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Expert Review of Molecular Diagnostics

ISSN: 1473-7159 (Print) 1744-8352 (Online) Journal homepage: https://www.tandfonline.com/loi/iero20

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To cite this article: Paul Dowling, Sandra Murphy, Margit Zweyer, Maren Raucamp, Dieter Swandulla & Kay Ohlendieck (2019) Emerging proteomic biomarkers of X-linked muscular dystrophy, Expert Review of Molecular Diagnostics, 19:8, 739-755, DOI: 10.1080/14737159.2019.1648214

To link to this article: https://doi.org/10.1080/14737159.2019.1648214

EXPERT REVIEW OF MOLECULAR DIAGNOSTICS
2019, VOL. 19, NO. 8, 739–755
https://doi.org/10.1080/14737159.2019.1648214

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REVIEW

Emerging proteomic biomarkers of X-linked muscular dystrophy

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ABSTRACT

Introduction: Progressive skeletal muscle wasting is the manifesting symptom of Duchenne muscular dystrophy, an X-linked inherited disorder triggered by primary abnormalities in the *DMD* gene. The almost complete loss of dystrophin isoform Dp427 causes a multi-system pathology that features in addition to skeletal muscle weakness also late-onset cardio-respiratory deficiencies, impaired metabolism and abnormalities in the central nervous system.

Areas covered: This review focuses on the mass spectrometry-based proteomic characterization of X-linked muscular dystrophy with special emphasis on the identification of novel biomarker candidates in skeletal muscle tissues, as well as non-muscle tissues and various biofluids. Individual sections focus on molecular and cellular aspects of the pathogenic changes in dystrophinopathy, proteomic workflows used in biomarker research, the proteomics of the dystrophin-glycoprotein complex and the potential usefulness of newly identified protein markers involved in fibre degeneration, fibrosis and inflammation.

Expert opinion: The systematic application of large-scale proteomic surveys has identified a distinct cohort of both tissue- and biofluid-associated protein species with considerable potential for improving diagnostic, prognostic and therapy-monitoring procedures. Novel proteomic markers include components involved in fibre contraction, cellular signalling, ion homeostasis, cellular stress response, energy metabolism and the immune response, as well as maintenance of the cytoskeletal and extracellular matrix.

ARTICLE HISTORY
Received 7 June 2019
Accepted 23 July 2019

KEYWORDS
Biofluid; biomarker; Duchenne muscular dystrophy; dystrophinopathy; liquid biopsy; muscle biopsy

1. Introduction

Progressive forms of inherited or acquired human disorders that are associated with primary or secondary skeletal muscle wasting are characterized by loss of contractile strength and decreased adaptability to changed physical demands [1]. These neuromuscular symptoms can have a devastating impact on the quality of life, especially muscular disorders of early childhood [2]. In primary diseases of the skeletal musculature, functional deficiencies in contractile tissues are often accompanied by alterations in cellular signalling mechanisms, physiological homeostasis, energy metabolism and fibre type distribution [3]. The most frequently inherited neuromuscular disorder of early childhood is Duchenne muscular dystrophy, which is associated with a substantial economic and caregiver burden [4]. X-linked muscular dystrophy is a mono-genetic disorder due to primary abnormalities in the largest human gene, the Xp21-located and 79-exon spanning *DMD* gene [5]. This highly complex gene encodes proteins ranging from approximately 71 to 427 kDa whereby the alternative usage of seven promoters, which are linked to unique first exons, is the basis of the tissue-specific production of a variety of distinct transcripts and corresponding protein products [6], as outlined in Figure 1. The full-length Dp427-M isoform of dystrophin belongs to the class of giant muscle proteins, which have been extensively characterized by biochemical and mass spectrometric analyses [7].

Skeletal muscle dystrophin is located in the subsarcolemmal cytoskeleton in voluntary contractile fibres and contains amino-terminal and central actin-binding domains, multiple spectrin repeats and a unique carboxy-terminal dystroglycan binding domain [5]. The most progressive form of dystrophinopathy is related to a great variety of patient-specific mutations, including mostly large deletions or duplications, but also small deletions, small insertions, splice site mutations, point mutations and mid-intronic mutations [8]. These genetic abnormalities result in the almost complete loss of the Dp427-M isoform and cause a multi-system pathology that primarily affects the skeletal musculature, but is also characterised by severe secondary dysfunctions in the cardio-respiratory system, the central nervous system and metabolism, as well as accompanying complications due to scoliosis [9]. Ideally, a panel of robust and highly sensitive biomarkers would be available to evaluate the various clinical features of dystrophinopathy and improve differential diagnostic, prognostic and therapy-monitoring procedures.

A variety of non-proteinaceous biomarker candidates of muscular dystrophy, such as single nucleotide polymorphisms, lipids, metabolites and extracellular microRNAs [10], as well as skeletal muscle imaging technologies [11], have been evaluated and shown considerable potential to improve diagnostics [12]. Building on previous reviews on the usage of mass spectrometric techniques in muscular dystrophy research

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Article Highlights

- Duchenne muscular dystrophy is a highly complex multi-system disorder that is characterized by chronic skeletal muscle wasting
- Mass spectrometry-based proteomics has identified new biomarker candidates of dystrophinopathy
- Novel proteomic biomarkers include tissue-associated proteins involved in a large variety of cellular processes.
- New biofluid-associated biomarkers have a great potential for establishing superior diagnostic methods in the field of muscular dystrophy.
- Promising new biomarker candidates include titin fragments in urine and a variety of increased muscle proteins in serum, such as fibronectin, carbonic anhydrase, myosin light chain, haptoglobin, fatty acid binding proteins, myomesin and troponin.
- Newly identified biomarkers of muscular dystrophy have now to be established in clinical laboratory practice
- Robust biomarkers may then be useful for the improved evaluation of new treatment approaches, such as stem cell transfer or gene therapy.

[13–15], this article focuses on emerging proteomic biomarkers of X-linked muscular dystrophy. General background information is based on established publications on the dystrophin-glycoprotein complex and muscular dystrophy. Literature screening was carried out with the *Pubmed* database and focused on mass spectrometry-based proteomic studies using combinations of a variety of search terms, i.e. 'proteomics', 'mass spectrometry', 'Duchenne muscular dystrophy', 'dystrophinopathy', 'dystrophin', 'dystrophin-glycoprotein complex', 'diagnosis' 'prognosis' 'therapy' and 'biomarker'. Publications were then further screened for their suitability based on the specific topic of this review, taking into account the suggested total number of references.

Major findings on novel proteomic biomarker candidates associated with skeletal muscle tissues, non-skeletal muscle tissues and various biofluids and their potential relevance to improve molecular diagnostics are discussed in individual sections. Illustrative figures are presented to summarize the complex pathology of X-linked muscular dystrophy, the bioanalytical workflow for the identification of novel disease markers using mass spectrometry, the proteomic characterization of the wider dystrophin-associated protein complex and the biochemical diversity of recently identified proteomic biomarker candidates of chronic fibre degeneration, reactive myofibrosis and sterile inflammation. A summary of the main findings from proteomic studies that have focused on the identification of the dystrophin complex and biomarker discovery in dystrophic skeletal muscles, secondarily affected non-skeletal muscle tissues and biological fluids are listed in individual tables. A certain degree of overlap exists between these listings due to the fact that some proteomic investigations have been concerned with both the basic biochemical characterization of dystrophin complexes and the comparative analysis of dystrophic tissues.

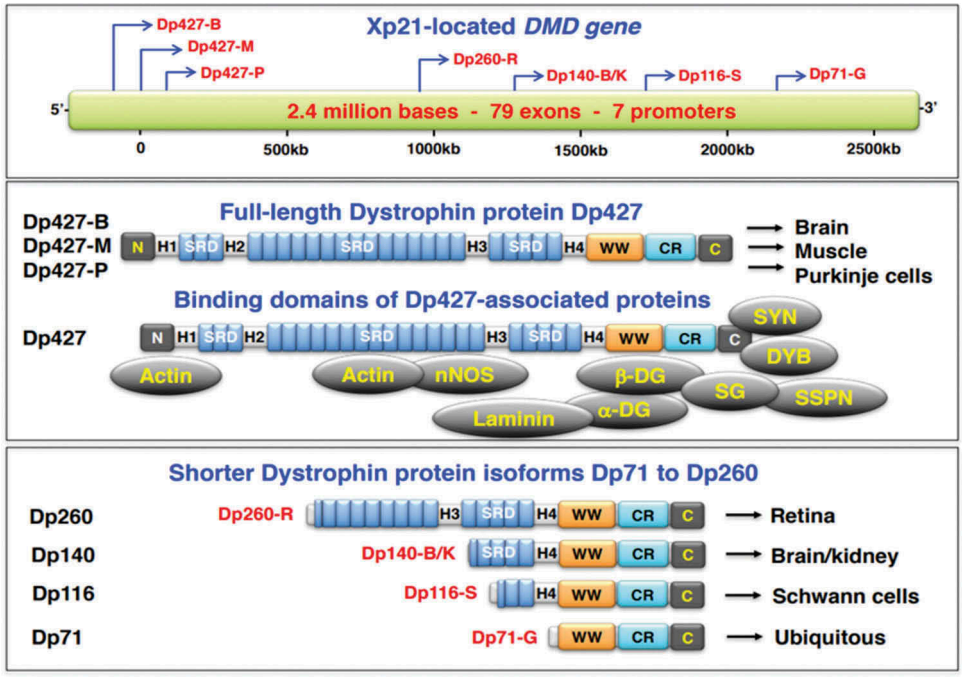

Figure 1. Schematic presentation of the *DMD* gene with tissue-specific promoters and domain structure of dystrophin protein isoforms. Shown is the overall organization of the dystrophin gene, which represents the largest gene in the human genome and contains 7 different promoters for the tissue-specific expression of full-length dystrophin isoforms Dp427-B (brain), Dp427-M (muscle) and Dp427-P (Purkinje cells), as well as the shorter dystrophin isoforms Dp260-R (retina), Dp140-B/K (brain/kidney), Dp116-S (Schwann cells in peripheral nerves) and Dp71-G (ubiquitous). The dystrophin protein products are characterized by a highly complex domain structure within tissue-specific protein isoforms. The diagram provides information on Dp427-associated domains for direct interactions with dystrophin-binding proteins, such as β-dystroglycan (β-DG), neuronal nitric oxide synthase (nNOS), syntrophins (SYN) and dystrobrevins (DYB). Indirectly associated proteins belonging to the core dystrophin-glycoprotein complex are also marked, including α-dystroglycan (α-DG), sarcoglycans (SG), sarcospan (SSPN), laminin and cortical actin.

