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SPECIAL REPORT

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Potential proteomic biomarkers for monitoring clinical studies in Duchenne/Becker muscular dystrophy

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ABSTRACT

Introduction: Clinical proteomics is an evolving discipline that aims to identify new biomarker candidates of human disease to improve diagnosis, prognosis, treatment monitoring and the discovery of novel therapeutic targets. This article outlines the pathoproteomic characterization of dystrophinopathies, which are classified as muscle wasting diseases due to mutations in the *DMD* gene.

Areas covered: The Special Report focuses on the proteomic profiling of progressive Duchenne muscular dystrophy of early childhood and more benign and later-onset Becker muscular dystrophy. The literature search on proteomics and biomarker discovery in dystrophinopathies was conducted with the standard scientific literature databases PubMed and Google Scholar for the preparation of the general text of this article.

Expert opinion: This report has outlined the biomedical value of the systematic proteomic characterization of tissue specimens and associated biofluids to establish novel biomarkers for monitoring clinical studies in muscular dystrophy. Both, mass spectrometry-based profiling approaches and high-plex /high-throughput proteomics platforms were shown to be suitable for the systematic study of complex changes versus adaptations in dystrophinopathy. The verification of promising disease indicators, especially minimally invasive biofluid markers of myonecrosis, chronic inflammation, disturbed energy metabolism and myofibrosis, using orthogonal methodology holds great potential for future clinical applications such as therapeutic monitoring.

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Biomarker; Duchenne/Becker muscular dystrophy; dystrophinopathy; mass spectrometry; neuromuscular disease; proteomics

1. Introduction

A better understanding of the molecular pathogenesis of human disease and the systematic identification of robust protein biomarkers to establish reliable and cost-effective assay systems for the monitoring of therapeutic success are major aims of clinical proteomics [1–3]. Ideally, a combination of proteomic biomarkers is used to create a characteristic protein signature of a pathophysiological process for the optimization of differential diagnostics, prognostics and the determination of therapeutic threshold [4–6]. The pathoproteomic identification of novel therapeutic targets is another important application of mass spectrometry (MS) based biochemical profiling [7–9], in addition to the systematic usage of high-plex /high-throughput proteomics platforms [10–12]. This Special Report discusses the potential of proteomic analysis for detailed studies of patient specimens and to establish pathobiochemical tools to evaluate clinical applications to treat the group of neuromuscular disorders named dystrophinopathies [13–15], which include the main phenotypic diseases of Duchenne muscular dystrophy (DMD) [16] and Becker muscular dystrophy (BMD) [17].

The standard scientific literature databases Pubmed and Google Scholar were employed for the preparation of the general text of this article, using a combination of search terms including 'Duchenne muscular dystrophy,' 'Becker muscular dystrophy,' 'dystrophinopathy,' 'dystrophin,' 'mass spectrometry,' 'biomarker' and 'proteomics.' Identified publications on the proteomic identification and characterization of altered protein species in dystrophic skeletal muscles and associated biofluids were further screened for their relevance to the development of a biomarker signature of dystrophinopathy in order to comply with the reference limitations imposed for this type of article.

2. Dystrophinopathies

Dystrophinopathies are due to primary abnormalities in the *DMD* gene that cause an abnormal abundance and/or size of the dystrophin protein isoform Dp427-M [18]. Figure 1 outlines the multi-promoter and 79-exon spanning *DMD* gene, which encodes eight dystrophin protein isoforms of varying molecular mass and tissue location, including Dp427-B (brain), Dp427-P (cerebellar Purkinje cells), Dp427-M (skeletal, cardiac and smooth muscle), Dp260-R (retina),

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Article highlights

- Duchenne muscular dystrophy is a highly progressive muscle wasting disease of early childhood.
- Mass spectrometric screening and high-plex/high-throughput analysis platforms are ideal tools for the characterization of dystrophic muscle tissues and associated biofluids.
- Comparative tissue proteomics has revealed considerable changes in proteins involved in the excitation-contraction-relaxation cycle, signaling pathways, cellular stress response, the extracellular matrix and the cytoskeletal network.
- Biomarker discovery by biofluid proteomics has identified a variety of promising candidates for minimally invasive analysis.
- The future integration of multi-omics datasets and systems biological analyses will help to establish a comprehensive biomarker signature of X-linked muscular dystrophy.

Dp140-B/K (brain and kidney), Dp116-S (Schwann cells), Dp71-G (ubiquitous) and Dp40 (ubiquitous; enriched in the central nervous system) [19–21]. Notably, a variety of *DMD* gene mutation types lead to abnormalities in the full-length dystrophin isoform Dp427-M in muscle fibers and shorter dystrophin isoforms in other types of tissues [22]. As recently reviewed by Okubo [23], the broad genetic spectrum of dystrophinopathies is characterized by distinct *DMD* gene variants, such as small exonic deletions, large exonic deletions, exonic duplications, small exonic insertions or large exonic insertions, as well as small *DMD* gene variants, including missense point mutations, splice site mutations, nonsense point mutations or mid-intronic mutations [24–26] (Figure 1). Thus, different mutations influence muscle dystrophin protein size and expression and can result in its absence, truncation or partial preservation [27–29]. Highly progressive DMD is characterized by out-of-frame mutations [16,18] and the almost complete loss of dystrophin [19,27–29], which is associated with extensive proteomic disturbances [30,31]. In contrast, in-frame deletions cause BMD [17,18] and are characterized by shortened but partially functional dystrophin molecules [19,27–29] and milder proteomic disturbances [32,33].

Severe forms of advanced dystropathology are characterized by chronic skeletal muscle wasting and impaired regenerative capacity [34–36], as well as multisystemic and body-wide abnormalities (Table 1), which are currently under investigation using proteomics [37]. These include late-onset cardiomyopathy, respiratory impairments, renal failure, fatty liver disease, bone fragility, scoliosis and gastrointestinal abnormalities, as well as neurological and behavioral deficiencies in a subset of DMD patients [22]. Non-skeletal muscle abnormalities might be associated with secondary body-wide disturbances, such as weakened cardiovascular function and the chronic shedding of muscle proteins into the circulatory system, or primary alterations in the expression of non-skeletal muscle dystrophins that directly trigger multisystem issues in a variety of tissues.

