Duchenne Muscular Dystrophy: recent advances in protein biomarkers and the clinical application

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ABSTRACT

Introduction: Early biomarker discovery studies have praised the value of their emerging results, predicting an unprecedented impact on health care. Biomarkers are expected to provide tests with increased specificity and sensitivity compared to existing measures, improve the decision-making process, and accelerate the development of therapies. For rare disorders, like Duchenne Muscular Dystrophy (DMD) such biomarkers can assist the development of therapies, therefore also helping to find a cure for the disease.

Area covered: State-of-the-art technologies have been used to identify blood biomarkers for DMD and efforts have been coordinated to develop and promote translation of biomarkers for clinical practice. Biomarker translation to clinical practice is however, adjoined by challenges related to the complexity of the disease, involving numerous biological processes, and the limited sample resources. This review highlights the current progress on the development of biomarkers, describing the proteomics technologies used, the most promising findings and the challenges encountered.

Expert opinion: Strategies for effective use of samples combined with orthogonal proteomics methods for protein quantification are essential for translating biomarkers to the patient’s bed side. Progress is achieved only if strong evidence is provided that the biomarker constitutes a reliable indicator of the patient’s health status for a specific context of use.

1. Introduction

Molecular biomarkers are measurable properties or characteristics eg. proteins, that are specific, accurate, and sensitive indicators of disease states. In clinical setup, biomarkers are routinely analyzed in biological samples, and development of novel biomarkers holds promise to radically change the way diseases are diagnosed, treated, and cured. Blood, for example, mirrors pathological changes that occur in the body, as it accesses different organs and accommodates molecular markers originating from different tissues [1,2]. Thus, blood samples constitute a valuable source of information for clinical management of disorders [1,3]. This review focuses on the current knowledge regarding discovery and validation of protein biomarkers for DMD, in blood, and highlights the challenges encountered in translating such markers to clinical applications.

2. The value of biomarkers

Numerous efforts have explored the promised land of biomarkers searching for new and/or better biomarkers, in a quest for health care improvement and development of novel therapies. Biomarkers are expected to significantly contribute to precision medicine by providing not only improved diagnostics but also new indicators for disease prediction [3]. Similarly, they are anticipated to provide information regarding the individual variation in terms of eg. response to treatment, to improve the decision-making process for selection of therapies and allow adaptation of clinical interventions to the patient’s need [4,5]. In addition, biomarkers benefit the patient not only by improving management of the diseases with already existing treatments but also by facilitating the development of novel therapies. For incurable disorders, biomarker aid preclinical research and clinical trials, by ensuring appropriate study design and patient recruitment, as well as evaluation of treatment outcome and toxic effects. Considering the many aspects biomarkers cover and the numerous applications they are used for, classification and standardization of definitions are essential. Biomarkers are classified in: (1) diagnostic, (2) pharmacodynamic/response, (3) disease progression monitoring, (4) prognostic, (5) predictive, and (6) susceptibility/risk [6,7]. This classification captures the disparity between biomarkers, ensures unambiguous communication, and facilitates translation of research results to clinical practice [7].

3. Duchenne Muscular Dystrophy

One of the most severe forms of muscular dystrophy is Duchenne muscular dystrophy (DMD) affecting 1:3,500 to 1:5,000 boys [8–11]. Affected individuals experience a progressive disease, in which loss of muscle mass and, consequently function, occurs over a period of many years...
with, fatal outcome [12, 13]. The disease causes initially, delay of motor milestones and as the disease progresses, loss of motor function, resulting in a complete loss of ambulation at age between 8 and 14 years [11], severe dilated cardiomyopathy and respiratory insufficiency [14]. The median life expectancy estimated across 12 countries, is between 19.6 and 20.4 years, for patients that have not received respiratory support and between 28.9 and 30.6 years for patients using respiratory aid [15].

DMD is an X-linked chromosomal-inherited recessive disease, caused by mutations in the DMD gene. The dystrophin gene encodes for a large protein, of 427 kDa, with a key role in muscle contraction and function [16–18]. Dystrophin provides a flexible connection between the actin filaments and the sarcolemma through four major functional domains: the actin-binding N-terminal domain interacting with γ-actin filaments, the a flexible connection between the actin filaments and the support and between 28.9 and 30.6 years for patients using respiratory function, resulting in a complete loss of ambulation at age between 8 and 14 years [11], severe dilated cardiomyopathy and respiratory insufficiency [14]. The median life expectancy estimated across 12 countries, is between 19.6 and 20.4 years, for patients that have not received respiratory support and between 28.9 and 30.6 years for patients using respiratory aid [15].

