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Histopathology of Duchenne muscular dystrophy in correlation with changes in proteomic biomarkers

Margit Zweyer[1], Hemmen Sabir[1], Paul Dowling[2,3], Stephen Gargan[2,3], Sandra Murphy[4], Dieter Swandulla[5] and Kay Ohlendieck[2,3]

[1]Department of Neonatology and Pediatric Intensive Care, Children's Hospital, University of Bonn, Bonn, Germany, [2]Department of Biology, Maynooth University, National University of Ireland, [3]Kathleen Lonsdale Institute for Human Health Research, Maynooth University, Maynooth, Kildare, Ireland, [4]Charles River Laboratories, Chesterford Research Park, Saffron Walden, UK and [5]Institute of Physiology, University of Bonn, Bonn, Germany

Summary. Duchenne muscular dystrophy is an inherited disorder of early childhood that affects multiple systems in the body. Besides late-onset cardio-respiratory syndrome and various body-wide pathophysiological changes, X-linked muscular dystrophy is primarily classified as a disorder of the skeletal musculature. This is reflected by severe histopathological alterations in voluntary contractile tissues, including progressive fibre degeneration, fat substitution, reactive myofibrosis and chronic inflammation. The underlying cause for dystrophinopathy are genetic abnormalities in the *DMD* gene, which can result in the almost complete loss of the membrane cytoskeletal protein dystrophin, which triggers the collapse of the dystrophin-associated glycoprotein complex and disintegration of sarcolemmal integrity. This in turn results in an increased frequency of membrane micro-rupturing and abnormal calcium ion fluxes through the impaired plasmalemma, which renders muscle fibres more susceptible to enhanced proteolytic degradation and necrosis. This review focuses on the complexity of skeletal muscle changes in X-linked muscular dystrophy and outlines cell biological and histological alterations in correlation to proteome-wide variations as judged by mass spectrometric analyses. This includes a general outline of sample handling, subcellular fraction protocols and modern proteomic approaches using gel electrophoretic and liquid chromatographic methods for efficient protein separation prior to mass spectrometry. The proteomic profiling of the dystrophic and highly fibrotic diaphragm muscle is described as an example to swiftly identify novel proteomic markers of complex histopathological changes during skeletal muscle degeneration. The potential usefulness of new protein markers is examined in relation to key histopathological hallmarks for establishing improved diagnostic, prognostic and therapy-monitoring approaches in the field of dystrophinopathy.

Key words: Dystrophinopathy, Fibrosis, Inflammation, Mass spectrometry, Muscle degeneration, Skeletal muscle

Corresponding Author: Prof. Kay Ohlendieck, Department of Biology, Maynooth University, National University of Ireland, Maynooth, Co. Kildare, Ireland. e-mail: kay.ohlendieck@mu.ie

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Introduction

The biological uniqueness of skeletal muscles is reflected by superlatives in the domains of genes, proteins, biomolecular heterogeneity, developmental processes, cellular signalling, the myogenic stem cell niche, physiological adaptability and cellular structures (Lepper et al., 2011; Egan and Zierath, 2013; Morgan and Partridge, 2020; Mukund and Subramaniam, 2020; Novak et al., 2021). For example, the X-chromosomal *DMD* gene with its 79-exon structure is one of the largest and most complex genes in the human genome that undergoes a highly complex process of pre-mRNA splicing to produce a 14kb mRNA (Tuffery-Giraud et al., 2017). A variety of promoters within the *DMD* gene are responsible for the tissue-specific expression of dystrophin proteins that range in molecular mass from approximately 45 kDa to 427 kDa (Muntoni et al., 2003). One of the full-length protein products encoded by the *DMD* gene, the Dp427-M isoform of dystrophin, represents a crucial component of the membrane cytoskeletal system in skeletal muscle fibres (Hoffman, 2020a).

In normal skeletal muscles, this large dystrophin proteoform interacts with several sarcolemmal glycoproteins in contractile fibres, i.e. dystroglycans, sarcoglycans, sarcospan, dystrobrevins and syntrophins (Ohlendieck, 1996; Michele and Campbell, 2003), and forms a tight membrane assembly that is crucial for

maintaining the structural integrity of the muscle surface membrane during excitation-contraction-relaxation cycles (Murphy and Ohlendieck, 2015). In addition to this membrane stabilising function, the dystrophin-glycoprotein complex is involved in lateral force transmission at costamers, the anchoring of surface receptors, the organization of the cytoskeletal network and the maintenance of signalling mechanisms at the sarcolemma (Dowling et al., 2021b).

Mutations in the *DMD* gene can result in the almost complete loss of dystrophin and a concomitant reduction in all dystrophin-associated glycoproteins in the skeletal musculature of patients afflicted with X-linked muscular dystrophy (Ohlendieck et al., 1993). Deficiency of dystrophin can cause severe forms of skeletal muscle degeneration (Mercuri et al., 2019; Thompson et al., 2020). In contrast to the more benign Becker type of dystrophinopathy, Duchenne muscular dystrophy is a highly progressive muscle wasting disease of early childhood (Flanigan, 2014; Dowling et al., 2021a) and is characterized by myonecrosis, reactive myofibrosis, fat substitution and chronic inflammation (Holland et al., 2013; Guiraud et al., 2015; Ohlendieck and Swandulla, 2017; Tidball et al., 2018). This review outlines the recent advances made in our general understanding of the molecular and cellular pathogenesis of dystrophinopathy using mass spectrometry-based proteomics.