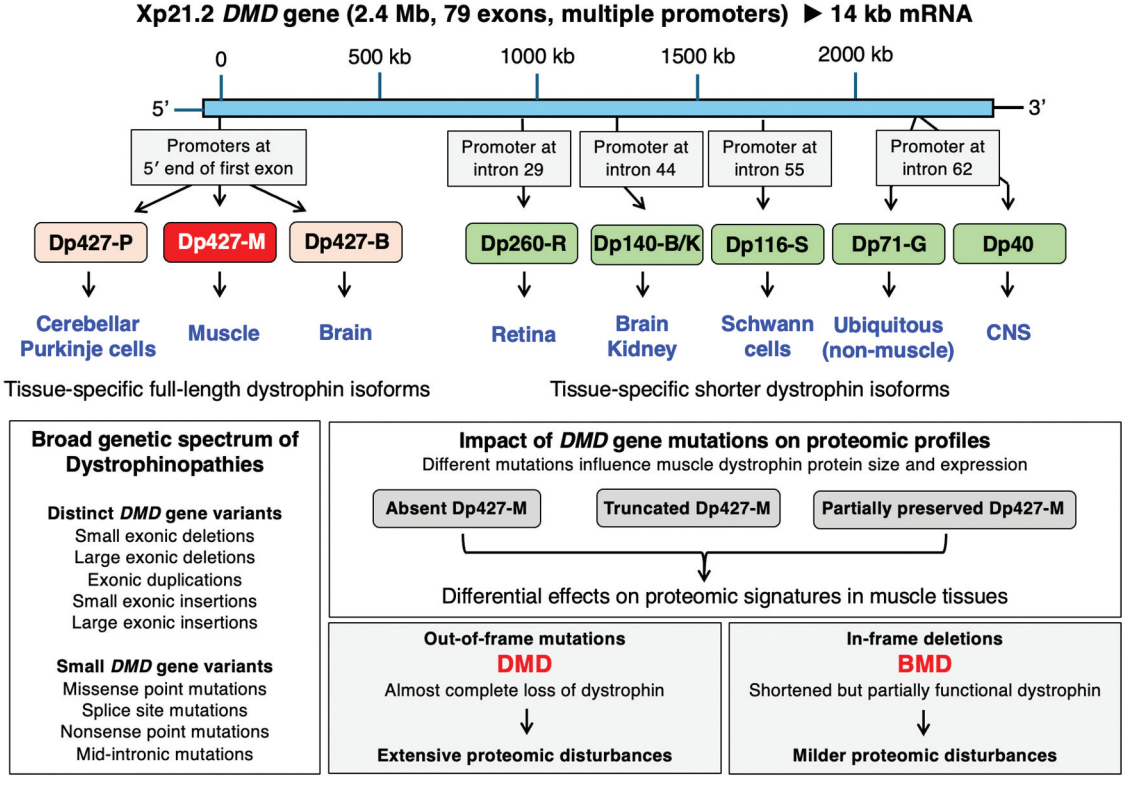

Figure 1. Overview of the *DMD* gene and dystrophin isoforms and their involvement in dystrophinopathy.

The diagram outlines the basic organization of the extremely large *DMD* gene and its promoter structure. Shown are the various tissue-specific dystrophin isoforms ranging in molecular mass from approximately 40 kDa to 427 kDa. The broad genetic spectrum of dystrophinopathies is presented, as well as the impact of *DMD* gene mutations on proteomic profiles.

Abbreviations used: BMD, Becker muscular dystrophy; CNS, central nervous system; DMD, Duchenne muscular dystrophy; Dp427-M, full-length muscle-specific dystrophin protein isoform of 427 kDa.

Table 1. Overview of primary skeletal muscle pathogenesis and multisystemic dysfunction in dystrophinopathy.

Organ system abnormalities	Pathobiochemical mechanisms	Cellular and physiological dysfunctions
Primary skeletal muscle degeneration	Primary deficiency in dystrophin isoform Dp427-M causes collapse of the dystrophin-associated glycoprotein complex	Disintegration of sarcolemmal integrity promoting contraction-induced membrane rupturing which renders contractile fibers more susceptible to necrosis
Skeletal muscle inflammation	Chronic myonecrosis evokes a sustained immune response involving the activation of macrophages and related immune cells	Skeletal muscle tissue damage linked to a chronic inflammatory response and the continuous release of inflammatory cytokines and signaling factors
Skeletal muscle fibrosis	Inflammation related triggering of reactive myofibrosis with increased collagen deposition	Skeletal muscle tissue scarring and matrisomal remodeling causing loss of muscle elasticity and cellular adaptability
Skeletal muscle adipogenesis	Chronic muscle wasting is associated with gradual fat substitution	Metabolic disturbances and unfavorable fat-to-muscle ratios in affected patients
Late-onset cardiomyopathy	Dystrophic phenotype is characterized by a disintegrating basement membrane and interstitial fibrosis	Cardiomyocyte dysfunction resulting in arrhythmias and heart failure
Cardio-respiratory syndrome	Cardiac and respiratory muscle weakness	Respiratory insufficiency and restrictive lung disease
Central nervous system abnormalities	Primary defects in brain dystrophins negatively affect neurons, synapses and glial cells	Cognitive deficiency influencing language, attention, emotions, social interactions and memory
Peripheral nervous system abnormalities	Defective neuromuscular connections	Impaired transmission at neuromuscular junctions
Liver disease	Fibrosis, atrophy and ectopic fat deposition	Metabolic dysfunction due to liver steatosis
Kidney failure	Impaired renal filtration and ectopic fat deposition	Dysfunction of the renal/urinary tract system
Endocrine disease	Hormonal disturbances and mild glucose intolerance	Endocrine imbalance negatively affects development and growth
Gastro-intestinal tract abnormalities	Gut motor dysfunction	Delayed gastric emptying, constipation
Skeletal disease	Abnormal bone-muscle crosstalk and decreased bone density	Fragile bones and spinal deformations