DMD is an X-linked chromosomal-inherited recessive disease, caused by mutations in the DMD gene. The dystrophin gene encodes for a large protein, of 427 kDa, with a key role in muscle contraction and function [16–18]. Dystrophin provides a flexible connection between the actin filaments and the sarcolemma through four major functional domains: the actin-binding N-terminal domain interacting with γ-actin filaments, the central rod domain acting as a linker, the cytosine-rich domain interacting with the β-dystroglycan at the sarcolemma, and the C-terminal domain. DMD is caused by mutations, which disrupt the reading-frame, with premature translation termination and alteration or absence of the actin or the β-dystroglycan binding sites, as a consequence. The absence of a functional dystrophin protein alters the composition and structure of the dystrophin-glycoprotein complex (DGC) with consequences on structural stability of muscle fibers, signaling, and regeneration of muscle fibers subsequent injury. In DMD muscles, the DGC’s role as a link between the cytoskeleton and the extracellular matrix is compromised resulting in increased fragility of the sarcolemma during intensive contractile activity [19]. The increased membrane fragility, alters its permeability to otherwise impermeable molecules with implication on calcium homeostasis, as Ca\(^{2+}\) ions enter through membrane breaks. The reoccurring muscle fiber damage solicits recruitment of inflammatory cells for removal of damaged fibers and subsequent muscle fiber regeneration [20, 21]. Restoration of damaged fibers is not fully compensated due to alteration in muscle satellite cells proliferation and differentiation [22, 23]. Thus, as the disease progresses, patients experience increasing muscle fiber degradation, inflammation, fibrosis, and fat infiltration. As a consequence of membrane breaks and/or increase deregulation of vesicle trafficking, muscle-specific proteins are most likely released into the blood stream [24–26]. In addition, inflammatory cells also contribute to accumulation of pro-inflammatory molecules and cytokines in blood [27].

The assessed diagnostic tests for clinical management of muscular dystrophies include a wide range of physical tests, blood and biopsy tests, performed by a multi-disciplinary team including but not limited to experts in neuromuscular medicine, pediatrics, genetics. The 6-minute walk test (6MWT), 10 meter walk test (10MWT), Northern Star Ambulatory Assessment (NSAA), and performance of upper limb (PUL) are commonly used measurement tools in motor dysfunctional children. Physical tests very often rely on the patient’s capability to collaborate with the clinicians, follow instructions, and ability to perform and complete the entire test. In addition, the tests are influenced by other factors than the disease eg. patient’s motivation, physical exercise, age, psychological-disorder, and/or attention deficiency. These aspects have consequences on the sensitivity and accuracy of the functional tests. Beside physical tests, skeletal muscle composition in terms of Magnetic Resonance Imaging (MRI) measured fat fraction is currently used as an exploratory biomarkers for DMD [28, 29]. The fat fraction in muscles correlates with functional outcomes [30], physical tests, and predict deterioration of patient mobility [29, 31, 32]. MRI itself is noninvasive and feasible, even in young children. However, imaging is not part of the routine and long-term clinical follow-up, due to the lack of standardized protocols for image acquisition and data analysis, high cost, and long duration of the scans [33].

4. Protein biomarkers for DMD

International efforts have explored blood components eg. proteins, miRNA to identify biomarker candidates for DMD. Proteomics studies have reported several muscle-specific proteins as well as proteins involved in energy metabolism, fibrosis, and inflammation as potential biomarker candidates [34–37] (Table 1). Similarly, circulating microRNAs eg. miR-29 c-3 [38], miR-1 and miR-206 [39, 40] have been shown to be potential biomarkers for early detection of DMD or monitoring disease progression and treatment outcome [41, 42]. In addition, amino acid eg. arginine, glutamine, and histidine [43] as well as metabolites eg. creatine has been identified as potential biomarker candidates [44, 45]. Additional studies of miRNA and metabolites in longitudinally collected samples can provide essential evidence to promote their use as blood biomarkers in a clinical setup [46].