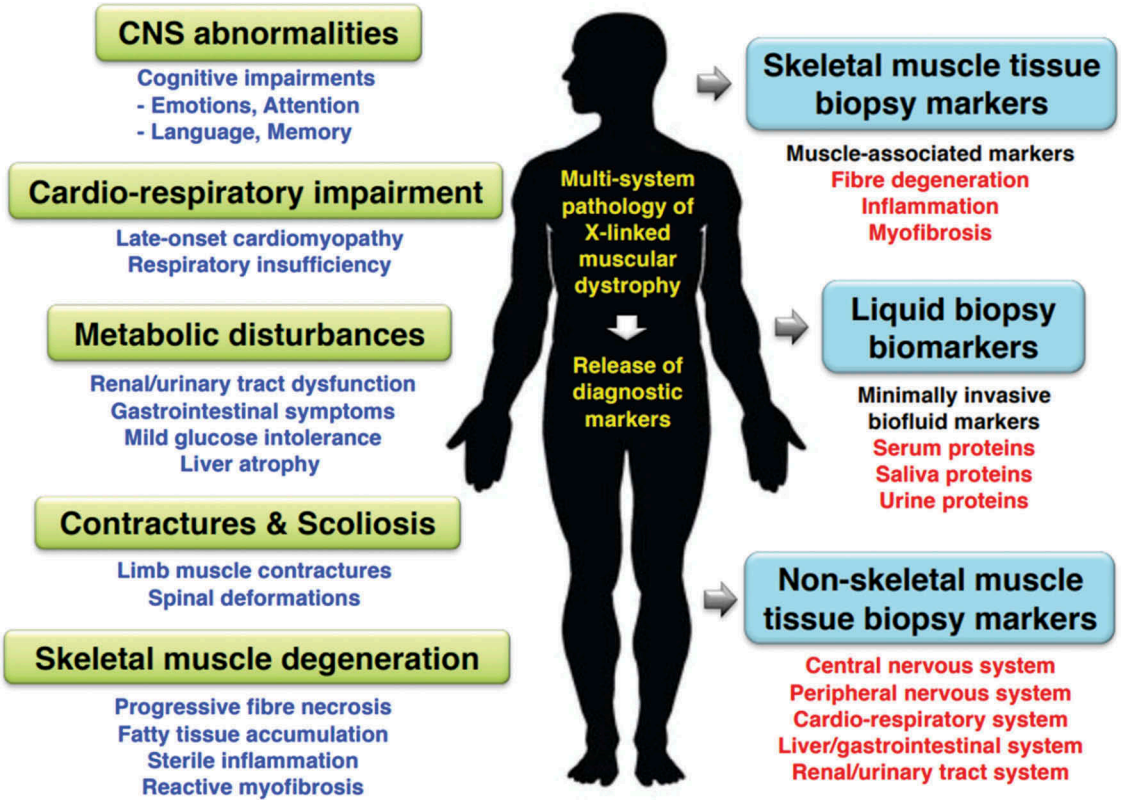

Figure 2. Overview of the multi-system disease process of Duchenne muscular dystrophy. Shown are the affected tissues, organs and biofluid systems, as well as the concept of tissue versus liquid biopsy biomarkers that may be useful for diagnostic, prognostic and therapy-monitoring purposes.

2. Biomarker discovery for the molecular evaluation of muscular dystrophy

Since only supportive treatments are available to address the devastating effects of progressive muscle degeneration and associated impairments of cardio-respiratory and cognitive functions in X-linked muscular dystrophy, there is an urgent need to improve diagnostic and prognostic methods to better evaluate novel therapeutic approaches to counter-act chronic muscle fibre wasting. Figure 2 summarizes the multi-system disease process of dystrophinopathy and the affected tissues, organs and biofluids, as well as the concept of tissue versus liquid biopsy markers that may be useful for diagnostic purposes. Dystrophic changes in the skeletal musculature are highly complex and include progressive fibre degeneration, reactive myofibrosis and sterile inflammation [16–18]. Cellular features of dystrophic muscles include increased fibre branching, more rounded cellular shapes, altered myofibre size, central nucleation due to degeneration-regeneration cycles, necrosis and fatty deposition.

Importantly, the immune response that usually supports repair mechanisms in acutely damaged skeletal muscle tissue was shown to be aberrant in the context of chronic muscle wasting. This involves both amplification of the innate immune system and activation of the acquired immune response, as extensively reviewed by Tidball et al. [18]. A perturbed crosstalk between the immune system and damaged muscles appear to amplify the dystrophic phenotype due to altered signalling patterns by immuno-modulators. Some of the most prevalent cell types of the innate immune response are present during the initial non-specific inflammatory response, including macrophages, neutrophil granulocytes and mast cells. However, more specific reactions also occur and are mediated by eosinophils, as well as key cells of the adaptive immune response such as cytotoxic CD8 + T-lymphocytes. This dysregulation of the immune response in association with chronic fibre injury is involved in promoting profibrotic changes that eventually cause severe myofibrosis [19]. Importantly, a longitudinal and multi-parametric analysis of fibrotic changes in dystrophic muscles highlighted the fact that endomysial fibrosis is the only significant myopathological characteristic that reliably correlates with the weakening of contractile strength [20]. Hence, the excessive accumulation of collagen and associated components of the extracellular matrix appears to be directly involved in the gradual decline in skeletal muscle elasticity and reduced motor functions in X-linked muscular dystrophy [21].

A major goal of current proteomic studies of muscular dystrophy is to identify novel protein biomarkers that are characteristic for individual aspects of the diverse pathobiochemical changes observed in dystrophinopathy. In general, biomarker types can be divided into (i) susceptibility markers that are

useful to estimate the risk for disease initiation in otherwise healthy individuals, (ii) diagnostic markers for the initial differential and reliable detection of a specific disease process, (iii) prognostic markers that can be useful for envisaging disease progression and adverse clinical events, (iv) predictive markers that are capable for differential patient screening and their individual sensitivity towards therapeutic interventions, (v) pharmacodynamic markers that reflect the response of the body to a specific treatment, (vi) therapy-monitoring markers that are suitable for the repeated assessment of potential changes in pathological status due to therapy, and (vii) safety-related biomarkers that are capable of measuring cytotoxic side effects in association with aggressive treatments [12]. The availability of robust biomarker signatures is especially crucial for the evaluation of new experimental treatments.

Previous pharmacological trials have focused on modulating calcium homeostasis, cellular growth, energy metabolism, the immune response, inflammation, oxidative stress and blood flow. Currently the feasibility of various innovative therapeutic approaches is tested in preclinical or clinical trials. These new methodologies include stem cell therapy, myoblast transfer, utrophin substitution therapy, enhancement of the cellular stress response, transcutaneous electrical nerve stimulation and improved steroid therapy, as well as a variety of direct or indirect genetic approaches including plasmid transfer, viral transfer, naked DNA transfer, mini-dystrophin delivery, stop codon read-through therapy, exon-skipping therapy and CRISPR/Cas9-based partial genome editing [22–24]. Potentially dangerous side effects due to *de novo* dystrophin expression following gene therapy were recently highlighted by the immunological evaluation of exon skipping in the *mdx* mouse [25]. Newly synthesised dystrophin molecules were shown to trigger both cell-mediated and humoral immune responses, which suggests that exon skipping therapy might be complicated by a long-term autoimmune response and associated cellular damage [25].

In general, the detailed biomedical value and associated complications of these types of new therapies are not easily measured. Findings from clinical strength tests, which are partially based on psychological motivation, can be misleading. Routine testing of serum enzymes, such as creatine kinase or other general markers of muscle damage, are not specific enough for the reliable evaluation of a specific neuromuscular disorder, such as X-linked muscular dystrophy. To overcome this shortage of suitable disease markers, comparative proteomics suggests itself as an ideal screening tool for the high-throughput comparison of normal versus dystrophic samples. The flow diagram in Figure 3 outlines the general bioanalytical workflow used in the standardized mass spectrometry-based proteomic identification of novel biomarker candidates.