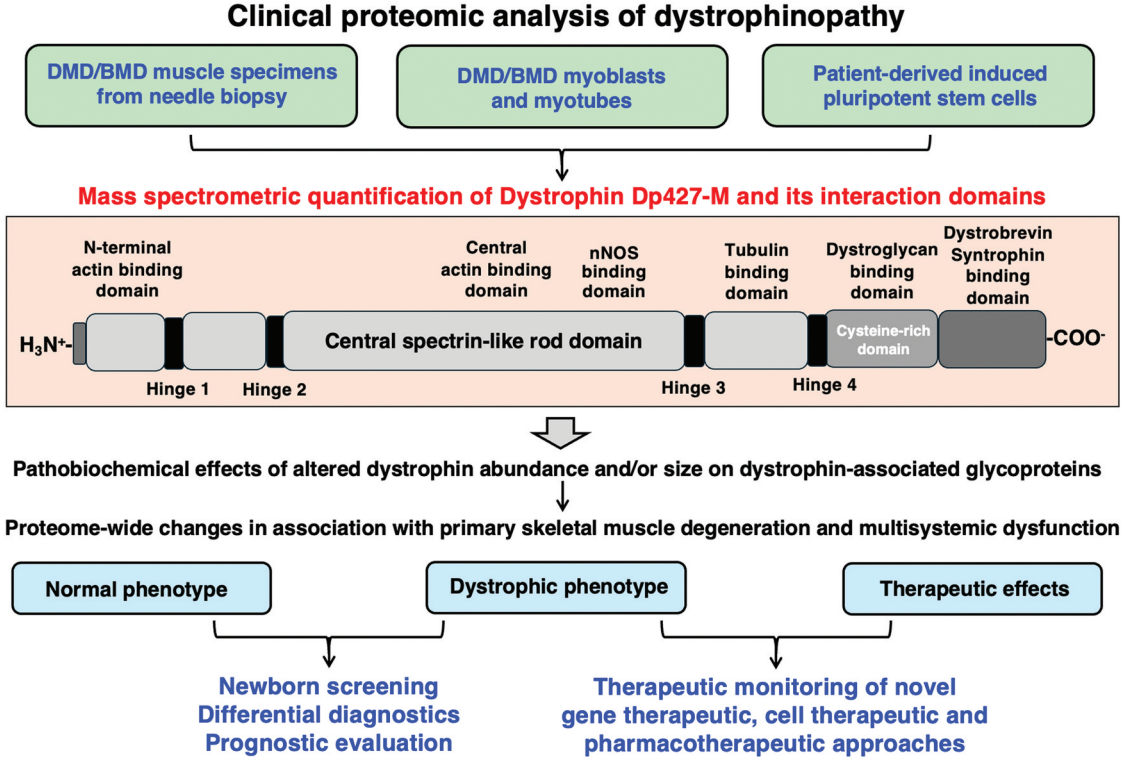

Figure 2. Overview of bioanalytical applications using clinical proteomics to investigate dystrophinopathies.

Listed is the starting material for proteomic investigations and a diagram outlining the basic domain structure of full-length muscle dystrophin and its binding sites, as well as a flowchart of proteomic analysis platforms to study dystrophinopathies.

Abbreviations used: BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy; Dp427-M, full-length muscle-specific dystrophin protein isoform of 427 kDa; nNOS, neuronal nitric oxide synthase.

3. Clinical proteomics and biomarker discovery in DMD research

An important application of MS-based methodology in DMD/BMD research is the assessment of dystrophin levels in normal versus dystrophic muscle, as well as the monitoring of potential changes in dystrophin following therapeutic interventions. Notably, a variety of proteomic approaches have been optimized for the quantitation of dystrophin isoform Dp427-M in human skeletal muscle biopsy specimens [38–42] (Figure 2). Interestingly, the protein turnover of dystrophin and its associated dystroglycan complex following experimental exon skipping therapy was successfully determined by proteomics [43]. For a better understanding of the complex pathogenesis of DMD and BMD, systematic MS-based proteomic surveys of dystrophic skeletal muscle specimens [30–33], DMD myoblasts/myotubes [44], induced pluripotent stem cell (iPSC) derived DMD cell cultures [45], 3D-anchored DMD muscle cells [46] and biofluids (serum and urine) [47–59] have been carried out. Complementary results were achieved by the application of proteomic multi-plex/high-throughput analysis platforms, such as aptamer-based SOMAscan and ProteoMiner beads, for the screening of biofluids from corticosteroid-naive [60–64] versus corticosteroid-treated DMD/BMD patients [65–67]. The serum biomarker signature of dystrophinopathy [59] was confirmed by biofluid proteomics using established animal models [68].

The comprehensive cataloging of proteome-wide alterations in dystrophin-deficient muscle cells and tissues is essential for improving our pathobiochemical understanding of the cellular mechanisms that underlie dystrophinopathy [30–33,44,45]. Discussions of key findings from proteomic studies that have focused on the characterization of DMD/BMD are available in reviews that give an overview of major achievements in dystrophinopathy research [5,37,59] including articles with a focus on individual biomarker candidates such as fatty acid binding protein FABP3 [69], carbonic anhydrase CA3 [70], and annexin isoform ANXA2 [71]. Importantly, for the establishment of a clinically useful biomarker signature of neuromuscular pathogenesis, especially protein changes in easily accessible biofluids, such as blood (serum/plasma), saliva or urine, are of considerable biomedical interest. It is encouraging that DMD biofluid analyses have established large cohorts of minimally invasive protein biomarkers that are suitable for repeated sampling and the monitoring of disease stage-specific alterations [59].