4.1. Proteomic technologies and biomarker discovery and validation

State-of-the-art technologies within the proteomics field have contributed to the discovery and validation of biomarkers for the most common diseases eg. cancer, metabolic, and cardiovascular disorders [61–63]. This development is also beneficial for biomarker discovery in the context of rare disorders which comprises chronic, incurable diseases [46, 64]. The initial studies exploring serum...
composition from patients with DMD focus mainly on diagnostic biomarkers and resulted in the discovery of creatine kinase [65,66] through enzymatic assays. In addition, muscle-specific enolase [67,68] and carbonic anhydrase III (CA3) [69,70] are two additional serum biomarkers identified using enzymatic or radio-immunoassays. From the initial studies, CK measurements have been translated to the clinical practice [71].

In time, biomarker discovery has transitioned from single target analysis to multiplex measurements using both mass spectrometry-based methods and affinity-based methods. Proteins associated with the disease are identified through protein profiling of healthy and dystrophic tissues in mouse and canine models [34,72–75] and further explored in samples collected from patients. The unprecedented development of high-throughput technologies has resulted in discovery of blood biomarkers through systematic protein profiling of a subset of the proteins associated with DMD or untargeted protein analysis [36,76,77]. Protein profiling using mass spectrometry has benefit from the increased resolution and throughput and resulted in the discovery of serum biomarkers eg. factor XIIIa in mdx mice [76] using MALDI-MS, myomesin-3 (MYOM3) in DMD patients using a tandem mass spectrometry approach LC/MS-MS [77] and fibronectin (FN1) detected by MS/MS [37]. However, serum and plasma comprise complex mixtures of proteins at concentration within a wide range, with albumin at concentration of 35–50 mg/ml being one of the most abundant proteins and IL-6 at a concentration of 0–5 pg/ml [2]. This complexity has consequences on the detection of relevant targets. The presence of high abundant peptides causes decreased ionization of rare peptides with consequences on the detection of least abundant proteins [78], restricting the detection limit to above 50 ng/ml [79]. The complexity of serum and plasma samples can be diminished through depletion of the most abundant proteins eg. albumin and IgG [80] or the 12 most abundant proteins [77] or enrichment of low abundant proteins [37]. However, depletion of such proteins may alter the abundance of other proteins and increase the standard error of the analysis [81,82]. To tackle with the complexity, samples are also fractionated based on hydrophobicity, charge, affinity, or size of the proteins [37,76,77,80]. The methods of choice in biomarker discovery studies vary resulting in discrepancies with respect to which biomarkers are detected. However, it should be noted that results obtained by mass spectrometry are confirmed with additional immune-based methods eg. ELISA and Western Blot in the case of eg. FN1 and MYOM3, respectively, [37,77].

The increasing availability of validated antibodies for protein profiling [83] enabled discovery of potential biomarkers using a suspension bead array platform [36,84]. This technology allows antibody-mediated capturing of protein targets to unique color coded beads and detection of the captured molecule through a covalently bound fluorescent label [36,85]. Besides a high multiplexing capacity, that allows analysis of as many as 500 analytes simultaneously, this technological platform uses only microliter amount of sample and has a sensitivity of pg/ml range for analysis of proteins in complex samples [86]. In addition, this technology also offers the possibility to mimic the ELISA concept, by detecting the captured target with a second antibody, making it attractive for the development of sensitive and accurate biomarker quantification assay for translation to clinical use.

Being indispensable for proteomics studies, antibodies are both praised and condemned, and their performance continuously scrutinized in particular since, antibody specificity is context and application dependent [87]. Academic efforts like the The Human Protein Atlas project [83] and commercial producers ensure development and access to large numbers of antibodies, validated according to developed guidelines [88,89]. In particular, the guidelines for enhanced validation of antibodies designed by the International Working Group for Antibody Validation (IWGAV), an ad-hoc formed group of leading researchers, promotes rigorous validation of such reagents and has been adopted by researchers and leading antibody producers [89–91]. Beside antibodies development of short single-stranded oligonucleotides as affinity reagents and the SomaLogic technology enabled researchers to analyze more than 1,000 targets in cross-sectional studies in the context of DMD [92–96]. The assay relies on the capture of the target in its native conformation to immobilized protein-specific Slow Off-rate Modified DNA aptamers (SOMAmers). The SOMAmer-target complex is isolated from the sample and subsequent dissociation of the complex, the SOMAmer quantified. Quantification is achieved through hybridization of the SOMAmers to an array using the SomaScan platform. The platform has a high multiplexing capacity that allows analysis of 5,000 targets simultaneously. Although the high-throughput screening methods are useful biomarker discovery tools, confirmation of results with more targeted assays eg. ELISA is necessary.