The article focuses on the identification of proteomic markers in skeletal muscle tissues and their usefulness to study histopathological changes in Duchenne muscular dystrophy. This includes the elucidation of the pathophysiological complexity of skeletal muscle degeneration due to deficiency in dystrophin and attempts to correlate cell biological and histological alterations in muscular dystrophy with proteome-wide changes as judged by systematic mass spectrometric analysis.

Histopathological hallmarks of Duchenne muscular dystrophy

Dystrophinopathies are characterized by primary abnormalities in the *DMD* gene that cause drastically changed expression levels or an abnormal size of the membrane cytoskeletal protein dystrophin (Okubo et al., 2016). The most progressive form of dystrophinopathy, Duchenne muscular dystrophy (Duan et al., 2021), represents the most commonly inherited neuromuscular disease in humans (Crisafulli et al., 2020). Early clinical symptoms in children affected with Duchenne muscular dystrophy include developmental delays, abnormal gait patterns and Gower's sign. Following the loss of unassisted ambulation, contractile weakness extends to the musculature of the upper body including dysfunction of the diaphragm (Messina and Vita, 2018).

Key histopathological changes in dystrophin-deficient skeletal muscle tissue include (i) a significantly greater variability in myofibre size, (ii) a high number of contractile fibres with a round appearance in the transverse direction, (iii) an increased frequency of central nucleation, (iv) longitudinal branching of myofibres, (v) fibre necrosis, (vi) large clusters of invading inflammatory cells such as monocytes, (vii) hypercontractility, (viii) fat substitution and (ix) extensive zones of reactive myofibrosis (Flanigan, 2014; Alic et al., 2020; Duan et al., 2021). Fig. 1 illustrates the histopathological hallmarks of a biopsy specimen from a patient afflicted with Duchenne muscular dystrophy, as previously described in detail (Holland et al., 2013).

Of note, severe dystrophinopathy is a multi-systems disease that also affects many other organ systems besides the skeletal musculature, including the central nervous system, the heart, lungs, liver, kidneys and the gastrointestinal tract, as recently reviewed by Ohlendieck and Swandulla (2021). Especially late-onset cardiomyopathy in combination with respiratory failure constitutes a serious co-morbidity in Duchenne patients (Florczyk-Soluch et al., 2021) and may trigger the cardio-respiratory syndrome that frequently necessitates cardiac drug treatment and ventilatory assistance (Meyers and Townsend, 2019). The routine pharmacological treatment of Duchenne patients therefore aims at delaying cardiomyopathy and prolonging ambulation. This includes the application of angiotensin-converting enzyme inhibitors, beta-blockers and oral corticosteroids (Hoffman, 2020b). Hope for future therapeutic options lie with the development of myoblast transfer therapy, gene replacement approaches, stop-codon read-through therapy, genome editing and exon skipping (Partridge, 2011; Mackenzie et al., 2021; Stephenson and Flanigan, 2021; Yao et al., 2021).

Histopathology of the *mdx*-type model of dystrophinopathy

Due to the limited amount of patient biopsy material being available for detailed biochemical and cell biological analyses, the utilization of genetic animal models plays a central role in the area of dystrophinopathy research (McGreevy et al., 2015; Zaynitdinova et al., 2021). This includes basic investigations into the molecular and cellular pathogenesis of muscular dystrophy (Durbeej and Campbell, 2002), as well as applied studies to evaluate new diagnostic biomarkers of pathophysiological changes (Grounds et al., 2020) and the effectiveness of novel therapeutic interventions (Zaynitdinova et al., 2021). Established animal models of Duchenne muscular dystrophy with differing degrees of disease progression and severity of contractile weakness include the *mdx* mouse and its genetic variations (Partridge, 2013), the golden retriever GRMD dog (Kornegay, 2017) and the DMD pig (Fröhlich et al., 2016). In addition, a few other model systems, such as Sapje-type *Danio rerio*, *Caenorhabditis elegans* and *Drosophila melanogaster*, with a differing dystrophic phenotype to mammalian species are employed for large-scale screening studies

(Zaynitdinova et al., 2021). As outlined in detail in a study by Duddy et al. (2015), although the *mdx* mouse exhibits an overall milder phenotype as compared to Duchenne patients, this mouse model shows nevertheless a severe myopathic pathology including hypotrophy, hypertrophy and hyperplasia in its skeletal musculature. Onset of muscular dystrophy in the severe *D2-mdx* model is driven by transforming growth factor TGF-beta and characterized by deficits in regenerative capacity (Mázala et al., 2020).

In normal skeletal muscle fibres, dystrophin is located to the internal membrane cytoskeleton of the sarcolemma (Watkins et al., 1988; Ohlendieck et al., 1991) and is almost completely lost in dystrophic fibres (Ohlendieck and Campbell, 1991). Fig. 2 outlines the deficiency of the full-length dystrophin isoform Dp427-M in the *gastrocnemius* muscle of the *mdx-4cv* mouse (Murphy et al., 2015), an *mdx*-type model that was generated by chemical mutagenesis (Im et al., 1996). In contrast to the spontaneous *mdx* mouse that harbours a point mutation in exon 23 of the *DMD* gene (Partridge, 2013) and contains a large number of revertant and dystrophin-positive fibres, the *mdx-4cv* model is characterised by a nonsense mutation in exon 53 and a drastically reduced number of revertant fibres (Danko et al., 1992). In analogy to dystrophic muscles from human patients, dystrophin-deficient *mdx*-type muscles are also characterized by an increased variability in fibre diameter, a round appearance of most muscle cells in the transverse direction and a high number of myofibres with central nucleation due to ongoing cycles of degeneration and regeneration (Ohlendieck and Campbell, 1991). This is illustrated in Fig. 2 which displays the immunofluorescence labelling of the key contractile protein alpha-actin and myonuclei in wild type versus dystrophic *mdx-4cv gastrocnemius* muscle.

