinical studies and clinical trials.

Another current challenge in monitoring response to therapies in DMD is that different outcome measures are required at different stages of the disease, and these measures can sometimes be subjective and less sensitive. In this context, molecular biomarkers are expected to be less subjective, more robust and sensitive and could be implemented throughout disease stages and patient ages.

It is well known that accelerated approval by the FDA or EMA would also benefit of the availability of surrogate endpoints expected to anticipate clinical benefit, thus accelerating the commercialization.

However, a biomarker can be considered a surrogate endpoint only if it directly and specifically correlates with a clinical outcome. This correlation is often very difficult to demonstrate and, in fact, very few surrogate endpoints have been approved so far.

A twofold effort should be performed to identify biomarkers with a proven surrogate endpoint: I) replicate identified molecular targets and pathways in large cohorts in order to discover biomarkers that correlates to a clinical endpoint; II) explore the potential of the candidate biomarker as surrogate endpoint in '*ad hoc*' studies aiming at integrating molecular and clinical outcomes.

Reported data made MMP-9 suitable as a surrogate endpoint in clinical trials [32, 33]; however, in a study comprising samples from two independent clinical trials, serum MMP-9 was not found to be a predictive biomarker for treatment response with Drisapersen [34].

Similarly, circulating miRNAs have been shown to be potential biomarkers for early detection of DMD or monitoring disease progression and treatment outcome. Over the last decade, numerous studies have highlighted the importance of these non-coding RNAs, critical regulators of myogenesis, muscle homeostasis and with a significant role in muscle-associated diseases, sarcopenia and cancer cachexia [99].

For DMD, dystrophin protein expression is the only approved surrogate pharmacodynamic biomarker for therapies aiming at restoring/inducing dystrophin synthesis [100].

Genetic modifiers are very appealing for sub-phenotypes categorization and for patients' stratification in clinical trials; however, ethnicity and different genotype or haplotype assortment may affect the accuracy of association studies, and therefore the validation of patient cohort studies.

Novel approaches, like the identification of epigenetic markers, which are completely unexplored, as well as the free availability of clinical data for research, will increase dataset informativeness and facilitate the clinical translation of biomarkers.

CONCLUSIONS

Biomarkers and genetic modifiers may provide deeper insights into DMD pathogenesis, stratify disease subtypes, and enable early diagnostics and early therapeutics.

The development of biomarkers is also crucial in accelerating the development of innovative therapies.

There is an urgent need for a reliable surrogate biomarker or set of biomarkers for DMD, ideally based on readily accessible and measurable

molecules. The potential of these candidates as surrogate endpoints needs to be evaluated in '*ad hoc*' studies where molecular and clinical outcomes can be compared.

While the work done so far has made it possible to discover several candidate biomarkers, their translation into clinical practice is still limited and major improvements are still needed.

DISCLOSURES

ACKNOWLEDGMENTS

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