The main strength of biomedical proteomics is the user-friendly usage of MS techniques and/or protein arrays as convenient and robust high-throughput analysis platforms which have been optimized and streamlined over the last decade [3,72]. Widely employed bottom-up proteomics takes advantage of the biochemical properties of small peptides which can be easily separated by a variety of liquid chromatography methods, such as strong cation exchange and reversed-phase columns, used alone (1D-LC), in combination (2D-LC), as split-flow applications (SF-2D-LC) or in series (3D-LC). In MS-based analysis, small peptides ionize well and their fragmentation patterns are relatively predictable. This enables the routine analyses of complex protein mixtures from clinical specimens for the efficient identification and quantification of distinct protein species and their potential role in pathophysiological processes. A cost-effective standard method of proteomics is presented by label-free quantification MS.

Disadvantages of proteomics are associated with both technical and biological limitations [72]. The initial purchase of state-of-the-art mass spectrometers and protein separation equipment is expensive, and considerable running costs are associated with proteomic analysis suites. The processing of samples involves a multi-step procedure that can be labor-intensive and be complicated if elaborate pre-fractionation processing is required or the removal of interfering chemicals, such as detergents. Poor protein identification can be due to unfavorable physicochemical properties of the analytes, incomplete proteolysis of the starting material and/or interfering contaminants. Routine bottom-up proteomics can result in only limited information on amino acid sequences which might not be comprehensive enough to properly identify a distinct proteoform, especially if sample size is restricted. Low-abundance proteins may be under-represented in bottom-up proteomics, and peptides derived from abundant proteins can weaken signals from low-copy number proteins. However, this issue can at least partially be addressed by immunoaffinity methods that deplete the most abundant proteins prior to proteomic analysis [72]. A difficult biological issue is the highly variable and complex proteome of any given biological system with a considerable dynamic range of protein concentrations and ongoing changes in post-translational modifications [7].

4. Muscle cell and tissue proteomics

As outlined in Figure 3, proteomics has been instrumental in the characterization of dystrophic muscle tissues and the concomitant identification of biofluid-associated disease indicators. The MS-based profiling of dystrophic skeletal muscle samples from DMD/BMD patients has great potential to reveal proteome-wide changes in myofibres and its tissue environment (Table 2). Studies by Capitanio et al. [30,32] using DMD and BMD skeletal muscle biopsies identified disturbances in the expression of proteoforms that are involved in the maintenance of the intracellular cytoskeleton and the extracellular matrisome, the provision of contractile function, the regulation of Ca^{2+}-homeostasis and protein metabolism, the cellular stress response, the inflammatory reaction to tissue damage and the regeneration of damaged contractile fibers. In addition, da Silva and coworkers [31] could show the concomitant decrease in sarcomeric protein species and a drastic increase in ubiquitination-related proteins, as well as alterations in proteoforms involved in key bioenergetic pathways in DMD. Of note, the imaging mass cytometric analysis of skeletal muscle specimens from BMD patients revealed different phases of muscle tissue degeneration with striking dissimilarities in the levels of specific collagen isoforms of the matrisome in fibrotic versus non-fibrotic parts, in combination with alterations in markers of regenerative processes, inflammation and adipogenesis [33].

Proteomic investigations using DMD myoblasts and myotubes revealed changes in myogenic mechanisms, mRNA processing, cellular adhesion processes and organellar

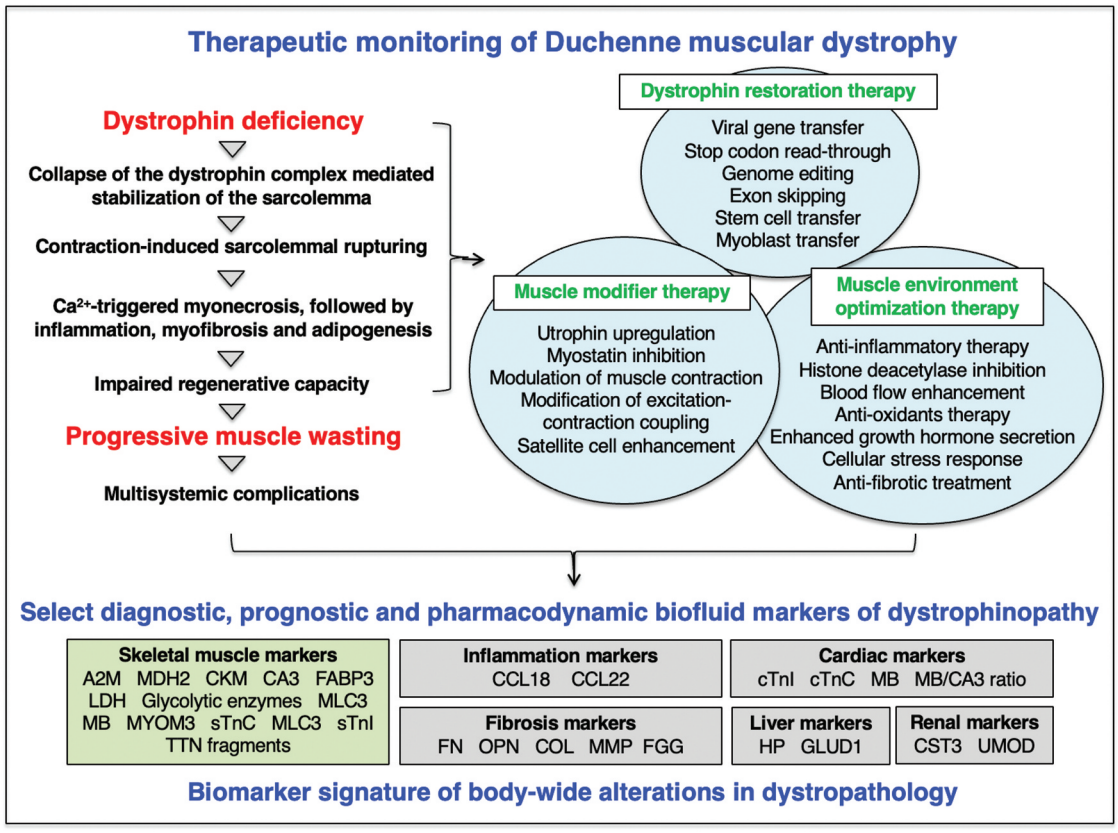

Figure 3. Summary of select biomarker candidates to improve the therapeutic monitoring of dystrophinopathy.