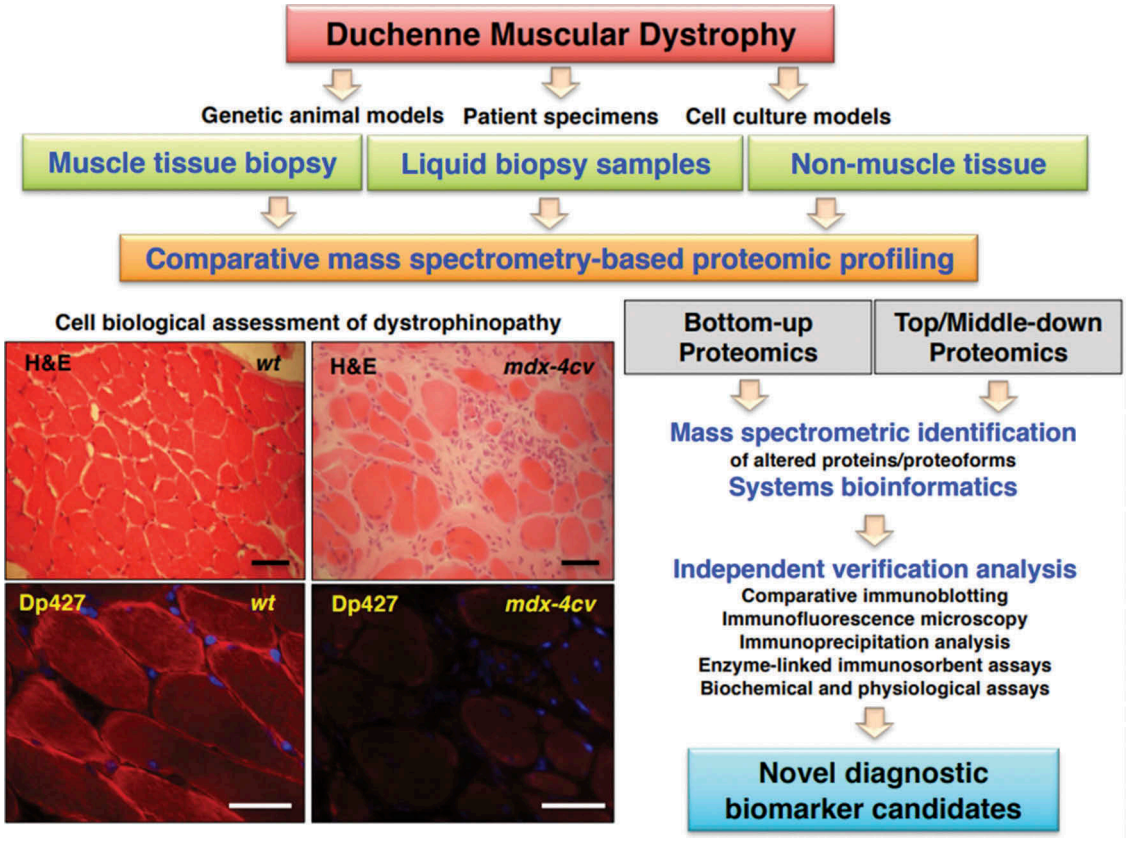

Figure 3. Bioanalytical workflow of the mass spectrometry-based proteomic identification of novel biomarker candidates of X-linked muscular dystrophy. Shown is a diagram that summarizes the various bioanalytical steps that are used for the routine evaluation of protein markers. Transverse cryosections of diaphragm muscle from wild type (*wt*) versus the dystrophic *mdx-4cv* mouse model of dystrophinopathy show typical histopathological features of dystrophin-deficient skeletal muscle fibres. Bar equals 50 µm.

Patient specimens and cell culture models can be used to study both skeletal muscle and non-muscle tissues, as well as suitable biofluids. However, due to high levels of inter-individual differences between patients and the extremely restricted amounts of human tissue biopsy specimens that are available for large-scale screening studies, most proteomic studies of muscular dystrophy have initially been carried out with samples from genetic animal models of this frequently inherited human neuromuscular disorder [26]. The histological and immunofluorescence microscopical analysis shown in Figure 3 illustrates the suitability of the dystrophic *mdx-4cv* strain, which arose from chemical mutagenesis [27], for proteomic analyses. Transverse cryosections of the diaphragm from this animal model show typical features of dystrophin-deficient muscle fibres, such as differing fibre sizes, central nucleation, sterile inflammation and reactive myofibrosis.

For proteomic analyses of dystrophic tissues, proteins are routinely extracted from organs, tissues, cells, subcellular fractions, isolated protein assemblies or biological fluids and then separated by one-dimensional gel electrophoresis, two-dimensional gel electrophoresis and/or liquid chromatography [13–15]. Typical bottom-up proteomics is based on the enzymatic digestion of protein mixtures followed by peptide mass spectrometry for the identification of individual protein species. In contrast, top-down or middle-down proteomics usually starts with isolated proteoforms that have been isolated to homogeneity by biochemical purification methods or high-resolution two-dimensional gel electrophoresis. Intact proteins or large protein fragments are then treated in a similar way as in bottom-up approaches for the characterization of their peptide sequence and post-translational modifications. In comparative proteomics, systems bioinformatics plays a crucial role in the establishment of proteome-wide changes in particular protein families, biochemical pathways, cellular signalling cascades and protein interaction networks. The existence of large databanks of already catalogued and characterized skeletal muscle-associated proteins is extremely helpful for more refined proteomic investigations [28–31]. Routine cell biological, immunochemical and physiological assays are used for the independent verification of proteomic findings and the confirmation of new biomarker candidates. These tests often include combinations of comparative immunoblotting, enzyme-linked immunosorbent assays, immunofluorescence microscopy, immunoprecipitation, biochemical binding assays, physiological measurements and enzyme assays.

3. Proteomic profiling of muscular dystrophy

The proteomic analysis of cells, tissues and organs that are directly or secondarily involved in the multi-system pathology of dystrophinopathy has been carried out with a focus on the characterization of (i) full-length dystrophin Dp427-M and related isoforms, (ii) its tightly bound dystrophin-associated glycoprotein complex, (iii) the wider dystrophin complexome including components of the sarcolemma, intracellular cytoskeletal networks and the extracellular matrix, (iv) subtypes of dystrophic skeletal muscles with varying degrees of progressive degeneration, including predominantly slow-twitching versus faster twitching limb muscles, naturally protected extraocular muscles and highly fibrotic diaphragm muscle and (v) non-skeletal muscle tissues, such as the heart, brain and liver.

3.1. Proteomic characterization of skeletal muscle dystrophin and the dystrophin-associated sarcolemmal protein complex

The dystrophin-glycoprotein complex forms a trans-sarcolemmal bridge between the intracellular cytoskeletal network and the basal lamina component laminin and its associated extracellular matrix, which is crucial for lateral force transmission and stabilisation of the fibre periphery during repeated excitation-contraction-relaxation cycles [32]. In addition, the dystrophin complex functions as a molecular scaffold for anchoring signalling proteins and plasmalemmal receptors [33]. The proteomic profile of full-length dystrophin isoform Dp427-M and its tightly bound dystrophin-associated core complex, as well as the indirectly linked components that belong to the laminin-dystroglycan-actin axis are shown in Figure 4. Both, focused studies on the mass spectrometric identification and biochemical characterization of the dystrophin-glycoprotein complex, as well as systematic cataloguing studies of muscle tissue and cell lines, have been carried out over the last decade. A summary of major proteomic studies on dystrophin and the dystrophin complexome is provided in Table 1 [28–30,34–45].

Building on the initial biochemical characterization of the dystrophin complex by density gradient ultracentrifugation and chemical crosslinking analysis [32,46,47], proteomic studies could confirm the close linkage of dystrophin with the integral glycoprotein β-dystroglycan and its associated extracellular laminin-binding protein α-dystroglycan [34,38–45]. Cortical actin, syntrophins, dystrobrevins, the α,β,γ,δ-sarcoglycan complex and laminin subunits are routinely identified by mass spectrometric surveys [28–30]. However, the integral protein sarcospan is often only identified by low sequence coverage using peptide mass spectrometry, even in highly enriched subcellular fractions from skeletal muscle [45], which is probably due to its low abundance and highly hydrophobic properties. Components of the wider dystrophin complex include caveolin, the desmoglein/desmoplakin complex, desmin, actinin, synemin, tubulins, vimentin and plectin on the intracellular side, and various collagen isoforms, fibronectin and biglycan on the extracellular side of muscle fibres [38–44] (Figure 4).

3.2. Proteomic profiling of dystrophic skeletal muscles

The pathobiochemical complexity of the dystrophic phenotype, which is characterized by progressive fibre degeneration, cycles of regeneration, fat substitution, reactive myofibrosis and inflammation in the skeletal musculature, complicates the interpretation of proteomic surveys. However, the usage of subcellular fractionation procedures, biochemical enrichment strategies, model systems from different animal species

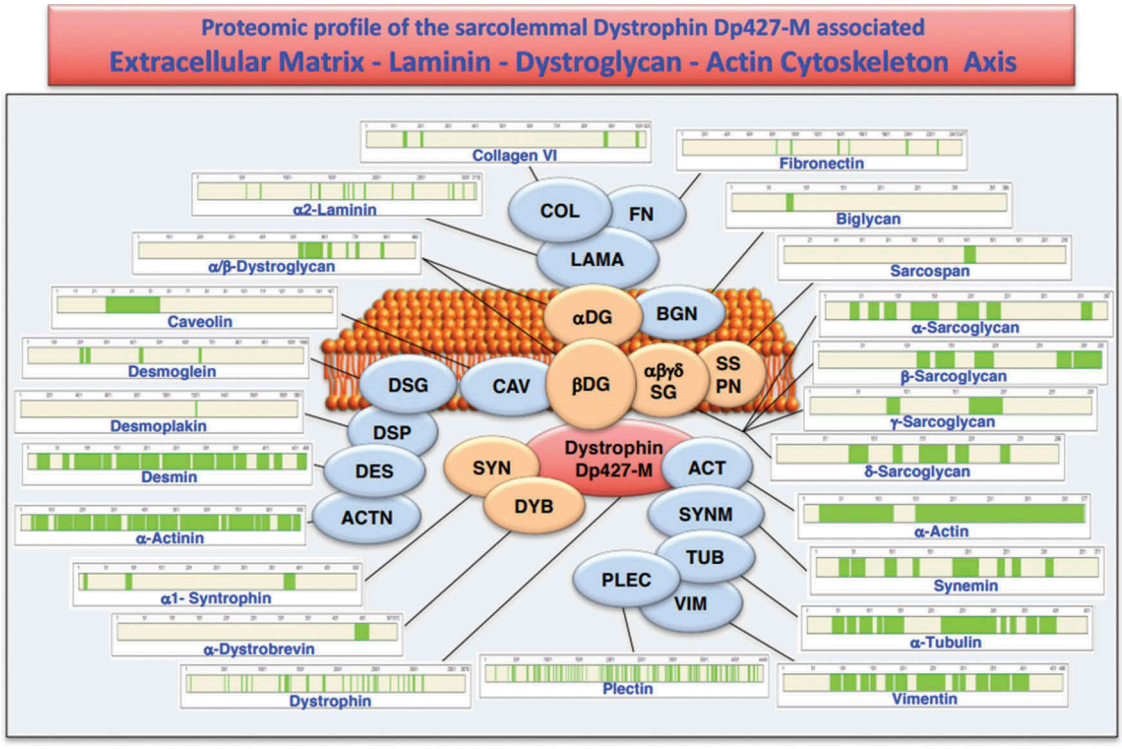

Figure 4. Proteomic profile of full-length dystrophin isoform Dp427-M and its tightly bound dystrophin core complex, as well as the indirectly associated components that belong to the laminin-dystroglycan-actin axis. The proteomic fingerprint of individual proteins is symbolized by green bars [45].