### 4.2. Biomarkers for DMD

One of the muscle-specific proteins released into the blood stream of DMD patients is creatine kinase (CK). CK is elevated in DMD patients and declines as the diseases progresses [36,84,97,98]. Its increased activity has also been used as an indicator of muscle damage in clinical practice [71]. As a biomarker, serum CK is influenced by other factors than the disease, as shown in both DMD patients and mouse model studies [99–101]. Alone CK is not specific enough to be used as a diagnostic or prognostic biomarker [102]. However, CK has been used for new born screening to facilitate early detection of muscle injury diseases as DMD [103–105]. Beside CK, several proteomics studies performed in the context of DMD, reported elevated blood levels of other muscle-specific proteins eg, carbonic anhydrase 3 (CA3), malate dehydrogenase 2 (MDH2), myosin light chain 3 (MYL3), cardiac muscle troponin I (TNNI3), titin (TTN) and proteins involved in energy metabolism eg. beta enolase, fructose-bisphosphate aldolase, electron transfer flavoprotein A, fibrosis eg. fibronectin and inflammation eg. interleukin [46,64,106] (Table 1).

The majority of the identified biomarker candidates are muscle-specific proteins elevated in DMD patients in comparison to controls. Over time, these biomarkers have declining abundance trajectories, as the disease progresses [107] most likely correlating with the decreasing muscle mass. The serum and/or plasma levels of CA3, MDH2, MYOM3, myosin, and
Table 1. List of DMD biomarkers identified. The biomarkers are ranked based on the number of publications providing evidence for association of the biomarker with DMD. Biomarkers identified in both human and animal models are listed first, followed by biomarkers identified only in DMD patients and subsequently in only DMD animal models.