The diagram outlines on the top left the main pathophysiological steps that lead to dystrophinopathy, and summarizes therapeutic approaches that aim at dystrophin restoration, muscle modification and muscle environment optimization at the top right of the figure. Select diagnostic, prognostic and pharmacodynamic biofluid marker candidates of dystrophinopathy are listed in the lower part of the figure.

Abbreviations used: A2M, alpha-2-macroglobulin; CA, carbonic anhydrase; CCL, C-C motif chemokine; CKM, creatine kinase M-type; COL, collagen; CST, cystatin; cTnC, cardiac troponin subunit C; cTnI, cardiac troponin subunit I; GLUD, glutamate dehydrogenase; FABP, fatty acid binding protein; FGG, fibrinogen gamma; FN, fibronectin; HP, haptoglobin; Hsp, heat shock protein; LDH, lactate dehydrogenase; MB, myoglobin; MDH, malate dehydrogenase; MLC, myosin light chain; MMP, matrix metalloproteinase; MYOM, myomesin; OPN, osteopontin; sTnC, skeletal muscle troponin subunit C: sTnI, skeletal muscle troponin subunit I; TTN, titin; UMOD, uromodulin.

Table 2. The potential of proteomics to improve differential diagnostics and the monitoring of clinical studies in Duchenne muscular dystrophy.

Clinically relevant analysis	Specimens	Methodology
Diagnostic quantification and mapping of dystrophin molecule	Skeletal muscle biopsy, cell lysates	Biochemical and mass spectrometry-based top-down analysis using immuno precipitation and/or gel electrophoretic separation
Characterization of the dystrophin-glycoprotein complex and its associated elements of the cytoskeleton and extracellular matrix	Skeletal muscle biopsy specimens	Mass spectrometry-based screening in combination with immuno precipitation, lectin agglutination, gel electrophoresis and/or density gradient ultracentrifugation
Comparative proteomic analysis of the dystrophic skeletal muscle phenotype following pharmacotherapy	Biofluids (serum, saliva, urine) and muscle biopsies	Biochemical and mass spectrometry-based bottom-up analysis of crude extracts, enriched preparations or subcellular fractions focusing on myonecrosis, myofibrosis and chronic inflammation
Comparative proteomic analysis of cardio-respiratory integrity following pharmacotherapy	Biofluids (serum, saliva, urine)	Mass spectrometry-based bottom-up analysis of total extracts or enriched preparations using cardiac-specific biomarkers
Comparative proteomic analysis of the dystrophic phenotype following gene therapy	Biofluids (serum, saliva, urine) and muscle biopsies	Mass spectrometry-based bottom-up analysis of crude extracts, enriched preparations or subcellular fractions following exon-skipping, viral transfer, genomic editing, stop codon read-through therapy or stem cell/myoblast transfer therapy
Comparative proteomic analysis of multisystemic and body-wide dysfunction in dystrophinopathy	Biofluids (serum, saliva, urine) and tissue biopsies	Mass spectrometry-based bottom-up analysis of crude extracts, enriched preparations or subcellular fractions using tissue-specific biomarkers

organization [44]. The analysis of DMD-derived iPSC preparations showed proteomic changes in the contractile apparatus, the matrisome, the cytoskeletal network and cell adhesion complexes [45]. The findings from this still limited number of comprehensive proteomic profiling studies of human dystrophic muscle cells agree with the vast knowledge that was gained from investigating many different subtypes of skeletal muscle preparations derived from dystrophic animal models of DMD [73–76]. Overall, proteomics could show that the deficiency in dystrophin and associated reduction in its sarcolemmal glycoprotein complex triggers massive alterations in the skeletal muscle proteome, which in turn reflects the

pathophysiological severity of DMD/BMD pathology. A large number of novel tissue-associated biomarker candidates of myonecrosis, inflammation and reactive myofibrosis could be identified [30–33]. Studies on the cardiac proteome and dystrophinopathy-associated cardiomyopathy have mostly focused on animal models of DMD, such as the DMD pig and *mdx*-type mice. Of note, these investigations have established marked differences between skeletal and cardiac muscle proteomic signatures [77–79].

5. Biofluid proteomics

Biofluid proteomics of DMD [59] revealed altered levels of both previously established general marker proteins of skeletal muscle damage and novel indicators of dystrophinopathy-related myofiber degeneration and myofibrosis. Proteomic screening studies of DMD serum samples by Spitali et al. [61] who used aptamer-based SOMAscan screening and Johanssen et al. [58] who employed immuno assays combined with peptide quantification by parallel reaction monitoring MS analysis identified a considerable number of biomarker candidates. These investigations and a large number of other proteomic studies of biological fluids from patients and animal models [47–56,58–68] identified an impressive array of promising biomarker candidates.