Table 1. Mass spectrometric identification of dystrophin and its tightly associated protein complex. Listed are major proteomic studies with a focus on the mass spectrometric characterization of dystrophin isoform Dp427-M and its closely associated sarcolemmal protein complex, as well as comprehensive cataloguing studies of skeletal muscles that have identified the various members of the dystrophin-glycoprotein complex. Abbreviations used: DIGE, difference in-gel electrophoresis; FASP, filter-aided sample preparation; IP, immunoprecipitation; LC-MS/MS, liquid chromatography tandem mass spectrometry; MALDI ToF, matrix assisted laser desorption/ionization time-of-flight; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin; XL, chemical crosslinking.

Species/Tissue	Methods	Proteomic identification	Reference
C2C12 cell line	FASP, LC-MS/MS analysis of assessable muscle cell proteome	Coverage of dystrophin complex in muscle protein databank	[28]
Mouse *soleus* and *extensor digitorum longus*, mechanically extracted fibres	FASP, off-gel fractionation, in-stage tip method, LC-MS/MS of single fibres	Comprehensive coverage of the core dystrophin-glycoprotein complex and associated protein species	[29]
Mouse diaphragm	LC-MS/MS analysis of assessable diaphragm proteome	Comprehensive coverage of dystrophin-glycoprotein complex	[30]
Rabbit skeletal muscle	SDS-PAGE, On-membrane digestion, LC-MS/MS	Dystrophin Dp427-M, sarcoglycan subcomplex	[34]
C2C12 myoblast lysate	SDS-PAGE, LC-MS/MS	MS-based mapping of dystrophin molecule	[35]
Human muscle, SILAC mouse *gastrocnemius*	SDS-PAGE, LC-MS/MS	MS-based quantitation of dystrophin Dp427-M	[36]
Human embryonic kidney 293T cells	Expression of recombinant mouse α-DG, MALDI-ToF MS	Glycopeptide profiling of α-dystroglycan	[37]
Cardiac muscle from various mouse strains (*mdx-5cv*, nNOS-KO); human cardiac biopsy	IP using antibodies to dystrophin, LC-MS/MS	Differential proteomic profiling of heart versus muscle dystrophin complex	[38]
Quadriceps and *gastrocnemius* muscles from various mouse strains (*mdx-23*, Galgt2 transgenic)	IP using antibodies to β-dystroglycan, LC-MS/MS	Coverage of dystrophin complex and identification of new dystroglycan-associated proteins	[39]
Hind limb muscle from various mouse strains (*mdx-5cv, Dag1*)	IP, WGA lectin-based precipitation; LC-MS/MS	Identification of novel dystroglycan complexes in skeletal muscle	[40]
Mouse and rabbit skeletal muscles	Sucrose gradient ultracentrifugation, IP, LC-MS/MS	Detailed proteomic analysis of the muscle dystrophin-glycoprotein complex	[41]
Rabbit skeletal muscle and mouse *quadriceps femoris*	Ultracentrifugation, SDS-PAGE, on-membrane digestion, LC-MS/MS	Characterization of the core dystrophin complex and co-purifying proteins	[42]
Rabbit skeletal muscle	WGA lectin affinity purification, LC-MS /MS	Subcellular localization of dystrophin-glycoprotein complex	[43]
Mouse skeletal muscle	IP, XL, LC-MS/MS	Proteomic profiling of β-dystroglycan subcomplex	[44]
Mouse hind limb muscle	WGA lectin affinity agglutination of sarcolemma, LC-MS/MS	Comprehensive profile of dystrophin complexome and laminin-dystroglycan-actin axis	[45]

and the comparative analysis of different subtypes of skeletal muscles has helped in the establishment of differential effects of dystrophin deficiency on individual skeletal muscles. A listing of overlapping protein hits from individual proteomic studies has recently been provided in a supplementary file of a review on muscular dystrophy [15], but these tables have excluded crucial information from gel-based proteomics. High-resolution two-dimensional gel electrophoresis, especially in combination with differential fluorescent tagging of entire proteomes, is a highly effective way of analysing skeletal muscle tissue [31]. Thus, for an updated and inclusive presentation of promising proteomic markers, this article lists studies that were conducted with both gel-based and gel-free methodologies. This information could be extremely helpful for the future testing and clinical establishment of novel and more robust biomarkers of Duchenne muscular dystrophy.

Table 2 provides an overview of the diversity of protein changes that have been identified by the systematic comparison of various skeletal muscles using highly sensitive mass spectrometric techniques [30,39–42,44,45,48–78]. As recently reviewed [3], proteomic studies revealed that the dystrophin-deficient skeletal musculature undergoes only moderate fibre type shifting on the level of bioenergetic pathways and the contractile apparatus. Although degenerative processes are observed in fast-twitching type IIb fibres prior to slower twitching fibre populations, and a considerable number of fibres in Duchenne patients appear to contain mostly the adult slow MyHC isoform of myosin heavy chain, proteomic studies indicate only modest alterations in the overall sarcomeric apparatus [14,15,17]. Table 2 lists the subtypes of skeletal muscles investigated in individual proteomic studies, and therefore correlates types of muscle, such as fast versus slow and glycolytic versus oxidative fibre populations, with key proteome-wide changes. The table also lists the main techniques used during proteomic surveys of specific skeletal muscles and therefore puts the main proteomic findings reported in a specific publication in the context of muscle subtypes.

The proteomic identification of new protein candidates using gel-free versus gel-based approaches is usually confirmatory or at least complementary in nature. Two-dimensional gel electrophoresis has the advantage of separating specific proteoforms by their unique combination of isoelectric point and molecular mass [48–55,57–59,61,63,65,70,78]. This type of top-down proteomics visualizes the actual protein species prior to digestion and is thus especially useful for the comprehensive analysis of the various isoforms of the actin-myosin apparatus and metabolic proteins. In contrast, integral membrane proteins, very large protein species and molecules with extreme isoelectric points are often underrepresented in 2D-gel systems. These types of muscle proteins are more appropriately analyzed by one-dimensional gel electrophoretic techniques that can be combined with on-membrane digestion and mass spectrometry [34,43,72,77] or affinity purification and immunoprecipitation approaches [39,41,44,77]. Alternatively, skeletal muscle proteins that are present in crude tissue extracts or subcellular fractions can be conveniently analyzed by bottom-up proteomics, which employs trypsination of total protein complements in combination with liquid chromatography and mass spectrometry [30,45,56,60,62,64,66–69,71,73–76,78].

The majority of comparative proteomic analyses of dystrophic skeletal muscle tissues has shown a greater number of increased versus decreased protein species, which is probably due to the occurrence of degeneration/regeneration cycle in the absence of dystrophin. Overlapping proteomic hits suggest that a variety of skeletal muscle-associated proteins are suitable candidates for the establishment of a biomarker signature of X-linked muscular dystrophy. This includes marker proteins involved in fibre contraction, energy metabolism, cytoskeletal maintenance, the cellular stress response and ion homeostasis (Table 2). The initial gel-based proteomic screening of *mdx-23* skeletal muscles identified a decreased concentration of adenylate kinase isoform AK1 [48], which was confirmed by several studies using both two-dimensional gel electrophoresis and liquid chromatography [50–52,56,59]. Lower levels of adenylate kinase may be linked to abnormal nucleotide metabolism in muscular dystrophy [50]. Another striking characteristic of dystrophic fibres are decreased levels of the cytosolic Ca^{2+}-binding protein regucalcin [51,52] and the luminal Ca^{2+}-sequestering proteins calsequestrin of the terminal cisternae region and sarcalumenin of the longitudinal tubules [45,49,61,64]. Changes in theses abundant Ca^{2+}-binding proteins were identified in both the highly fibrotic diaphragm and less severely affected leg muscles, indicating a general role in the molecular pathogenesis of dystrophinopathy. The enhanced susceptibility of dystrophin-lacking muscle fibres to micro-rupturing of the fragile sarcolemma causes an increased influx of Ca^{2+}-ions, which in turn results in higher levels of proteolytic degradation [16]. The additional impairment of efficient Ca^{2+}-buffering in the cytosol and lumen of the sarcoplasmic reticulum appears to exacerbate these physiological disturbances of excitation-contraction coupling in muscular dystrophy [49].