| Biomarker candidate                                    | Sample        | Species                                      | Proteomics method                  | Reference                      |
|--------------------------------------------------------|---------------|----------------------------------------------|------------------------------------|--------------------------------|
| Creatine kinase                                        | Serum, plasma | Human, mdx, GRMD                             | Immuno-, mass spectrometry-based   | [34,47–52,80,98,101] |
| Carbonic anhydrase 3                                   | Serum, plasma | Human, mdx-4cv                               | Immuno-, mass spectrometry-based   | [36,77,80,92,94,96,107,124] |
| Fatty acid binding protein 3                           | Serum         | Human, mdx-4cv, mdx-23, mdx-52              | Immuno-, mass spectrometry-based   | [34,80,92,93,106,142] |
| Myoglobin                                              | Serum         | Human, mdx-23, mdx-52                        | Immuno-, mass spectrometry-based   | [34,84,92,107,144] |
| Cardiac muscle troponin I                             | Serum         | Human, mdx-23                               | Immuno-, mass spectrometry-based   | [92–94,107,144] |
| Calcium/calmodulin-dependent protein kinases           | Serum, plasma | Human, mdx-23                               | Immuno-, mass spectrometry-based   | [92–94,107,144] |
| Lactate dehydrogenase                                 | Serum, plasma | Human, mdx-4cv                               | Immuno-, mass spectrometry-based   | [47,77,80,93,124] |
| Myosin light chain 1/3                                 | Serum, plasma | Human, mdx-23, mdx-52, mdx-4cv              | Immuno-, mass spectrometry-based   | [34,36,55,80,142] |
| Myostatin                                              | Serum         | Human, mdx-23                               | Immuno-, mass spectrometry-based   | [34,53,80,94,107] |
| Haptoglobin                                            | Serum, plasma | Human, mouse mdx-4cv                         | Immuno-, mass spectrometry-based   | [77,80,119,120] |
| Fibrinogens                                            | Serum, plasma | Human, mouse mdx-23, mdx-52                 | Immuno-, mass spectrometry-based   | [34,58,94,96] |
| Malate dehydrogenase 2                                 | Serum, plasma | Human, mdx-4cv                               | Immuno-, mass spectrometry-based   | [36,80,144] |
| Enolase αβ                                             | Serum, plasma | Human, mdv-4cv                               | Immuno-, mass spectrometry-based   | [34,36,80] |
| Adiponectin                                            | Serum         | Human, mdx                                 | Immuno-, mass spectrometry-based   | [34,56,77] |
| Cytochrome-c                                           | Serum, plasma | Human, mouse mdx-23, mdx-4cv                | Immuno-, mass spectrometry-based   | [34,80,144] |
| Pyruvate kinase                                        | Serum         | Human, mdx-23, mdx-52, mdx-4cv              | Mass spectrometry-based            | [34,77,80] |
| Fructose biphosphate aldolase                          | Serum         | Human, mdx-23, mdx-52, mdx-4cv              | Mass spectrometry-based            | [34,77,80] |
| Titin                                                  | Serum         | Human, mdx                                 | Immuno-, mass spectrometry-based   | [34,54,77] |
| Tumor necrosis factor receptor                         | Serum, plasma | Human, mouse mdx-23                         | Immuno-, mass spectrometry-based   | [59,118,121] |
| Interleukins (IL-6, IL-10, IL-13)                      | Serum, plasma | Human, Mouse mdx                            | Immuno-, mass spectrometry-based   | [94,121] |
| Myomesin-3                                             | Serum         | Human, mdx-23, mdx-52                       | Mass spectrometry-based            | [34,77] |
| Transforming growth factor β                            | Plasma        | Human, mdx                                 | Immuno-based                       | [55,121] |
| Tissue inhibitor TIMP-1 of metalloproteinase           | Plasma        | Human, mdx                                 | Immuno-based                       | [55,112] |
| Filamin C                                              | Serum         | Human, mdx-23, mdx-52                       | Mass spectrometry-based            | [34,77] |
| Fibronectin                                            | Serum         | Human,mdx-4cv                              | Mass spectrometry-based            | [37,80] |
| A disintegrin and metalloproteinase with thrombospondin motifs 5 | Serum         | Human, mdx                                 | Aptamer-, mass spectrometry-based   | [144] |
| N-terminal fragment of α dystro-glycan                 | Serum         | Human, mdx                                 | Immuno-based                       | [57] |
| Phosphoglycerate mutase                                | Serum         | Human, mouse mdx-23                        | Immuno-, mass spectrometry-based   | [144] |
| Matrix metalloproteinase MMP-9                         | Serum, plasma | Human, mdx-23, mdx-52                       | Immuno-, mass spectrometry-based   | [95,112,113] |
| Interleukin 1 Receptor-Like 1 Protein                  | Serum         | Human, mdx                                 | Immuno-, mass spectrometry-based   | [95] |
| Calmodulin-like protein                                | Serum         | Human, mdx                                 | Mass spectrometry-based            | [54] |
| Electron transfer flavoprotein A                       | Serum, plasma | Human                                        | Immuno-based                       | [36] |
| Ankyrin repeat domain 2                                 | Serum, plasma | Human                                        | Immuno-based                       | [84] |
| Carbonic anhydrase 1                                   | Serum         | Human                                        | Mass spectrometry-based            | [54] |
| Fibulin 1                                               | Serum         | Human                                        | Mass spectrometry-based            | [54] |
| Gelsolin                                               | Serum         | Human                                        | Mass spectrometry-based            | [54] |
| Coagulation factor Xlla                                 | Serum         | Mouse mdx-23, mdx-3cv                       | Mass spectrometry-based            | [54,76] |
| Tumor necrosis factor-alpha                            | Plasma        | Mouse mdx                                  | Immuno-based                       | [60,121] |
| Interferon-γ                                            | Plasma        | Mouse mdx                                  | Immuno-based                       | [60,121] |
| Glutathione peroxidase                                 | Plasma        | Mouse mdx-23                               | Mass spectrometry-based            | [58] |
| Lumican                                                | Serum         | Mouse mdx-23, mdx-52                        | Mass spectrometry-based            | [34] |
| Leukemia inhibitory factor receptor                    | Serum         | Mouse mdx-23, mdx-52                        | Mass spectrometry-based            | [34] |
| Plasminogen                                             | Serum         | Mouse mdx-23, mdx-52                        | Mass spectrometry-based            | [34] |