Identified marker proteins derived from (i) the sarcomere (troponin subunit TnI of the regulatory complex of the actin-containing thin filament; myosin light chain MLC3 of the myosin-containing thick filament; titin TTN fragments of the gigantic half-sarcomere spanning titin filament; myomesin isoform MYOM3 of the M-line structure), (ii) the cytosol (a variety of glycolytic enzymes including phosphoglycerate mutase, lactate dehydrogenase and pyruvate kinase; CO_2-converting enzymes carbonic anhydrase isoforms CA1 and CA3; muscle-specific oxygen-binding protein myoglobin MB; muscle-associated fatty acid binding protein FABP3; M-type creatine kinase CKM), and (iii) mitochondria (mitochondrial malate dehydrogenase MDH2 of the citric acid cycle). Additional biomarker candidates are represented by alpha-2-macroglobulin (A2M), the muscle growth inhibiting myokine myostatin and tumor necrosis factor receptor. These proteins can be classified according to biological function and/or subcellular localization into contractile apparatus proteins (TTN, TnI, MLC3, MYOM3), cytosolic enzymes (CA1, CA3, CKM, glycolytic enzymes), mitochondrial proteins (MDH2) and metabolite transporters (FABP3, MB), plus various cellular stress proteins and signaling elements [49–68]. Biofluid proteomics also identified serum-associated markers of reactive myofibrosis, including collagens, fibronectin and osteopontin, as well as matrix metalloproteinases (MMP9) and their tissue inhibitors [47,48,59].

Based on the identification in numerous proteomic studies that have evaluated changes in DMD/BMD biofluids and being confirmed by verification analyses [80], especially promising markers (in conjunction with the already frequently used M-type creatine kinase) [81–83] are N- and C-terminal fragments of titin [49,84], the muscle-specific carbonic anhydrase isoform CA3 that is enriched in slow myofibers [70], the M-line protein myomesin MYOM3 [51], fatty acid binding protein 3 (FABP3) [69], mitochondrial malate dehydrogenase MDH2 [85], myosin light chain MYL3 and skeletal muscle troponin I (sTnI) [49–68]. Table 3 lists minimally invasive biofluid marker candidates and gives information on their protein name, their subcellular origin, monitoring potential and representative references. Most of these identified biomarkers are highly elevated in DMD, moderately increased in BMD and show relatively small fluctuations in normal individuals. A significantly increased concentration of A2M [58] and MYOM3 [51] is clearly associated with DMD progression. Both proteins can potentially be utilized as therapy-responsive markers that are sensitive to the restoration of dystrophin. Pharmacotherapeutic-responsive markers to corticosteroid treatment were identified as CKM, TnI, TnC, MLC3, MYOM3 and pro-inflammatory proteins [64–66]. Increased levels of serum CA3 and FABP3 correlate well with clinical milestones [69,70]. The characterization of respiratory performance has revealed cellular alterations in the dystrophic and highly fibrotic diaphragm, triggering weakness in this major muscle of inspiration. Severe diaphragm fibrosis was shown to be associated with elevated levels of fibronectin, osteopontin and collagens in muscle tissues [75,76] and is reflected by increased levels of ECM proteins in serum [47,48]. Cardiac cTnC and cTnI, listed in Table 3, are potential markers of dystrophinopathy-associated cardiomyopathy, and the CA3/MB ratio might be suitable to differentiate between the degree of cardiac versus skeletal muscle wasting [70].

In both, the analysis of patient specimens and animal models, cytokines, chemokines and myokines have been identified as potential markers of the immune response, degeneration-regeneration cycles and cellular disturbances in dystrophinopathy. The recruitment of macrophages, neutrophiles, eosinophiles and a variety of T-cells was shown to be associated with elevated levels of cytokines, including tumor necrosis factor TNFα and a large variety of interleukins [65,86–88].

6. Proteomic monitoring of future clinical DMD studies

As illustrated in Figure 3, a variety of novel therapeutic approaches have been evaluated in preclinical and clinical settings to counteract the main symptoms of dystrophinopathy, including advanced pharmacotherapy, cell-based therapy and gene therapeutic approaches at the level of both mRNA and DNA [16,21,89]. Standard care of DMD patients [90] includes cardiovascular management using treatment regimens with angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, beta-adrenergic receptor blockers and diuretics for cardioprotection [91]. This is accompanied by respiratory management, orthopedic interventions, physical therapy and optimized nutrition [92–94]. At later stages of DMD, ventilatory support has to be applied to address the cardiorespiratory syndrome and often life-threatening cardiomyopathic complications [95]. To address the primary skeletal muscle disease and preserve motor function, potent anti-inflammatory glucocorticosteroids, such as prednisolone, deflazacort and the more recently introduced agent vamorolone, form an integral part of supportive therapy in DMD [96–

Table 3. Promising and minimally invasive proteomic biomarker candidates for the improved differential diagnosis, prognosis and therapeutic monitoring of dystrophinopathies. Most listed studies have been carried out with serum specimens. An elevated concentration of fragments of the giant titin protein was also established in urine samples. Most identified biomarkers are highly elevated in DMD, moderately increased in BMD and show relatively small fluctuations in normal individuals. However, a variety of factors such as age, general disease status, side effects from pharmacotherapy, surgical procedures and the circadian rhythm can influence the concentration of certain muscle damage markers.