A very interesting biomarker candidate that has been repeatedly identified by mass spectrometry in dystrophin-deficient contractile tissues is the CA3 isoform of carbonic anhydrase [51,53,57,59,69–71]. This metabolic enzyme catalyses the reversible hydration of carbon dioxide to carbonic acid. The CA3 isoform is present in all muscle fibre types with the highest density in slow-twitching type 1 fibres. Altered CA3 levels and its leakage into the circulatory system may be related to fibre type-specific susceptibilities and accompanying changes in the dystrophic phenotype [3]. Consistent changes were also reported for annexin isoforms ANX1, ANX2 and ANX5, which may be related to disturbed Ca^{2+}-homeostasis and repair mechanisms within dystrophic fibres [42,45,50,61,64,67,71–74]. Potential compensatory mechanisms that are based on the up-regulation of cytoskeletal elements were confirmed by mass spectrometry. A drastically increased vimentin and desmin concentration seems to be a reliable indicator of cytoskeletal restructuring in Dp427-deficient fibres [51,52,54,55,57,58,63,66,67,77]. The fact that high levels of cellular stress is associated with muscular dystrophy was verified by the proteomic

Table 2. Proteomic profiling of dystrophic skeletal muscle tissues. Listed are major proteomic studies with a focus on the comparative mass spectrometric analysis of dystrophin-deficient skeletal muscle tissues. Abbreviations used: 2D-GE, two-dimensional gel electrophoresis; DIGE, difference in-gel electrophoresis; ICAT, isotope-coded affinity tag; ICPL, isotope-coded protein labeling; IEF, isoelectric focusing; IP, immunoprecipitation; iTRAC, isobaric tag for relative and absolute quantitation; LC-MS/MS, liquid chromatography tandem mass spectrometry; MALDI ToF, matrix assisted laser desorption/ionization time-of-flight; MudPIT, multidimensional protein identification technology; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SILAM, stable isotope labeling in mammals; Stains-All, cationic carbocyanine dye staining; TMT, tandem mass tag; XL, chemical crosslinking.

Species/Tissue	Methods	Key proteomic changes	Reference
Mouse *mdx-23* hind limb muscle	2D-GE, MALDI-ToF MS	Decreased adenylate kinase AK1	[48]
Mouse *mdx-23* hind limb muscle	2D-GE, MALDI-ToF MS, 2D Stains-All	Decreased calsequestrin CSQ1 and sarcalumenin	[49]
Mouse *mdx-23* hind limb muscle (cytosol)	2D-GE, MALDI-ToF MS	Increased creatine kinase, myosin light chain MLC2 and annexin ANX5; decreased adenylate kinase AK1	[50]
Mouse *mdx-23* diaphragm	2D-DIGE, MALDI-ToF	Increased heat shock protein cvHSP, desmin and vimentin; decreased adenylate kinase AK1, regucalcin, carbonic anhydrase CA3	[51]
Mouse *mdx-23* diaphragm	2D-GE, MALDI-ToF MS	Increased vimentin, desmin, albumin; decreased regucalcin, adenylate kinase AK1, alphaB-crystallin	[52]
Mouse *mdx-23* and antisense PMO-treated diaphragm	2D-DIGE, MALDI-ToF	Reversal of expression changes in heat shock protein cvHSP, adenylate kinase AK1 and carbonic anhydrase CA3	[53]
Mouse *mdx-23* extraocular muscle	2D-DIGE, LC-MS/MS	Minimal changes; increased desmin	[54]
Mouse *mdx-23 gastrocnemius*	2D-DIGE, MALDI-ToF MS	Increased vimentin, various heat shock proteins	[55]
Dog GRMD *vastus lateralis*	ICAT, phosphoprotein enrichment, LC-MS/MS	Increased phosphorylated tropomyosin-4; decrease in PGC-1α regulated metabolic proteins	[56]
Mouse *mdx-23 quadriceps* and *gastrocnemius*	IP using antibodies to β-dystroglycan, LC-MS/MS	Increased levels of utrophin and ankyrin-1 in *mdx-23* dystroglycan complexes	[39]
Mouse *mdx-23* diaphragm	2D-DIGE, LC-MS/MS	Increased desmin, vimentin; decreased adenylate kinase AK1 and carbonic anhydrase CA3	[57]
Mouse *mdx* diaphragm	2D-GE, LC-MS/MS	Increased collagen VI, dermatopontin, vimentin, alphaB-crystallin; decreased myozenin-1	[58]
Mouse *mdx-23 tibialis anterior*	2D-GE, LC-MS/MS	Increased carbonic anhydrase CA3 during *mdx-23* aging	[59]
Dog GRMD *cranial sartorius*	LC-MS/MS	Increased spectrin; decreased laminin-α2	[60]
Mouse *mdx-23* diaphragm	2D-DIGE, LC-MS/MS	Increased heat shock protein cvHSP, dermatopontin, decreased parvalbumin, calsequestrin CSQ1, adenylate kinase AK1, annexin ANX5	[61]
Mouse *mdx-23 gastrocnemius*	SILAM mouse analysis, LC-MS/MS	Complex changes in pathways related to kinases, cytoskeleton, energy metabolism and calcium homeostasis	[62]
Mouse *mdx-23 soleus, extensor digitorum longus, flexor digitorum brevis* and *interosseus*	2D-DIGE, LC-MS/MS	Differential protein changes in individual muscles; increased alphaB-crystallin, vimentin, desmin; decreased parvalbumin	[63]
Mouse *mdx-23* diaphragm versus extraocular muscle	TMT isobaric mass tagging, MudPIT, LC-MS/MS	Differential expression levels; increased annexin ANX1 and ANX5, decreased calsequestrin CSQ1 in *mdx* diaphragm	[64]
Mouse *mdx-23* hind limb muscles (microsomes)	2D-GE, LC-MS/MS	Increased heat shock protein Hsp70, transferrin, ferritin	[65]
Mouse *mdx-23* diaphgram	LC-MS/MS	Increased periostin, dermatopontin, desmin, obscurin and vimentin	[66]
Mouse *mdx-23* diaphragm, *soleus, extensor digitorum longus, flexor digitorum brevis* and *interosseus*	LC-MS/MS	Increased annexin ANX2 and ANX5, lamin A/C-B and vimentin	[67]
Mouse *mdx-23* and antisense PMO-treated *tibialis anterior*	iTRAC, IEF, LC-MS/MS	Partial restoration of *mdx-23* proteome following exon skipping, including dystrophin-associated proteins	[68]
Mouse *mdx-4cv* hind limb muscles (microsomes)	LC-MS/MS	Decrease in dystrophin complex, carbonic anhydrase CA3; increased anti-trypsin, myoferlin and periostin	[69]
Mouse *mdx-23 quadriceps* following low-intensity endurance exercise	2D-GE, MALDI-ToF MS	Differential effect of exercise on carbonic anhydrase CA3 and superoxide dismutase SODC	[70]
Mouse *mdx-4cv* hind limb muscles	LC-MS/MS	Decrease in dystrophin complex, carbonic anhydrase CA3, parvalbumin; increased collagens, fibronectin, biglycan, dysferlin and annexins	[71]
Dog GRMD *biceps femoris* following allogenic MuStem cell application	ICPL, SDS-PAGE, LC-MS/MS	MuStem cell-induced additional increase in annexin ANX1 and ANX5, ferritin, haptoglobin	[72]
Mouse *mdx-4cv gastrocnemius* and C2C12 myoblasts	LC-MS/MS	Increased annexin ANX1 and ANX5, periostin, lumican, albumin	[73]
Pig DMD *biceps femoris*	LC-MS/MS	Increased caveolin; decreased fast troponin TnC, endoplasmin	[74]
Mouse *mdx-23* skeletal muscles	Sucrose gradient ultracentrifugation, IP, LC-MS/MS	Disruption of the dystrophin-glycoprotein complex in mutants	[41]
Mouse *mdx-23* and *mdx-52 gastrocnemius*	SILAM mouse analysis, LC-MS/MS	Increased dysferlin, annexin ANX1 and ANX2; alterations in mitochondrial proteins	[75]
Mouse *mdx-23 quadriceps femoris*	Ultracentrifugation, SDS-PAGE, on-membrane digestion, LC-MS/MS	Decreased desmoglein and FABP1; increased annexin ANX1, biglycan and fibronectin	[42]
Mouse *mdx-4cv* skeletal muscles	Lectin agglutination of sarcolemma, LC-MS/MS	Increased integrin and neural cell adhesion molecule NCAM1	[76]

(*Continued*)

Table 2. (Continued).

Species/Tissue	Methods	Key proteomic changes	Reference
Mouse *mdx-23* skeletal muscles	IP, XL, LC-MS/MS	Charcterization of β-dystroglycan and caveolin subcomplexes in Dp427-deficient muscle	[44]
Mouse *mdx-4cv* hind limb muscles (microsomes)	XL, SDS-PAGE, LC-MS/MS	Increased oligomerization of myoferlin, caveolin, vimentin and tubulin	[77]
Mouse *mdx-4cv* hind limb muscles following treadmill exercise	2D-GE, MALDI-ToF MS	Exercise-induced increase in fast troponin TnT and TnI, myozenin, glycolytic enzymes	[78]
Mouse *mdx-4cv* skeletal muscles	Lectin affinity agglutination of sarcolemma, LC-MS/MS	Increased myoferlin, dysferlin and annexins; decreased periaxin and myelin PO	[45]
Mouse *mdx-4cv* diaphragm	LC-MS/MS	Increased myosin-8 and vitronectin; decreased parvalbumin and fatty acid binding protein FABP3	[30]

identification of a variety of molecular chaperones, including the heat shock proteins cvHsp, αB-crystallin, Hsp70 and Hsp90 [51,52,55,58,61,63,65].