troponins in DMD have been confirmed in several studies as potential disease progression monitoring biomarkers [34,36,68,70,84,92,108]. In addition, serum MDH2 has also been identified as a predictor of loss of ambulation and associated with response to steroid treatment in a longitudinal study [84] and MYOM3 as a therapy responsive biomarker in antisense oligonucleotide-mediated exon-skipping treated mdx mice [77]. Beside proteins that are ubiquitously expressed in skeletal muscle, proteins specifically expressed in eg. heart provides information about cardiac status. TNNI3 and Interleukin 1 Receptor-Like 1 Protein are associated with cardiac degeneration and potential biomarkers for cardiac injury.
in both patients and DMD models [95,109,110]. The use of membrane sealant in dystrophic dogs to ameliorate cardiac injury resulted in decrease of TNNI3 [111]. Matrix metalloproteinase-9 (MMP9) which is involved in the degradation of the DGC protein has been shown to increase in DMD patients as the disease progresses [112,113]. Similar studies did not corroborate these findings raising questions regarding the differences between the cohorts analyzed in terms of eg. age, mutations, baseline levels in healthy individuals, etc. [114–116]. Besides muscle-specific proteins, other targets are identified as biomarker candidates. Leptin (LEP) which is produced mostly by adipocyte is identified as a serum biomarker associated with severity of metabolic syndrome [117]. Furthermore, LEP together with the C reactive protein (CRP) are also inflammatory markers [118]. Several reports show that the degree of inflammation is evaluated by plasma haptoglobin levels [119,120]. Elevated serum levels of haptoglobin are also identified in dystrophic mdx-4cv mice [80]. Beside inflammation, DMD disease progression is also associated with increasing fibrosis. Proteins like IL-6, Interleukin 10 and IFN-γ and Interleukin 13 are suitable for monitoring fibrosis at different disease stages [92,94,118,121]. Biomarkers associated with muscle function, inflammation, and fibrosis may recapitulate disease progression but additional biomarkers are required for the development of therapies. Cystatin C (CST3) which is a clinical biomarker for kidney injury and altered glomerular filtration rate [122], has also been studied in the context of DMD [123]. The results indicate that serum CST3 can be used to monitor nephotoxicity of therapies in eg. clinical trials [123]. As a toxicity biomarker, CST3 has the advantage of being independent of age (above 1.5 years), ambulatory capacity, and type of steroid treatment [123].

4.3. Biomarker validation

Several identified biomarkers, have been analyzed in different patient cohorts and confirmed by several research groups. However, none has been approved by regulatory authorities like the Food and Drug Administration or the European Medicines Agency. The general consensus that biomarkers are difficult to translate from research to practical use is also valid for DMD [126,127]. The challenges encountered relate to providing

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**Figure 1.** Biomarker development for DMD requires (1) discovery, (2) analytical validation, (3) clinical validation, and (4) qualification of biomarker. The discovery phase comprises formulation of the context of use, assessment of feasibility in terms of eg. samples resources, selection of appropriate analytical method, etc., discovery of the biomarker and confirmation in additional samples collections to assess reproducibility in several cohorts. The analytical validation phase includes orthogonal validation of the biomarker using different analytical methods, development of biomarker quantification assays for targeted analysis and validation of the biomarker quantification assay, to evaluate biomarker sensitivity, specificity, dynamic range, and variation using the developed quantification assay and refine the context of use. Clinical validation requires testing the specificity and sensitivity of the biomarker in clinical setup, development of clinical assays, assess the context of use, robustness and variability, and its utility in clinical practice.
evidence that clearly defines how a biomarker is used in a specific context with the purpose of improving patient health care. The intended context of use varies and determines the burden of proof required to support translation of biomarkers to clinical practice [6]. For example, a higher false discovery rate is acceptable for a biomarker used in the context of newborn screening, in contrast to a biomarker used to measure the effect of therapy in non-ambulant patients eg. steroid treatment that does not cure the disease. The biomarker development process (Figure 1) is often described as a linear process comprising: (1) discovery, (2) analytical validation, (3) clinical validation, and (4) qualification of biomarker but the linearity is not entirely sustained. Analysis of three cross-sectional studies performed with the same technology reported an overlap of only 12% of the biomarkers analyzed, discriminating between DMD patients and controls in all datasets [107]. This indicates the necessity to confirm discovery studies in several cohorts. To discard irreproducible results early in the biomarker development process, orthogonal analytical validation strategies that use independent proteomics methods to confirm the value of biomarkers should be employed. Furthermore, the biomarker’s context of use defined during the discovery phase is not necessarily confirmed in the validation or qualification phase. The trajectory of muscle function associated biomarkers indicates that they are potential disease progression biomarkers that decrease as the disease progresses [84]. However, this raises the question whether such biomarkers are useful disease progression biomarkers throughout the course of the disorder, as their abundance may decrease below the limit of detection of the quantification assay. If this is the case more sensitive assays are needed or new biomarker candidates have to be developed. Similarly, in mdx mice interleukin 13 has been reported as a disease progression marker useful during the late stages of the disease whereas IL-6, INF-γ, and IL-10 during earlier stages of DMD [121]. Maintaining a circular process with feedback mechanisms ensures that biomarkers are continuously assessed, their context of use evaluated and refined in each phase, and necessary action prioritized.