Biomarker candidate	Subcellular origin	Monitoring potential	References
Titin; amino- and carboxy-terminal TTN fragments	Half-sarcomere spanning giant TTN-filament in myofibers	Disintegration of auxiliary sarcomeric TTN-filament	[49,54,61,84]
Carbonic anhydrase isoform CA3, enriched in slow type I skeletal muscles	Sarcosol	Disintegration of sarcolemmal barrier function and abnormal CO_2-removal	[50,54,57,61–63,70]
Fatty acid binding protein isoform FABP3 (heart-type hFABP), present in heart and skeletal muscle	Sarcosol	Disintegration of sarcolemmal barrier function and abnormal fatty acid transportation in muscles	[50,61–63,69]
Myomesin MYOM3	Sarcomeric M-line structure	Disintegration of the sarcomere	[51,54,58,61]
Myoglobin MB	Sarcosol	Disintegration of sarcolemmal barrier function and abnormal O_2-transport	[50,54,61]
Creatine kinase CKM (muscle type)	Sarcosol	General muscle damage	[50,54,58,61,63]
Malate dehydrogenase MDH2, mitochondrial	Mitochondrial matrix	Mitochondrial dysfunction; impaired citric acid cycle	[60,61,85]
Troponin sTnC and sTnI (slow and fast isoforms)	Skeletal muscle sarcomeric thin actin-containing filament	Disintegration of contractile actomyosin apparatus	[50,61–63]
Myosin light chain MLC3, slow skeletal muscle	Skeletal muscle sarcomeric thick myosin-containing filament	Disintegration of contractile actomyosin apparatus	[54,61]
Alpha-2-macroglobulin A2M	Plasma protein	Pharmacodynamic marker of dystrophin restoration	[58]
Muscle carbonic anhydrase CA3 / muscle and heart myoglobin ratio	Sarcosol; muscle-specific CA3 versus cardiac/muscle MB	Evaluation of cardiomyopathy versus skeletal muscle degeneration	[70]
Matrisomal markers (FN, COL-VI, MMP9, OPN)	Extracellular matrix (basal lamina, endomysium, perimysium)	Reactive myofibrosis characterized by structural remodeling and excessive collagen deposition	[47,48,59,61]
Fibrinogen FGG gamma chain	Extracellular matrix niche and perivascular areas of dystrophic muscles	Muscle tissue injury, myofibrosis and inflammation	[58,61–63]
Glycolysis-associated enzymes (GPI, PFK, ALDO, TPI, PGAM, ENO, PKM, LDH, PYGM)	Sarcosol	Bioenergetic disturbances of carbohydrate metabolism,	[54,57,58,61–63]
CKM, TnI, TnC, MYL3, MYOM3 and pro-inflammatory proteins	Sarcosol, contractile apparatus and inflammation-associated proteins	Evaluation of effect of corticosteroid treatment	[64–66]
Troponin cTnC, cTnI, cardiac muscle	Cardiac sarcomeric thin actin-containing filament	Cardiomyopathy	[50,61]

Abbreviations used: A2M, alpha-2-macroglobulin; ALDO, aldolase; CA, carbonic anhydrase; CKM, muscle-type creatine kinase; COL, collagen; cTnC, cardiac troponin subunit C; cTnI, cardiac troponin subunit I; ENO, enolase; FABP, fatty acid binding protein; FGG, fibrinogen gamma chain; FN, fibronectin; GPI, phosphoglucose isomerase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; MMP, matrix metalloproteinase; MYOM, myomesin; OPN, osteopontin; PFK, phosphofructo-kinase; PGAM, phosphoglycerate mutase; PKM, pyruvate kinase; PYGM, glycogen phosphorylase; sTnC, skeletal muscle troponiun subunit C; sTnI, skeletal muscle troponin subunit I; TPI, triosephosphate isomerase (TPI); TTN, titin.

98]. For DMD boys aged 6 and older, treatment with Givinostat (Duvyzat), a histone deacetylase inhibitor drug [99], was shown to have great potential to counteract inflammation and reactive myofibrosis, and exhibiting a positive effect on skeletal muscle regeneration [100]. Of note, the recent integration of open-access proteomic and transcriptomic datasets with *in silico* analyses has identify dysregulated pathways that might be reversed by existing drugs, such as inhibition of the sodium-glucose cotransporter-2 by the agent SGLT2i that could trigger anti-inflammatory and anti-fibrotic effects in dystrophic muscles [101].

The development of dystrophin restoration therapy for advanced DMD treatment includes adeno-associated virus (AAV) mediated gene transfer, stop codon read-through, genome editing, exon skipping, stem cell transfer and myoblast transfer [16,21,89]. Dystrophin quantification was successfully carried out by parallel reaction monitoring of human muscle biopsies [39]. Walsh and coworkers [42] have evaluated a potential DMD gene therapy drug during the early phase of a clinical study using MS-based methodology. The investigation aimed at assessing the usefulness of AAV-based gene replacement therapy using mini-dystrophin constructs. Immunoaffinity liquid chromatography-tandem MS analysis was employed for protein identification emphasizing the usefulness of clinical proteomics. Thus, surrogate biomarkers are suitable for testing the efficacy and safety of gene therapy. Similar studies are under way to determine the proteomic profile of dystrophic skeletal muscles following exposure to new agents used in gene therapy.

Muscle modifier therapies aim at utrophin upregulation to substitute for dystrophin deficiency, myostatin inhibition, modulation of muscle contraction, modification of excitation-contraction coupling and satellite cell enhancement [16,21,102]. Muscle environment optimization therapy involves histone deacetylase inhibition, anti-inflammatory therapy, blood flow enhancement, anti-oxidants therapy, enhanced growth hormone secretion, modulation of the cellular stress response and anti-fibrotic treatment [16]. The above described novel proteomic markers of DMD pathogenesis [4,5,30–33,44–75], in addition to indicators of abnormal whole-body multisystemic changes including indicators of chronic inflammation, liver disease, kidney failure and cardiomyopathy [22,37,77–79], should be ideal candidates for developing a robust biomarker signature for evaluating new DMD treatment protocols (Figure 3).

7. Conclusion

As outlined in this Special Report, proteomics is a suitable bioanalytical platform to study the underlying mechanisms of the molecular and cellular pathogenesis of neuromuscular disease, and identify and characterize novel biomarker candidates of myofiber degeneration versus adaptive responses. In the field of dystrophinopathy research, proteomics has been instrumental for the quantitation of dystrophin and its associated sarcolemmal glycoprotein complex, as well as the detailed analysis of the wider dystrophin complexome consisting of elements of the extracellular matrix and the intracellular cytoskeletal network. Comparative proteomic studies have elucidated in detail a variety of alterations in the heterogenous cellular and acellular skeletal muscle niche due to different types of *DMD* gene mutations. Primary genetic abnormalities lead to alterations in the abundance and/or size of the membrane cytoskeletal protein dystrophin which is intrinsically associated with various forms of dystrophinopathy, i.e. severe DMD or more benign BMD. Extensive MS-based screening and multiplex/high-throughput analyses have demonstrated that the class of mutation in the extremely large *DMD* gene has differential effects on the extend of proteomic changes in dystrophic muscle tissues. Besides considerable disturbances of the protein constituents of voluntary myofibers and their cellular environment due to myonecrosis, chronic inflammation, fat substitution and reactive myofibrosis, deficiency in Dp427-M also affects cardiac and smooth muscles in dystrophinopathy.