Reactive myofibrosis is a histopathological hallmark of X-linked muscular dystrophy and closely related to loss in motor functions [17,19–21]. Proteomics established an increased density of a variety of markers of the extracellular matrix, such as collagens, dermatopontin, the matricellular protein periostin, fibronectin, biglycan, lumican and asporin [42,58,61,66,69,71,73]. Additional biomarker candidates of dystrophinopathy are represented by albumin, myosin light chains, the actin binding-protein profilin, the lipid droplet-associated protein perilipin and various glycolytic and mitochondrial enzymes [30,39–42,44,45,48–78]. Figure 5 gives an overview of the wider dystrophin complexome of the muscle fibre periphery and its association with the extracellular matrix, the cytoskeletal network and the muscle membrane system consisting of the sarcolemma, transverse tubules and the sarcoplasmic reticulum, as revealed by proteomic studies [39–45]. The concentration of a large number of these directly or indirectly dystrophin-associated proteins is changed in dystrophic skeletal muscles and these pathobiochemical alterations can now be used as proteome-wide evidence for establishing a robust biomarker signature of dystrophinopathy (Table 2).

3.3. Proteomic analysis of non-skeletal muscle tissues

Although dystrophinopathies are primarily classified as muscle diseases with characteristic neuromuscular symptoms [5], Duchenne muscular dystrophy is a multi-system disorder with severe complications due to cardiac failure, respiratory dysfunction, scoliosis, impaired liver metabolism, renal abnormalities and cognitive impairments [9]. Most studies into proteome-wide changes in non-skeletal muscle tissues [79–86] have focused on preparations from the dystrophin-deficient heart [38,79–81,84], but muscular dystrophy-related effects on the brain [82,86], kidney [83] and liver [85] have also been studied by proteomics. Investigations using both gel-based and gel-free systems have established changed concentration levels in protein markers of energy metabolism, ion homeostasis, the extracellular matrix and cytoskeletal networks. Table 3 lists proteomic biomarker candidates from non-skeletal muscle tissues that are significantly changed in X-linked muscular dystrophy [79–86]. This includes cardiac-specific changes in the core dystrophin complex, mitochondrial enzymes, the basal lamina and associated extracellular matrix [38,79–81,84], which might be linked to the cardio-respiratory syndrome in X-linked muscular dystrophy [5].

An interesting finding in relation to hepatic alterations is the identification of elevated levels of fatty acid binding protein FABP5 in the *mdx-4cv* liver. Dystrophinopathies appear to be associated with secondary alterations that especially affect fatty acid transportation in liver tissue [85]. A useful indicator of potential side effects of exon skipping therapy on the kidney was identified as the renal marker protein meprin MEP-1 [83]. Since a subset of children suffering from X-linked muscular dystrophy is also affected by neuropathological complications that affect emotion, language, attention and memory [5], it is of interest that the proteomic analysis of the central nervous system from the *mdx-4cv* mouse model has established an increased concentration of glial fibrillary acid protein GFAP [82,86]. Since GFAP is an intermediate filament component of astrocytes in the brain, its elevated concentration implies neurodegeneration-associated astrogliosis in muscular dystrophy. Thus, gliosis may be a contributing factor that is involved in dystrophinopathy-related cognitive impairments and brain abnormalities.

4. Targeted biomarker discovery in biofluids

In paediatric muscle disease diagnostics, the routine usage of minimally- or non-invasive methods is usually advantageous over potentially more uncomfortable muscle biopsy techniques [87]. Importantly, in genetic disorders of early childhood, swift and robust diagnostic and prognostic procedures will be crucial for the reliable evaluation of new therapeutic strategies, such as gene or stem cell therapy. Thus, biofluid-associated biomarkers are excellent candidates for the implementation of liquid biopsy procedures in the field of muscular dystrophy.

4.1. Advances in biofluid array technology

Antibody arrays (or forward-phase protein assays; FPPAs) are mainly applied to quantify analytes that are present in complex samples. Antibody arrays are an established proteomic technology providing an exceptional methodology for various

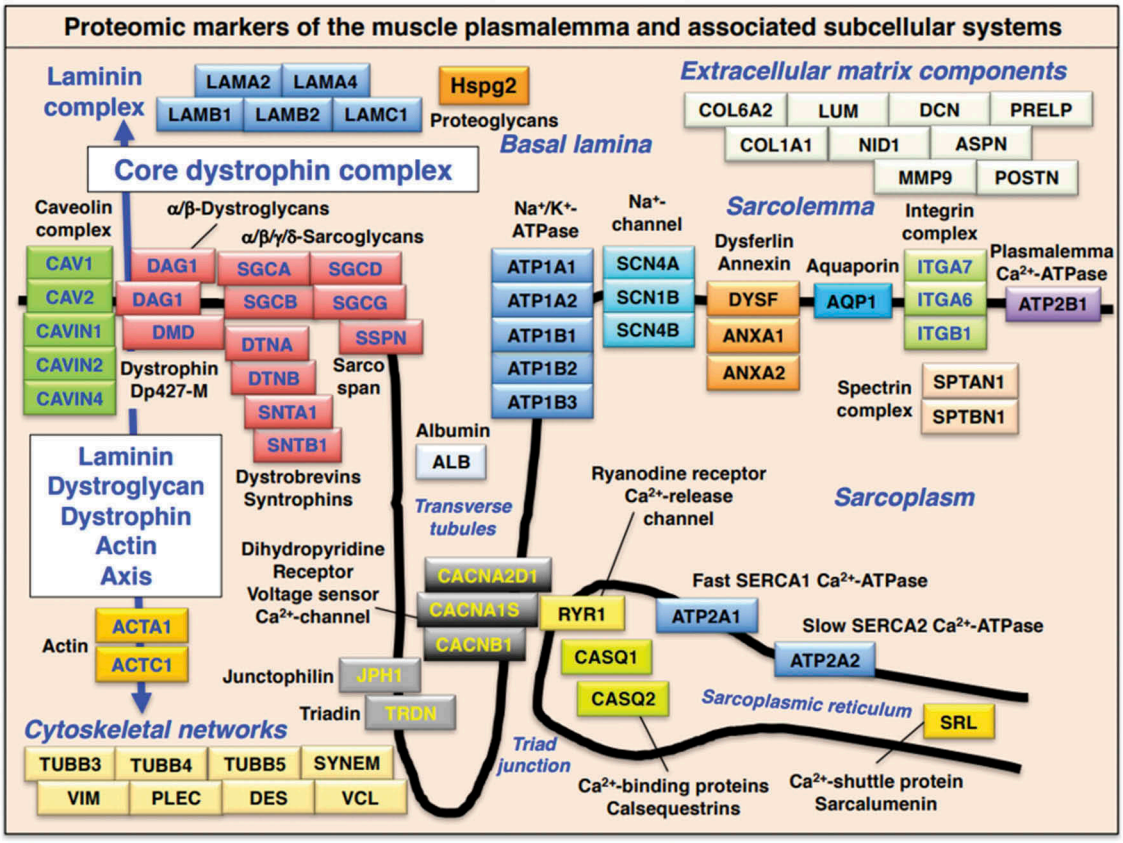

Figure 5. Overview of the wider dystrophin complexome and sarcolemmal protein markers as revealed by mass spectrometry-based proteomics. Shown are the various protein species that were shown to be linked to the wider sarcolemmal protein network and its associated subcellular systems, including the extracellular matrix, the cytoskeletal network, the plasmalemma, the transverse tubules and the sarcoplasmic reticulum.

applications, such as protein expression profiling and biomarker discovery (diagnostics/prognostics/therapy selection/disease monitoring) in samples ranging from cells, tissues, serum and conditioned media to more novel biofluids such as nipple aspirate fluid and tears [88]. An antibody array is a multiplex immunoassay that can utilise the ELISA (enzyme-linked immunosorbent assay) sandwich antibody pair principle, which allows researchers to detect potentially several hundred proteins within a single analysis. The antibodies are individually deposited in an ordered pattern, with each antibody printed as replicates to improve confidence in subsequent results. Positive and negative controls are included to maximize data reliability. Bead-based arrays of the Luminex type rely on colour-coded beads, pre-coated with analyte-specific capture antibody for the molecule of interest, with each antigen then being identified based on bead colour [89].

The typical workflow of an antibody microarray involves the choice of an appropriate antibody array platform (solid or bead), blocking of non-specific reactive groups, selection of suitable samples containing soluble proteins of interest and incubation with the antibody array, where targeted proteins from the sample are captured by the antibodies. The resulting binding events are reported precisely by fluorescent labelling of the sample or by the addition of a secondary detection reagent. Most of the antibody arrays can achieve pg/ml detection for specific antigens, similar to single ELISA detection methodology [90]. However, to overcome potential cross-reactivity and target protein abundance issues and concerns at detecting low/high abundant target proteins on the same array, more specialised arrays can be developed to mitigate for these trepidations. Current off-the-shelf antibody array panels include those specifically developed to detect and quantitate various cytokines, chemokines, adipokines, growth factors, proteases, soluble receptors and adhesion molecules. The custom option offers researchers the flexibility to design tailored panels to meet niche demands where novel biomarkers or marker molecules harbouring specific post-translation modifications need to be included as part of an antibody array panel.

Antibody array technology has proven to be a successful approach for identifying urgently needed biomarkers for muscular dystrophies. Using an antibody bead array platform with 384 antibodies, eleven proteins associated with muscular dystrophy were identified from the analysis of both serum and plasma patient samples [91]. Four proteins were established as having increased abundance levels in blood from Duchenne and Becker's muscular dystrophy patients compared to control groups, i.e. carbonic anhydrase CA3, myosin light chain MYL3, mitochondrial malate dehydrogenase MDH2 and electron transfer flavoprotein ETFA. If confirmed by longitudinal studies, these new protein signatures could then be used to

Table 3. **Proteomic profiling of non-skeletal muscle tissues in association with X-linked muscular dystrophy**. Listed are major proteomic studies with a focus on the comparative mass spectrometric analysis of non-skeletal muscle tissues, including heart, liver, kidney and brain specimens. Abbreviations used: LC-MS/MS, liquid chromatography tandem mass spectrometry; DIGE, difference in-gel electrophoresis; IP, immunoprecipitation; SILAM, stable isotope labeling in mammals.