Most mammalian skeletal muscles contain a mixture of muscle fibres, which however differ between animal species in their composition (Pette and Staron, 2000). Fibre types in an individual muscle may include slow-oxidative type I and the faster contracting types IIa, IIb and IIx/d, plus a variety of hybrid fibres (Schiaffino and Reggiani, 2011). Fibre type shifting occurs frequently in neuromuscular disorders (Ciciliot et al., 2013) including

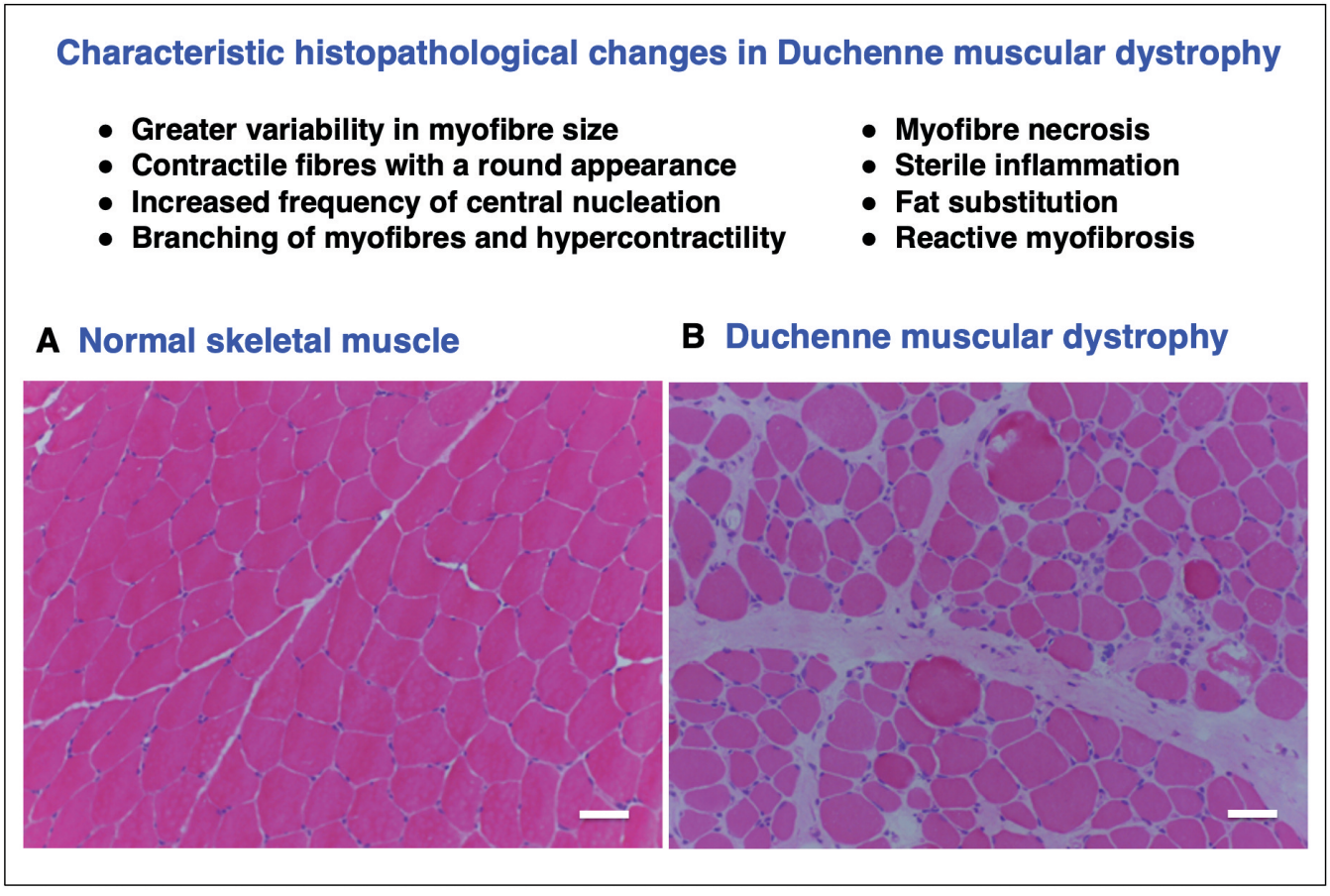

Fig. 1. Histological hallmarks of Duchenne muscular dystrophy. Shown is the histological staining of transverse sections from normal human muscle **(A)** versus dystrophic skeletal muscle from a patient afflicted with Duchenne muscular dystrophy **(B)** using haematoxylin and eosin. Permission of images for reproduction from an article by Holland et al. (2013) has been granted by Bentham Science Publishers. Scale bars: 40 µm.

muscular dystrophy as demonstrated by the analysis of animal models and skeletal muscle biopsy specimens from Duchenne patients (Dowling et al., 2016). The dystrophic skeletal musculature shows a higher susceptibility for cellular degeneration in a subset of fast myofibers, as compared to slower-twitching fibres (Webster et al., 1988) and this was shown to correlate with decreased levels of fast myosin heavy chain isoforms in dystrophin-deficient skeletal muscles (Marini et al., 1991; Pedemonte et al., 1999). The recent proteomic and transcriptomic analysis of dystrophin-deficient *mdx* skeletal muscle confirmed that fast-twitching myofibers with predominantly glycolytic metabolism are preferentially damaged in dystrophinopathy, and that fast-to-slow transitions are associated with elevated fibre respiration and enhanced protection from muscle injury due to eccentric contraction patterns (Hardee et al., 2021).