Establishment of the DMD diagnosis is well developed but approval of novel therapies is hindered by the lack of validated biomarkers for monitoring treatment outcome and disease progression. Collection and access to longitudinal samples are necessary to enable testing of already discovered biomarkers for new context of use and validation of disease progression biomarkers. Therapies that restore expression of a functional dystrophin protein are developed using different strategies (1) exon skipping, (2) stop codon readthrough, (3) gene addition, (4) genome editing, or (5) myoblast transplantation. Currently, the available therapies that restore expression of functional dystrophin provide treatment for only a limited number of patients (14% for Eteplirsen approved by FDA and 13% for Ataluren conditionally approved by EMA) [128,129]. To facilitate the development of new therapies biomarkers suitable for clinical trials remains to be developed. Biomarkers are required also for pre-clinical studies that test novel therapies in DMD model organisms. For a more effective development of therapies biomarkers should be usable in both clinical and pre-clinical studies both patients and model organisms.

4.4. Challenges

The scarcity of high-quality samples, the limited knowledge about disease phenotype and patient variability, makes muscular dystrophies a challenging research area. Within rare disorders, the number of samples mirrors the low prevalence of the disorders and the difficulty in retrieving samples from patients. Clinicians, care providers, and parents encounter difficulties in motivating collection of samples for other than diagnostics and/or treatment purposes. However, collection of samples and patient information has been standardized and procedures harmonized through several national and international efforts eg. EuroBioBank [130] (http://www.eurobiobank.org/index.html), RD-Connect (http://rd-connect.eu/about/) [131] Orphanet (https://www.orpha.net/consor/cgi-bin/index.php?lng=EN) [132]. The importance of sample collection and access to such resources has been acknowledged and platforms are designed for easy access, selection, and sharing of biological samples as well as integration of omics results with phenotypic information [132]. Another essential aspect is the quality of samples as blood components are subjected to alterations during retrieval, handling, and storage. Serum is prepared without additives through removal of the fibrin clot and blood cells, whereas plasma is prepared by addition of anticoagulants like EDTA, heparin, or sodium citrate. The preparation procedures influence the sample composition in terms of the total protein concentration, the volume, presence of additives, and stability [133]. To overcome this limitation and increase the statistical power of the studies, strategies have been designed for multi-cohort and concurrent serum and plasma analysis [36]. Biomarkers with concordant results in sample collections collected at different clinical sites and both serum and plasma represent most likely robust biomarkers not influenced by variation in sample collection and pre-analytical preparation procedures.

Another challenging aspect is the heterogeneity of the disease. Patients with severe DMD forms may loose ambulatory capacity at the age of 8 years whereas patients with milder DMD forms after the age 14 years. The patient variation in muscle function between the age of 8–14 can obscure correlation between biomarkers and functional tests like 10MWT, 6MWT, etc. In the light of these challenges studies are required to increase our understanding of the disease pathology and the different phenotypes on both cellular and tissue level.

The outcome of several clinical trials shows that tested therapies are promising but not always effective. This makes it difficult to develop pharmacodynamic biomarkers. For example, in a study comprising samples from two independent clinical trials, serum MMP9 was not found to be a predictive biomarker for treatment response with drisapersen [113]. The limited efficacy of such a treatment, might not be measurable regardless of the specificity or sensitivity of a biomarker measurements.
5. Translation of biomarkers beyond DMD