Species/Tissue	Methods	Key proteomic changes	Reference
Mouse *mdx-23* heart	2D-DIGE, LC-MS/MS	Differential changes in mitochondrial and metabolic proteins	[79]
Mouse *mdx-23* heart	2D-DIGE, LC-MS/MS	Increased lamin-A/C; decreased ATP synthase, fatty acid binding protein FABP3 and adenylate kinase AK1	[80]
Mouse *mdx-5cv* heart	IP using antibodies to dystrophin, LC-MS/MS	Altered cardiac dystrophin interactions with syntrophin/dystrobrevin	[38]
Mouse *mdx-23* heart	LC-MS/MS	Cardiac-specific decrease in laminin, nidogen and annexin ANX6	[81]
Mouse *mdx-4cv* brain	LC-MS/MS	Increased glial fibrillary acidic protein, calretinin, annexin ANX5 and vimentin	[82]
Mouse *mdx-23* kidney following antisense PMO treatment	SILAM mouse analysis	PMO-related increase in glutamyl aminopeptidase and kidney marker meprin MEP-1	[83]
Mouse *mdx-4cv* heart	LC-MS/MS	Increased periostin, asporin, lumican, cardiac-specific myosin light chain kinase; decreased laminin and sarcalumenin	[84]
Mouse *mdx-4cv* liver	LC-MS/MS	Increased fatty acid binding protein FABP5, ferritin and calumenin; decreased phospho-glycerate kinase and perilipin	[85]
Mouse *mdx-4cv* brain	LC-MS/MS	Secondary disturbances in fatty acid and carbohydrate metabolism	[86]

monitor disease progression and facilitate clinical management decisions for dystrophic patients. Ho and co-workers [92] demonstrated how a Luminex mouse 38-plex kit was used to determine if *mdx/mTRG2* mice may have developed a peripheral inflammatory cytokine signature following systemic delivery of micro-dystrophin vectors, an investigational therapy involving systemic gene replacement to introduce a functional dystrophin gene to skeletal and cardiac muscles. Proteomic findings indicated an overall decrease in abundance levels of IFN-γ, TNF-α, IL-6, and IL-17A within the vector-treated group as compared with the vehicle group, however these results were not statistically significant.

Lysate arrays (or reverse phase protein arrays; RPPAs) represent an innovative technology that combines high throughput analysis with minimal sample consumption [93]. Small amounts (low ng range) of protein (lysates from cells/tissue) are printed onto nitrocellulose or glass slides. Proteins of interest are then quantified using highly specific primary antibodies, with slides potentially containing hundreds of individual samples, with a specific pattern for each sample containing serial dilutions and replicates. Escher et al. [94] demonstrated how powerful RPPA approaches are when evaluating changes in protein expression between healthy and diseased muscle tissue, as well as cultured primary myotubes. This study established that the differential diagnosis of muscular dystrophies using RPPAs in combination with a set of diagnostic antibodies is a worthwhile strategy, as exemplified by increased sarcoglycan signals in limb-girdle muscular dystrophy of type LGMD2A as compared to controls.

4.2. Novel biofluid markers of dystrophinopathy

Several proteomic studies have characterized blood, urine and saliva samples for the establishment of novel biofluid-based biomarkers of dystrophinopathy. Many complex biological fluids are readily available and can be easily collected for systematic proteomic analysis [87]. If specific proteoforms are passively released or actively secreted in response to pathophysiological processes, these types of proteins are an excellent starting point for designing new diagnostic assays, establish superior prognostic tests and develop more reliable techniques for the continued monitoring of novel therapeutic approaches to treat X-linked muscular dystrophy. Table 4 lists biomarker candidates that have been identified through the systematic screening of suitable biofluids [91,95–113]. Importantly, glucocorticoids are routinely used for treating chronic inflammatory conditions, including dystrophinopathies, making biofluid proteomics an essential part of the establishment of biomarkers of the anti-inflammatory response to this category of drugs [114]. Systematic investigations with a focus on serum biomarkers for chronic corticosteroid treatment revealed new candidate pharmacodynamic markers for evaluating anti-inflammatory drug efficacy, including leptin and matrix metalloproteinase MMP3 [115].

Proteomic screening studies have used samples from established animal models of muscular dystrophy, as well as blood and urine specimens from Duchenne patients. The identification of proteomic biomarker candidates in the blood included the confirmation of general indicators of muscle damage, such as creatine kinase, myoglobin and glycolytic enzymes, as well as novel proteins involved in metabolism, the extracellular matrix and the sarcomeric structures [91, 95–98,100–110]. Significant changes included fibronectin, fibrinogen, carbonic anhydrase CA3, myosin light chain MLC3, mitochondrial malate dehydrogenase, titin, myomesin, troponin TnI, calcium/calmodulin-dependent protein kinases, fatty acid binding proteins and the inflammation-induced plasma protein haptoglobin. In addition, leptin was identified as a new biomarker candidate and is potentially linked to the metabolic syndrome in Duchenne patients [116,117].

Interestingly, urine samples from Duchenne patients contained increased levels of uromodulin, which is exclusively synthesised in renal tissue and presents the most abundant kidney protein in urine, and N- and C-terminal fragments of the giant muscle protein titin [99]. Titin is a stabilising half-sarcomere spanning component of the acto-myosin apparatus in skeletal muscle fibres [7] and the appearance of titin fragments in urine indicates the pathological release of this structural protein into the circulatory system due to muscular dystrophy. The recent mass spectrometric survey of whole saliva from the *mdx-4cv*

Table 4. Proteomics of biofluid changes in association with X-linked muscular dystrophy. Listed are major proteomic studies with a focus on the comparative mass spectrometric analysis of biofluids, including serum, urine and saliva. Abbreviations used: 2D-GE, two-dimensional gel electrophoresis; LC-MS/MS, liquid chromatography tandem mass spectrometry; DMD, Duchenne muscular dystrophy; ELISA, enzyme-linked immunosorbent assay; MALDI ToF, matrix assisted laser desorption/ionization time-of-flight; SILAC, stable isotope-labeled amino acids.

Species/Tissue	Methods	Key proteomic changes	Reference
Mouse *mdx-23* and *mdx-3cv* serum	Magnetic beads affinity purification, MALDI-ToF MS	Identification of differentiating N-terminal peptide of coagulation factor XIIIa	[95]
Mouse *mdx-23* plasma	2D-GE, LC-MS/MS	Increased fibrinogen, epidermal growth factor 2 receptor and glutathione peroxidase GPX3	[96]
Secretome of mouse *mdx-23* myotubes	SILAC labelling, LC-MS/MS	Enrichment of LAMP1/MLC-1 (lysosomal-associated membrane protein/myosin light chain) – positive vesicles	[97]
Human DMD serum	ProteoMiner beads enrichment, LC-MS/MS, ELISA	Increased fibronectin levels	[98]
Human DMD serum and plasma	Large-scale screening with antibody bead array platform	Increased carbonic anhydrase CA3, myosin light chain MLC3, mitochondrial malate dehydrogenase MDH2, electron transfer flavoprotein ETFA	[91]
Human DMD and mouse *mdx-23* urine	Sample concentration, LC-MS /MS	Increased uromodulin and N-/C-terminal titin fragments, decreased cubulin	[99]
Mouse *mdx-23* and *mdx-52* and human DMD serum	SILAC mouse analysis, LC-MS /MS	Increased titin, myosin light chain MLC1/3, myomesin MYOM3, filamin-C, myoglobin	[100]
Mouse *mdx-23* urine	SILAM mouse technology, antisense PMO treatment	Increase in clusterin, differential changes in gamma-glutamyltranspeptidase GGT1	[83]
Human DMD and mouse *mdx-23* serum	Immuno-depletion, LC-MS/MS, antisense PMO treatment	Increased myomesin MYOM3, pyruvate kinase, carbonic anhydrase CA3	[101]
Human DMD and mouse *mdx-23* serum	Aptamer-based SOMAscan screening	Identification of troponin TnI, phosphglycerate mutase PGAM1, calcium/calmodulin-dependent protein kinase CAMK2b and Cytochrome-c	[102]
Human DMD serum	Aptamer-based SOMAscan screening	Identification of troponin TnI, carbonic anhydrase CA3, myoglobin, fatty acid binding protein FABP3	[103]
Human DMD serum	2D-HPLC, MALDI-ToF MS	Identification of titin, calmodulin-like protein CALM-5 and carbonic anhydrase CA1	[104]
Human DMD serum	SOMAscan screening, LC-MS/MS	Overview of DMD serum biomarker candidates	[105]
Mouse *mdx-4cv* serum	Immuno-depletion, LC-MS/MS	Increased levels of inflammation-induced plasma marker haptoglobin	[106]
Mouse *mdx-23* and transgenic *mdx-23 Fiona* serum	Aptamer-based SOMAscan screening	Increased utrophin related identification of tumor necrosis factor receptor GITR, myosin-binding protein C and heat shock protein Hsp60	[107]
Human DMD and mouse *mdx-23* serum	Immuno affinity purification, LC-MS/MS	Decreased myostatin in DMD and other neuromuscular disorders	[108]
Human DMD serum	Aptamer-based SOMAscan screening	Identification of troponin TnI, carbonic anhydrase CA3, myoglobin and fatty acid binding protein FABP3	[109]
Mouse *mdx-4cv* saliva and serum	Immuno-depletion, LC-MS/MS	Identification of differential saliva/serum markers	[110]
Human DMD serum	Aptamer-based SOMAscan screening	Identification of calcium/calmodulin-dependent protein kinases CAMK2a and CAMK2b, carbonic anhydrase CA3 and fatty acid binding protein FABP3	[111]
Mouse *dKO-Hom* serum	LC-MS/MS	Dystrophin/utrophin double-KO related decrease in haptoglopin and increase in peroxiredoxin-2	[112]
Mouse *mdx-4cv* saliva	LC-MS/MS	Increased kallikrein Klk-1 and Klk1-related peptidases b1, b5 and b22	[113]

model of dystrophinopathy identified an increased concentration of kallikrein-1 and its related peptidases Klk1-b1, Klk1-b5 and Klk-b22 [113]. Significantly higher levels of kallikrein-1 were also found in *mdx-4cv* serum samples [110]. These altered concentrations of kallikreins in biofluids are probably related to muscular dystrophy-related changes in cellular signalling mechanisms, remodelling of the extracellular matrix and a modified immune response. The successful proteomic screening of plasma, serum, urine and saliva samples demonstrates the suitability of liquid biopsy methods for the evaluation of body-wide changes in dystrophinopathy.