Mass spectrometry-based muscle proteomics

Following the establishment of proteomics as a new analytical subdiscipline within protein biochemistry (Wilkins et al., 1996; Banks et al., 2000), the unprecedented advances in large-scale protein separation and highly sensitive peptide mass spectrometry were also applied to studying skeletal muscle tissues (Sanchez et al., 2001; Yan et al., 2001) and lead to the new field of skeletal muscle proteomics (Doran et al., 2007; Ohlendieck, 2011; Capitanio et al., 2017). Mass

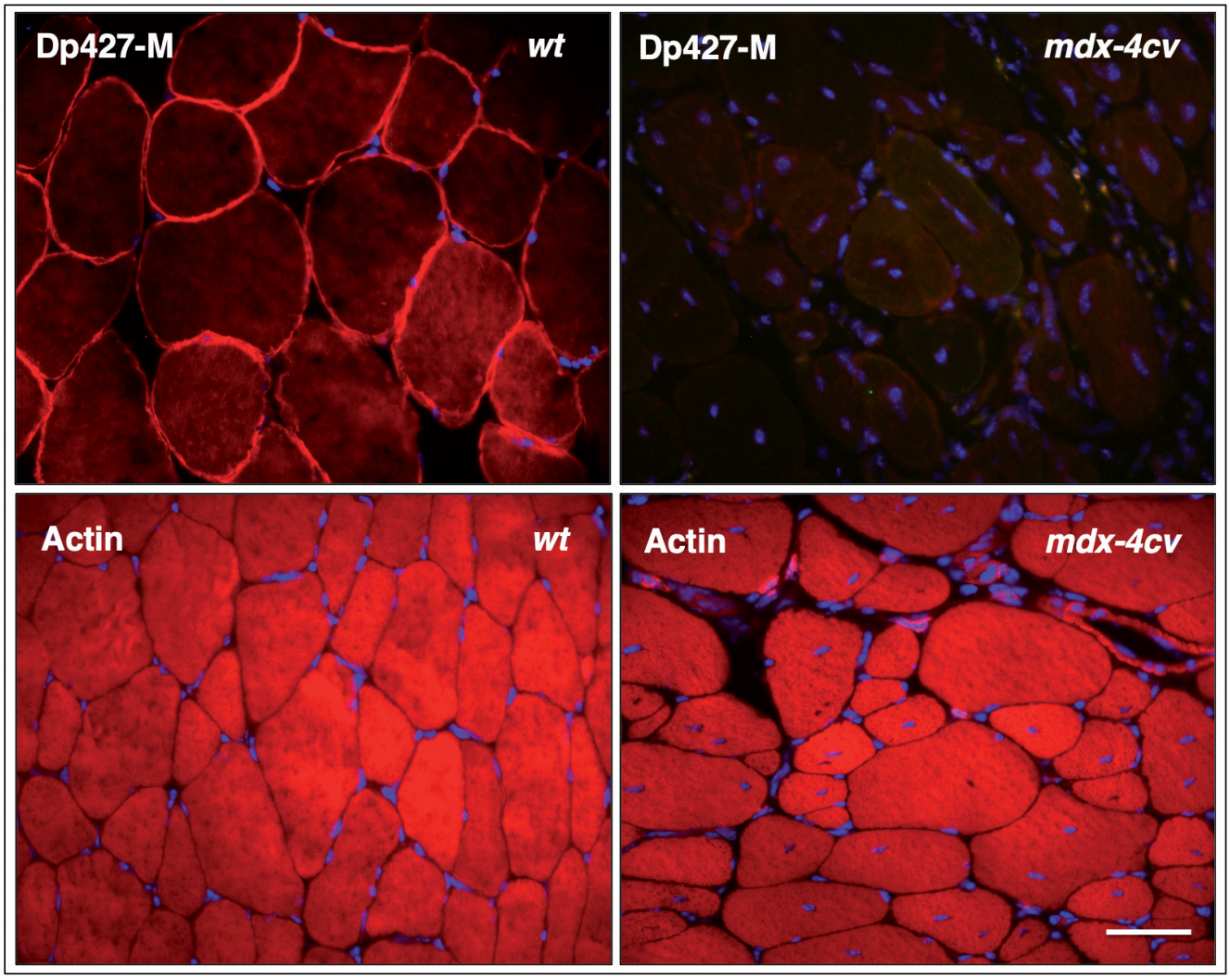

Fig. 2. Localization of dystrophin and actin in normal and dystrophic skeletal muscle using immunofluorescence microscopy. Shown is the labelling of the Dp427-M isoform of dystrophin using antibody NCL-DYS1, the contractile protein alpha-actin using antibody MA5-12542 and Hoechst-33342 staining of nuclei in 3-month old wild type (*wt*) versus age-matched dystrophic *mdx-4cv* gastrocnemius muscle. Methods of tissue preparation and immunofluorescence microscopy were optimized and carried out as previously described in detail (Murphy et al., 2019b; Mundegar et al., 2018). Scale bars: 40 µm.

spectrometry-based proteomic screening approaches usually start with isolated protein species using top-down proteomics or entire protein populations employing bottom-up proteomics (Dupree et al., 2020; Kang et al., 2020). Various one- and two-dimensional gel electrophoretic methods and liquid chromatography are routinely used to separate and isolate proteins. Top-down muscle proteomics is a proteoform-centric approach for the biochemical identification and characterization of intact protein species including the determination of protein mass and dynamic post-translational modifications (Dowling et al., 2019b). Bottom-up proteomics represents a proteolytic peptide-centric approach and is based on the initial extraction of large protein mixtures and their subsequent mass spectrometric analysis based on generated peptide populations (Manes and Nita-Lazar, 2018).