Future developments in the field of clinical proteomics will hopefully (i) upgrade the overall bioanalytical workflow, (ii) involve more streamlined sample handling, (iii) improve protein and peptide separation, (iv) use more sensitive protein identification by peptide MS analysis, (v) enhance the applications of systems bioinformatics leading to superior coverage of protein identification in small samples, such as single cell or subcellular preparations, and (vi) utilize artificial intelligence (AI) and Machine Learning (ML) algorithms for the improved interpretation of complex and high-throughput data, and thereby decisively accelerate the discovery rate of novel biomarkers and predicting clinical outcomes.

8. Expert opinion

The main analytical approaches of modern MS-based proteomics can be divided into top-down analyses, which focus on the detailed characterization of isolated proteoforms and their dynamic post-translational modifications, and bottom-up proteomics, which examines complex protein mixtures in a peptide-centric way for the identification of distinct protein species [72]. While top-down proteomics requires the purification of proteins of interest, the comparative screening by bottom-up proteomics can be carried out with crude tissue samples, isolated cells, subcellular fractions or complex biofluids following proteolytic digestion of the protein constituents. Both biochemical approaches can be employed in basic biomedical research, applied clinical chemistry and biomarker characterization. The biochemical knowledge from proteomics can now be used to widen the comprehensiveness of the biomarker signature of DMD/BMD pathogenesis, especially for the evaluation of future clinical studies and pharmacological trials. Since different *DMD* gene mutations have been shown to exhibit a considerable impact on proteomic profiles in skeletal muscles and other tissue types, the application of integromic research strategies would be helpful for a systems biological understanding of dystrophinopathy [37].

Proteomics-based biomarker discovery, as outlined in this article, holds promise to improve various clinically relevant aspects in the field of dystrophinopathy. However, proteomic screening for the routine analysis of patient specimens would not be a realistic undertaking due to its enormous costs and limited access to specialized equipment in the clinical setting, but novel biomarkers that have been identified by proteomics and then been verified by orthogonal techniques should be useful to improve many clinically relevant aspects. This could include sample screening, differential diagnosis, prognosis and therapeutic monitoring. High-plex/high-throughput proteomics or MS-based analyses are not suitable to be directly used as routine analysis tools, but the field of clinical chemistry can build on proteomic findings and use newly established markers as potential parameters that would improve clinical measurements. Proteomics can certainly be useful for studying variations in natural history clinical studies that investigate the progression of a pathophysiological process and its main symptoms. The medical impact and response to therapeutic interventions, including potential differences in phenotype/genotype correlations, would be another aspect that could be studied by proteomic surveys. That could include the determination of disease severity before full manifestation of skeletal muscle wasting in BMD versus DMD.

The successful application and long-term treatment viability of combination therapy to halt or even reverse skeletal muscle wasting and associated multisystemic complications, such as cardiomyopathy, respiratory weakness, renal failure, liver atrophy and scoliosis in DMD/BMD, will heavily depend on the robust monitoring with proper biomarker assay systems. Optimized protein markers should be highly useful to determine the biomedical strength versus potential weaknesses of innovative pharmacotherapy or advanced gene/cell therapy approaches. In this regard, proteomic markers are ideal biomolecules to improve newborn screening, differential diagnosis, clinical predictions and continuous therapeutic monitoring of dystrophinopathy. Following the proper validation of proteomic findings using orthogonal verification methodology, such as immunoblotting, confocal microscopy, enzyme assays, protein binding studies and physiological experiments, the best protein markers should be chosen to develop a comprehensive set of surrogate biomarkers [103]. These indicators of muscle disease progression and clinical endpoints of DMD pathogenesis should then be able to differentiate between the detrimental effects that are generated by myofiber degeneration, chronic inflammation, adipogenesis and reactive myofibrosis, and how these pathological mechanisms can be reversed by novel therapeutics.

In relation to drug utilization, protein biomarkers can be used to predict and scrutinize a drug's safety by quantifying biological responses and identifying inflammation, stress or

organ damage caused by therapeutic interventions. This is especially relevant for virus-associated gene therapy and potential body-wide complications [104]. An interesting cost-effective and translational approach for future therapeutic discovery to counteract dystrophin deficiency is a drug repurposing strategy that combines information from open-access proteomic data and transcriptomic results with *in silico* analyses [101]. The systematic application of integromics by combining multi-omics datasets with systems biological analysis should be able to identify dysregulated pathways and aberrant signaling mechanisms in DMD/BMD that can potentially be reversed by existing pharmacological agents and thereby mitigate disease progression in dystrophinopathies.

Alternative biomarker discovery methods to MS-based bottom-up proteomics would be the systematic screening of proteins of interest with (i) standardized biochemical techniques such as enzyme-linked immunosorbent assay systems, and immunochemical approaches using dot blots, immunoblots and/or immunoprecipitation, (ii) cell biological methodology, including immunofluorescence microscopy and flow cytometry, (iii) the utilization of computational biological tools for the bioinformatic assessment of multi-omics findings integrating results from genomics, transcriptomics, proteomics, metabolomics and cytomics, and (iv) commercial affinity-based proteomics platforms (e.g. Olink, SomaScan) with improved sensitivity and throughput of biofluid proteome analysis, allowing for the rapid and parallel measurement of thousands of proteins.

Declaration of interests

The authors have 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.

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Author contributions

CRediT: **Paul Dowling:** Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing; **Dounia Bouragba:** Writing – review & editing; **Elisa Negroni:** Writing – review & editing; **Capucine Trollet:** Funding acquisition, Writing – review & editing; **Margit Zweyer:** Writing – review & editing; **Dieter Swandulla:** Writing – review & editing; **Kay Ohlendieck:** Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing.

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