Figure 6 provides an overview of proteomic biomarker candidates that are associated with X-linked muscular dystrophy. The diagram lists (i) primary tissue markers of the disintegration of the core dystrophin complex, (ii) protein families that are secondarily affected in muscular dystrophy and (iii) biofluid markers that are found in the circulatory system due to passive protein leakage or active protein release.

5. Expert opinion

Since many chronic disorders, such as diabetes, neurological diseases, cardiovascular disorders or cancer trigger a variety of relatively general pathophysiological alterations in the composition of the circulatory system, it is crucial to establish a skeletal muscle disease-specific damage marker signature. Often changes in the levels of hormones, cytokines, metabolic enzymes or signalling proteins are not specific enough to properly evaluate a specific neuromuscular pathology. However, based on the systematic application of mass spectrometry-based proteomics in the field of muscular dystrophy research, select dystrophin-associated proteins, a large number of indirectly affected tissue components and a considerable array of biofluid-associated proteins can now be used to develop novel and hopefully more reliable diagnostic and prognostic marker assays. It should also be possible to exploit some newly established proteomic markers as therapeutic targets to treat progressive fibre wasting in X-linked muscular dystrophy.

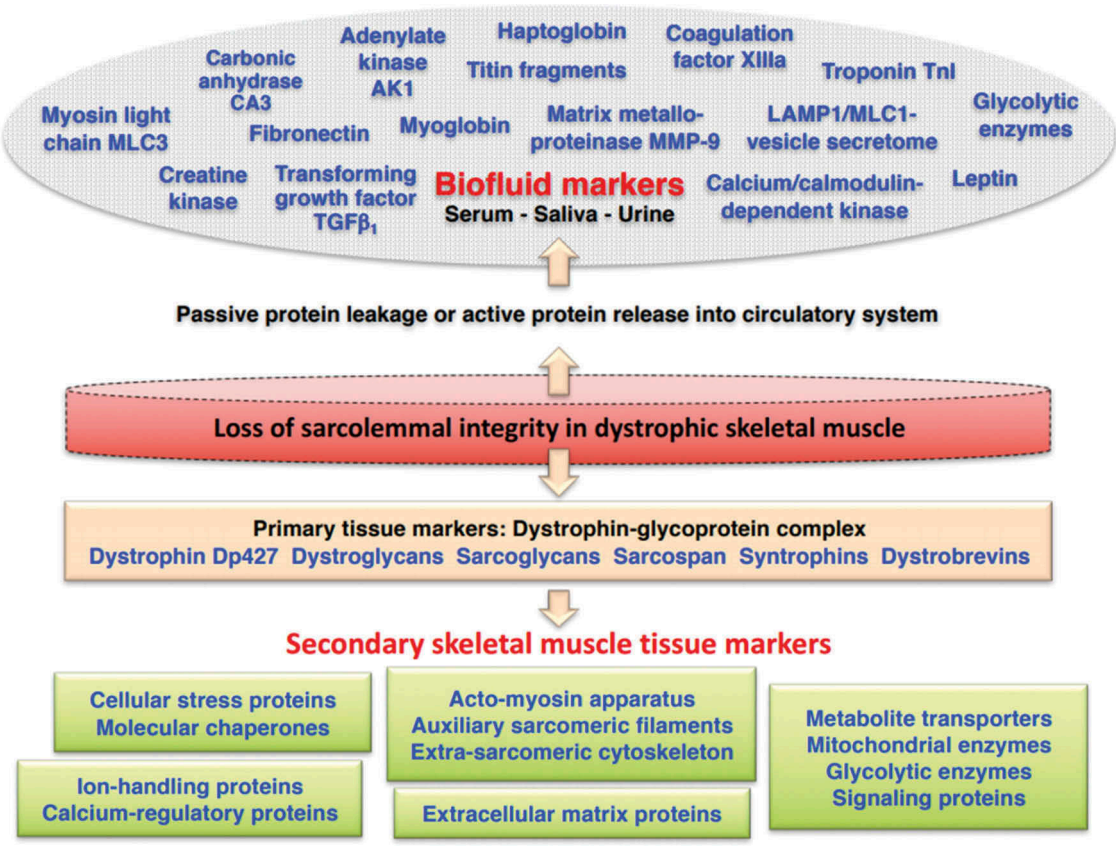

Figure 6. Schematic presentation of proteomic biomarker candidates that are associated with dystrophinopathy. The diagram lists primary tissue-associated markers of the sarcolemmal dystrophin-glycoprotein complex, protein families that are secondarily affected during chronic fibre wasting and biofluid markers that are passively leaked or actively released from dystrophic muscle tissue.

Especially liquid biopsy procedures that use independently verified protein biomarkers should be helpful to improve the diagnosis and therapy monitoring of X-linked muscular dystrophy. The recent identification of additional muscular dystrophy-specific enzymes in easily sourced biofluids, such as blood, urine and saliva, will be extremely useful for clinical applications that are based on the dependable measurement and analysis of disease progression and therapeutic response. Serum-associated fibronectin, carbonic anhydrase, myosin light chain, myomesin, troponin, fatty acid binding proteins and haptoglobin, as well as titin fragments in urine and saliva kallikrein have great potential to be useful biomarkers of dystrophinopathy. In contrast to the high costs, invasive character and clinical necessity of full asepsis of muscle biopsy procedures, non-invasive liquid biopsy procedures have the advantage of being more cost-effective, quicker and can be applied in a repeated manner for optimum therapy monitoring.

Over the next few years, it will be critical to verify the general biomedical suitability of the newly identified proteomic marker molecules that are associated with X-linked muscular dystrophy. In contrast to biofluid analyses from patients, the proteomic identification of tissue-associated marker molecules has almost exclusively been carried out with animal models of X-linked muscular dystrophy. Hence, there is an urgent need to extent proteomic studies of dystrophic skeletal muscle fibres to human tissue samples. For the translation of biomarker usage as a routine diagnostic tool in the clinical setting, potential problems with inter-individual differences in large patient cohorts have to be evaluated. Although it is difficult to obtain large numbers and quantities of muscle biopsy material from paediatric patients, it should be possible to perform comparative profiling studies with restricted amounts of human tissue by using advanced and highly sensitive mass spectrometric approaches. Both, miniaturized gel- and/or liquid chromatography-based procedures should be suitable for such analyses. Proteome-wide changes in newly identified biomarkers in tissues versus biofluids should then be pathobiochemically correlated. Subsequently new biomarker signatures have to be established in clinical laboratory practice for the superior evaluation of patient health status and the degree of skeletal muscular damage and contractile weakness. Ideally, longitudinal studies would be used to determine the robustness of new diagnostic markers and evaluate the modifying effects of routinely applied drug regimes, including pharmacological agents used in the supportive treatment of Duchenne patients, i.e. corticosteroids, beta blockers and angiotensin-converting-enzyme inhibitor.

In the long-term, simple dot blot methods, standardized enzyme-linked immunosorbent assays, antibody arrays or miniaturized immunoblotting techniques have to be developed for routine diagnostic procedures. These assay systems should ideally test protein changes within a broad biomarker signature, which covers complex alterations due to tissue degeneration, reactive myofibrosis and sterile inflammation in the affected skeletal musculature, as well as secondary changes in the heart, liver, kidney and the central nervous system. Testing of proteomic markers can also be combined with non-proteinaceous molecules, such as extracellular microRNAs, lipids and/or metabolites. Noteworthy, fatty substitution in dystrophic muscles and ectopic fat depots in other organs due to muscular dystrophy has not been extensively researched by modern large-scale methodologies. Since alterations in a number of fatty acid binding protein isoforms have been described in various tissues and biofluids, it is of considerable interest to better understand abnormal fatty acid metabolism. The systematic proteomic screening of the liver, kidney and pancreas in combination with blood and urine samples could improve our understanding of secondary metabolic dysfunctions in dystrophinopathy. The standardization of biomarker assays will be especially crucial for the testing of new therapeutic approaches in the field of muscular dystrophy, such as stem cell therapy, utrophin substitution, steroid applications, stop codon read-through therapy, exon-skipping and CRISPR/Cas9-related genome editing. Ideally new liquid biopsy-based biomarker assays will be combined with conventional examination methods, such as strength tests and skeletal muscle imaging, for the improved diagnosis and prognosis of Duchenne muscular dystrophy.

Funding

This manuscript was supported by the Health Research Board (Ireland), Muscular Dystrophy Ireland, the Higher Education Authority (Ireland) and Maynooth University.

Declaration of interest

Kay Ohlendieck discloses research funding from the Health Research Board (Ireland), Muscular Dystrophy Ireland, the Higher Education Authority (Ireland). Sandra Murphy discloses Postgraduate Hume Scholarship from Maynooth University. The authors have no other 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 apart from those disclosed.

Reviewers Disclosure

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

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