Based on the comprehensive mass spectrometric characterization of the pathophysiological crosstalk between the spleen and skeletal muscle in muscular dystrophy (Dowling et al., 2020a), a typical bottom-up proteomic workflow to analyse tissue specimens has recently been described (Dowling et al., 2020b). This method paper lists all essential experimental steps, chemicals, buffers and equipment that are necessary for the conduction of an optimum proteomic screening exercise (Dowling et al., 2020b). The interdependent stages involved in the bioanalytical assessment from individual proteoform to peptide level involve optimized tissue homogenization, reliable determination of protein concentration, reproducible protein digestion and the removal of potentially interfering chemicals prior to mass spectrometric analysis. A commonly employed approach to digest complex protein mixtures for mass spectrometric analysis is the filter-aided sample preparation method named FASP, for which experimental steps have been described in detail by Wiśniewski (2018).

Of central importance for proteomics is the detection sensitivity of the mass spectrometric analysis following protein separation (Aebersold and Mann, 2016), whereby different types of mass spectrometers are used for discovery proteomics as compared to targeted proteomics (Uzozie and Aebersold, 2018). Discovery proteomics is an unbiased screening tool that is based on large-scale protein separation from complex cellular preparations and focuses on the comparative evaluation of dynamic changes in protein concentration. In contrast, targeted proteomics is used for the mass spectrometric detection of select protein species and their absolute quantification. Peptide and protein mass spectrometry can be carried out with both label-free approaches or labelled proteins in comparative studies (Kang et al., 2020).

Commonly used labelling techniques for cell biological investigations are represented by stable isotope labelling by amino acids in cell culture (SILAC) and isobaric tagging for relative and absolute quantitation (iTRAQ) (Craft et al., 2013), as well as isotope-coded affinity tagging (ICAT) and tandem mass tagging (TMT) that has been used for serum profiling of young patients afflicted with dystrophinopathy (Alayi et al., 2020). New developments in mass spectrometric analysis include targeted labelling approaches that use parallel reaction monitoring (PRM) (Wilson et al, 2019) or multiple reaction monitoring (MRM) (Percy et al., 2017), which has recently been applied to the absolute quantification of dystrophin in skeletal muscle biopsy specimens (Canessa et al., 2020). In addition, label-free approaches have been established that are based on the sequential window acquisition of all theoretical mass spectra (SWATH) (Muench et al., 2020) and novel data-acquisition methods such as the BoxCar protocol (Meier et al., 2018), which promise an even higher level of detection sensitivity and protein identification in future proteomic studies.

Bioanalytical strategy to study muscular dystrophy

An overview of the general bioanalytical strategy to carry out cell biological, biochemical and proteomic studies of X-linked muscular dystrophy is provided in Fig. 3. As outlined in detail in below sections, this review attempts to correlate histopathological changes occurring in dystrophinopathy to biochemical alterations in proteomic markers. Although genetic testing and serum-based assays are becoming more prevalent in the field of diagnosing myopathies (Okubo et al., 2016), the usage of muscle biopsies still plays an important part in skeletal muscle pathology (Nix and Moore, 2020). The routine cell biological assessment of muscle biopsy specimens includes the usage of established histological stains, such as haematoxylin and eosin, to determine general changes in the numbers, shape and cross-sectional area of myofibres, as illustrated in above Fig. 1. In addition, histochemical staining procedures can be employed to test for a variety of enzyme activities within contractile fibres, as well as for differentiating slow versus fast fibre populations in cryosections of muscle biopsy material. Immunofluorescence microscopy is a relatively swift and efficient technique to evaluate the expression levels and subcellular localization of individual proteins in pathological muscle specimens (Mundegar et al., 2018), such as dystrophic tissues (Fig. 2), and determine histopathological levels of cellular degeneration, inflammation, myofibrosis, fat substitution and nuclei relocation, as well as potential changes in the number of satellite cells and fibre type shifting (Meola, 2005).

To complement cell biological and histological investigations, protein biochemistry has been instrumental to advance the field of diagnosis of Duchenne muscular dystrophy and its more benign form called Becker muscular dystrophy (Arahata et al., 1989; Hoffman et al., 1989; Beggs and Kunkel, 1990). With new developments in mass spectrometry, proteomics has also been applied to studying muscular dystrophy, as reviewed by Holland et al. (2013). Besides studying

entire skeletal muscle proteomes, mass spectrometric screening approaches are also suitable to characterize protein constituents in single fibre preparations and extracts from tissue sections, as well as cellular subproteomes, isolated protein complexes and purified protein species. Major biochemical separation methods involved in subproteomic investigations of skeletal muscles include (i) differential centrifugation for the isolation of crude subcellular fractions, (ii) biochemical affinity purification techniques for the isolation of highly enriched subcellular fractions or protein complexes, (iii) microdissection for the analysis of histological preparations such as single myofibres or muscle tissue sections, and (iv) gel electrophoretic methods used to separate complex protein mixtures. Of note, simple differential centrifugation steps can be employed to enhance the yield of low-abundance proteins for the proteomic profiling of dystrophic muscle samples (Carberry et al., 2014).