Muscular dystrophies represent a large group of genetic rare disorders caused by different genetic mutations, with progressive muscle weakness symptom as the common denominator. Several muscular dystrophies share phenotypic characteristics related to deterioration of muscle function, age of onset, severity of muscle wasting symptoms, loss of mobility, short life expectancy in severe cases, and mild to severe cardiac and respiratory impairment [134]. Among them the most common muscular dystrophies besides Duchenne DMD are limb-girdle muscular dystrophy (LGMD), facioscapulohumeral muscular dystrophies (FSHD) and myotonic dystrophy 1 (DM1) with a prevalence of 0.8–6.9, 3.2–4.6 and 1.7–4.2 per 100,000, respectively [135–139]. In addition, Becker muscular dystrophy (BMD) has similarities with DMD as it is caused by in-frame mutations in the dystrophin gene [140,141]. Patients affected by these muscular dystrophies also exhibit elevated serum levels of muscle proteins like CK and CA3. CA3 is a severity marker in DMD and BMD but also a serum biomarker candidate for FSHD, LGMD, congenital, and myotonic dystrophy [70,142]. Serum ratio of lactate dehydrogenase (LDH) isoforms in patients affected by DMD, FSHD, or LGMD has been reported to be disease-specific [143]. These reports support the hypothesis that muscular dystrophies are likely to share muscle wasting biomarkers in blood.

6. Conclusion

Available proteomics technologies, sample resources, under the umbrella of international collaborations promote discovery of biomarkers. The unmet need for translating discovered biomarkers to practical tools, however, still remains unachieved in spite of the impressive number of biomarkers identified. Biomarker associated with muscle function, inflammation, fibrosis, and cardiac status, that can potentially describe different disease states are within reach. Valorization of these findings will require large efforts to be invested in analytical validation and clinical qualification of biomarkers. As DMD diagnostics has been developed during the past decades, biomarkers for monitoring disease progression and pharmacodynamic/response to treatment, are in critical demand. Such biomarkers can facilitate the development of effective therapies to cure DMD. Concerted action from clinicians, researchers, pharma companies, and patient organizations is required to make the necessary progress.

7. Expert opinion

In spite of the constraints related to the sample accessibility and disease heterogeneity, biomarker development within DMD has benefit from a number of key aspects. Several networks eg. TREAT-NMD and RD-Connect have contributed to the development of a strong stakeholder communities and promoted new partnerships and collaborative projects. Majority of the reports during the past few years originate from the involvement of multi-disciplinary research teams rather than single laboratories scattered around the world [34,84,92,107,113,144]. Exchange of knowledge and experience between experts contribute to the development of common goals, well-defined research objectives, selection appropriate study design and evaluation of research outcome from different perspectives eg. medical, biological, statistical, and/or analytical. In addition, patient organizations eg. Parent Project Muscular Dystrophy (www.parentprojectmd.org), Duchenne World Organization (www.worldduchenne.org), have become instrumental in promoting patients benefit and shaped the research environment [145,146]. The organizations have advocated not only for an active involvement of patients in clinical trials and research but also for increased sharing of resources and data according to the FAIR guidelines [147,148].

The efforts invested in the establishing patient registries and biobanks have provided invaluable data and biological material for research while still preserving the patients’ legal rights [130,132,149]. Harmonized protocols for data and sample collection, handling, and storage, enable multi-cohort comparisons and meta-analysis. These type of analysis are imperative for rare disease studies in which the statistical power of the study is often low due to the limited number of cases affected. One aspect that can propel the development of biomarkers is a more active collaboration between academic research groups and pharmaceutical companies. In particular, accessibility to samples from pre-clinical and clinical trials, even if collected during clinical trials failing to prove a beneficial effect of the tested drug, can be used for research studies eg. evaluation of discovered disease progression biomarkers.

There is a general consensus that the development of biomarkers other than diagnostic is required within DMD. While biomarkers for monitoring disease progression are beneficial for clinical management of the disease, biomarkers for monitoring treatment outcome can aid the development of therapies, with positive impact on patients’ life quality and life expectancy. In addition, development of biomarkers for newborn screening are also imperative for early diagnostics and early therapeutic interventions. Evidently, several biomarkers are required to match the different requirements. In addition, majority of the most promising biomarkers have declining trajectories as the disease progresses making them more suitable to measure disease state changes during the early phase of the disease. Efforts should be invested in identifying biomarker signatures rather than single biomarkers, taking into consideration the individual variability and phenotype, enabling transition to precision medicine.

Funding

This paper was funded by the The French Muscular Dystrophy Association, MARK-MD project [Grant reference number 17724].

Declaration of interest

The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.
Reviewers disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

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