Experimental details of biochemical affinity purification techniques that are routinely used in skeletal muscle proteomics have been described for the usage of immunoprecipitation (Yoon et al., 2012), chemical crosslinking of protein complexes (Murphy et al., 2018a) and the lectin-mediated agglutination of sarcolemma vesicles (Murphy et al., 2019a). A gel electrophoretic technique that is frequently employed for the comparative analysis of normal versus pathological muscle samples is fluorescence two-dimensional in-gel electrophoresis, usually abbreviated as 2D-DIGE (Gelfi and Capitanio, 2018). Normal versus dystrophic muscle specimens are tagged with different fluorescent CyDyes and can then be separated within the same gel system. Gel electrophoretic separation is usually carried out by isoelectric focusing in the first dimension and sodium dodecyl sulphate polyacrylamide slab gel electrophoresis in the second dimension (Ohlendieck, 2018). Abundance changes in individual protein spots that exhibit a unique combination of isoelectric point and molecular mass can be determined by densitometric scanning and subsequently identified by peptide mass spectrometry (Carberry et al., 2013a). Although the 2D-DIGE method

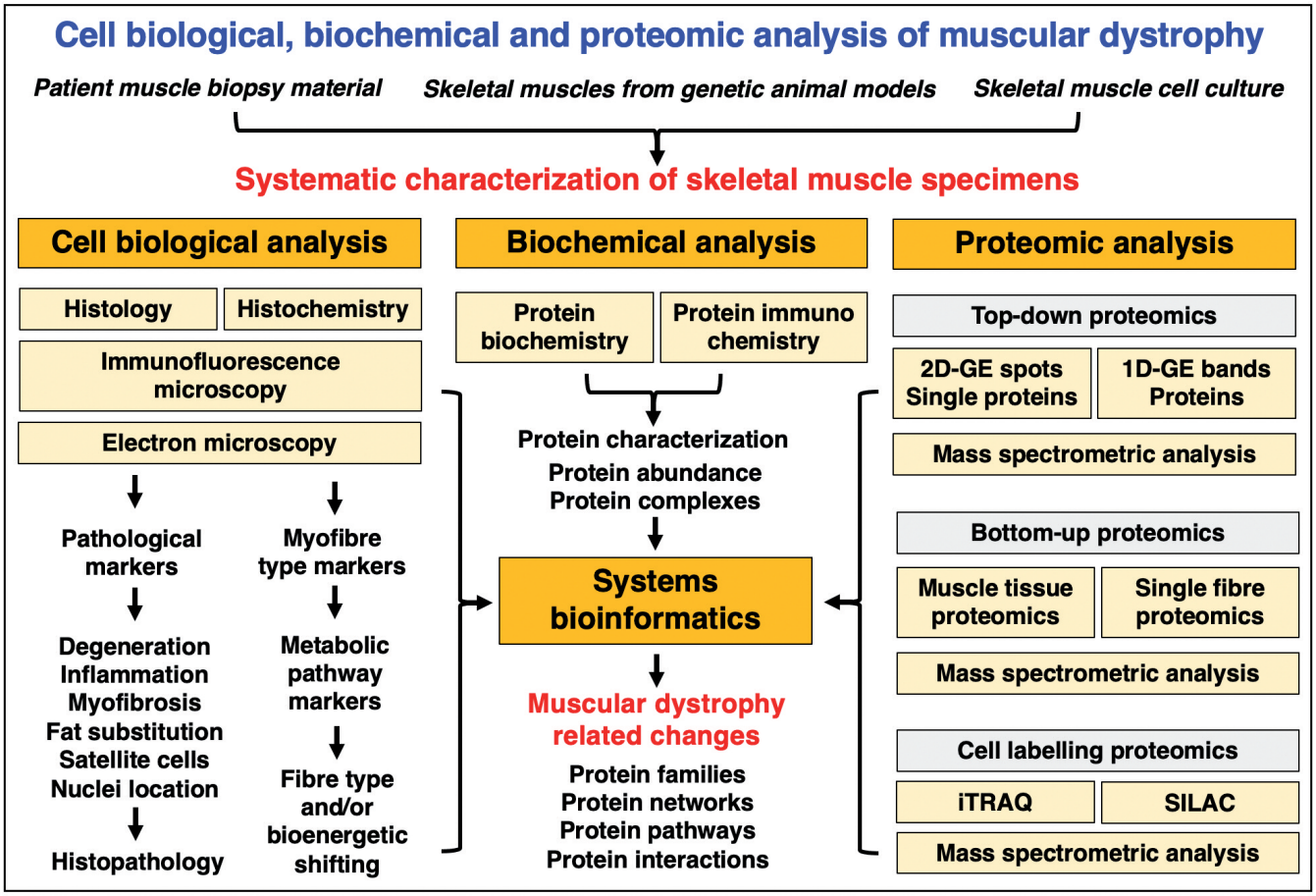

Fig. 3. Overview of the bioanalytical strategy to characterize muscular dystrophy. Listed are the main cell biological, biochemical and mass spectrometric workflows to identify and characterise novel factors involved in the molecular and cellular pathogenesis of Duchenne muscular dystrophy, as well as establish new proteomic markers of dystrophic changes. Abbreviations used: 1D, one-dimensional; 2D, two-dimensional; GE, gel electrophoresis; iTRAQ, isobaric tagging for relative and absolute quantitation; and SILAC, stable isotope labelling by amino acids in cell culture.

has the advantage of eliminating gel-to-gel variations during comparative protein analyses, it is not suitable to study certain types of proteins including molecular species that exhibit extreme isoelectric points, broad levels of dynamic post-translational modifications, an extensive number of hydrophobic domains and/or very high molecular masses. However, these types of muscle proteins can be conveniently studied by on-membrane digestion (Lewis and Ohlendieck, 2010; Murphy and Ohlendieck, 2017) or 1D-GeLC approaches that combine both one-dimensional gel electrophoresis and liquid chromatography for the optimum separation of complex mixtures of proteins, including integral membrane proteins and high-molecular-mass proteins (Murphy and Ohlendieck, 2018).

The systematic application of micro-proteomic sampling approaches (Alexovič et al., 2021) has greatly improved the mass spectrometric analysis of distinct cell types and their microenvironment (Mao et al., 2021). A key technique of spatial proteomics is presented by laser capture microdissection (Jensen, 2013). Laser-assisted microdissection techniques can be employed to isolate distinct cells under direct microscopical visualization of the tissue of interest (Espina et al., 2006) and produce through the subsequent analysis of the pure histological specimen a plethora of systems biological data (Datta et al., 2015). This allows the establishment of spatial molecular signatures of a distinct cellular layer within a targeted microscopic area (Espina et al., 2007). The micro-dissected sample can be processed for a variety of bioanalytical purposes, such as RNA transcript profiling, the generation of cDNA libraries, genotyping of DNA, protein pathway analysis, biomarker discovery and mass spectrometry-based spatial proteomics (Liotta et al., 2021). The usage of laser capture microdissection for enhancing cell biological, biochemical and proteomic analyses of skeletal muscle fibre populations has been recently described by Eggers et al. (2021) and been widely employed in the analysis of pathological muscle specimens (Maerkens et al., 2013).

Spatial proteomic tissue profiling has been instrumental for studying the regulation of myoferlin during skeletal muscle repair (Demonbreun et al., 2010), the protein profile of the myotendinous junction (Can et al., 2014), the abundance of myosin isoforms in human muscle fibres (Stuart et al., 2016) and mitochondrial DNA damage in human contractile fibres (Elstner et al., 2021), as well as proteome-wide changes in limb-girdle muscular dystrophy (Greenberg et al., 2012), the protein aggregation pathologies filaminopathy (Kley et al., 2013) and myotilinopathy (Maerkens et al., 2016), inclusion body myositis (Güttsches et al., 2017), necrosis and regeneration in muscular dystrophy (Morales et al., 2018) and mitochondrial myopathy (Murgia et al., 2019). The technical advances in the proteomic analysis of single muscle fibres have been described in studies by Murgia et al. (2015, 2017, 2021) and Deshmukh et al. (2021). The potential of single muscle fibre analysis with biochemical and molecular biological methods has long been realised (Schiaffino and Reggiani, 1994; Pette et al., 1999) and its potential to further revolutionize the field of skeletal muscle proteomics has recently been reviewed by Schiaffino et al. (2020).

How proteomic markers can be used for the characterization of normal versus dystrophic samples using standardized biochemical techniques, such as immunoblotting, is shown in Fig. 4. Both fibronectin and dysferlin are novel biomarker candidates of X-linked muscular dystrophy that have been identified by systematic mass spectrometric investigations, as reviewed by Dowling et al. (2019a). In contrast to laminin, which concentration is not majorly changed due to deficiency in dystrophin, both emerging biomarkers of dystrophinopathy are significantly increased in dystrophic *mdx* and *mdx-4cv* skeletal muscle preparations (Murphy et al., 2017). The increase in dysferlin, a proteomic marker of the sarcolemma membrane, was shown to occur in association with the up-regulation of crucial repair mechanism in dystrophinopathy (Demonbreun and McNally, 2016). Elevated levels of fibronectin, a proteomic marker of the extracellular matrix, agree with substantial levels of reactive myofibrosis, a key feature of Duchenne muscular dystrophy (Klingler et al., 2012). The importance of reactive myofibrosis in the progressive nature of X-linked muscular dystrophy is outlined in below section on the dystrophin-deficient diaphragm muscle.

The dystrophin-deficient *mdx*-type diaphragm as a model system to study progressive myonecrosis and reactive myofibrosis in dystrophinopathy

Despite the presence of the same type of mutation in the *DMD* gene, different subtypes of skeletal muscles are affected in distinctive ways in X-linked muscular dystrophy (Muntoni et al., 2003; Thompson et al., 2020; Duan et al., 2021). This is probably due to physiological, biochemical and anatomical dissimilarities between the hundreds of different skeletal muscles in the body, as well as diverse degrees of susceptibility to skeletal muscle damage and the select occurrence of naturally protective mechanisms in certain skeletal muscles. The detailed analysis of *mdx*-type mouse models of Duchenne muscular dystrophy has confirmed this (Partridge, 2013) and shown a mild phenotype in dystrophin-deficient extraocular fibres (Gargan et al., 2021), segmental necrosis in limb muscles (Torres and Duchen, 1987) and severe myonecrosis and fibrotic changes in the diaphragm muscle (Coirault et al., 2003; Murphy et al., 2019b). The comparative proteomic profiling of dystrophin-lacking *extensor digitorum longus, soleus, flexor digitorum brevis, interosseus* and diaphragm muscle has established annexin, lamin, and vimentin as universal proteomic markers of muscle tissue-associated changes in *mdx*-type mouse models of X-linked muscular dystrophy (Carberry et al., 2013b; Holland et al., 2015b) and confirmed the dystrophic

mdx-4cv diaphragm as one of the most severely affected types of skeletal muscle in dystrophinopathy (Murphy et al., 2019b). This makes the dystrophic and highly fibrotic diaphragm muscle from the *mdx-4cv* mouse and related disease models (Beastrom et al., 2011) an excellent cellular system for studying progressive skeletal muscle degeneration and the complex interplay between myonecrosis, myofibrosis and chronic inflammation.

The developmental formation and functional maturation of the diaphragm is highly complex and originates from the emigration of progenitor cells from cervical somites, which is followed by the recruitment of multiple cell populations. During diaphragm myogenesis, coordinated interactions between muscle precursor cells, the neural tube-derived phrenic nerve, the vasculature, connective tissue derived from the pleuroperitoneal folds and tendon form the functional respiratory muscle system (Sefton et al., 2018). Diaphragm dysfunction and accompanying respiratory deficiency is associated with a variety of neuromuscular disorders (Howard, 2016), including cardiopulmonary failure at the more advanced stages of Duchenne muscular dystrophy (Barnard et al., 2019). Deficiency in dystrophin clearly leads to progressive diaphragm degeneration and oxidative stress (Kim et al., 2013), which in turn causes pulmonary malfunction during the progression of dystrophinopathy (Pennati et al., 2020). Thus, the pathophysiological imbalance between required muscular force via the diaphragm and respiratory load plays a key role in dystrophinopathy. Importantly, sleep disordered breathing may be already present in young Duchenne patients (Sawnani, 2019) and the resulting oxygen deprivation, apnea and hypercapnia during periods of sleep can progress to hypoventilation and respiratory failure during wakefulness (LoMauro et al., 2017). In analogy to dystrophic patients, the diaphragm in animal models of X-linked muscular dystrophy is also characterized by progressive fibre degeneration, reactive myofibrosis, chronic inflammation and severe deficiencies in force generation (Beastrom et al., 2011; Burns et al., 2019; Loehr et al., 2018).

The histological and immunofluorescence microscopical analysis of transverse cryosections from the *mdx-4cv* diaphragm clearly show the disease status of muscular dystrophy (Murphy et al., 2019b). In contrast to a regular cell shape, peripheral nucleation and relatively sparse extracellular matrix regions between myofibres in wild type muscle, the dystrophic phenotype is characterized by more round shaped myofibres that exhibit a greater variety in cellular diameter, as well as

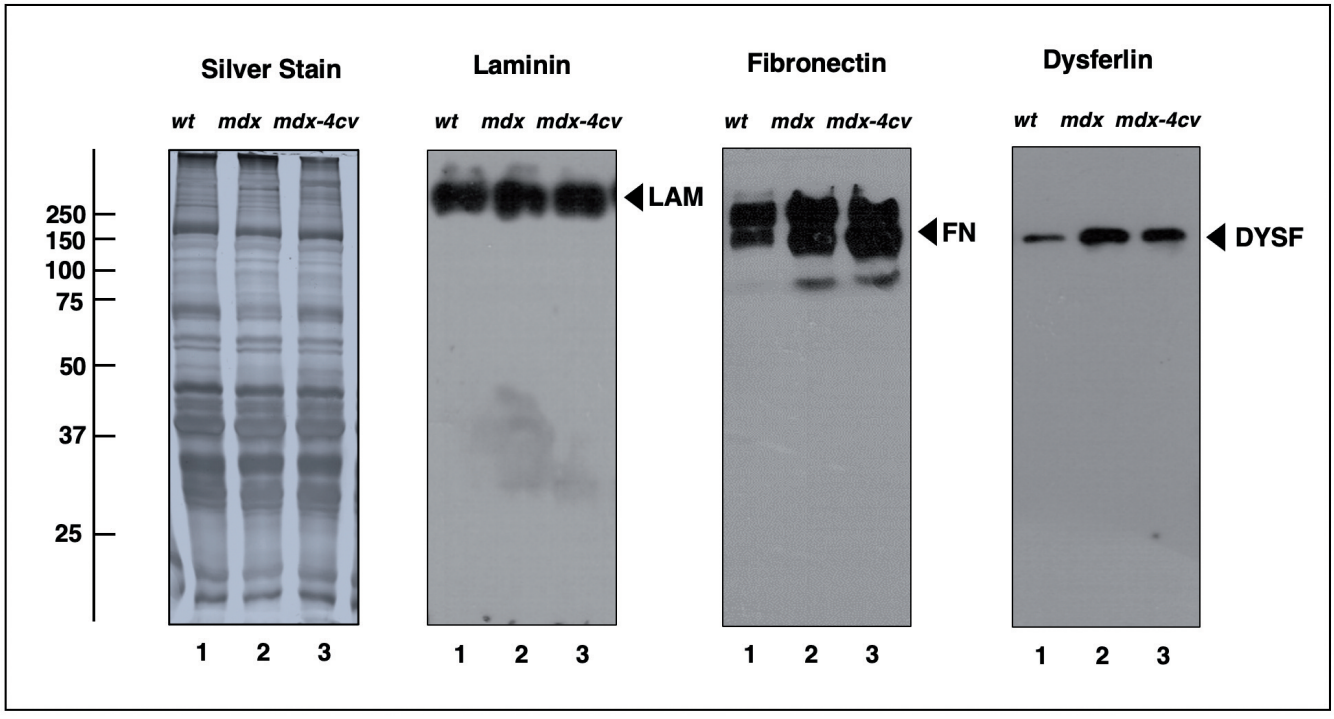

Fig. 4. Immunoblot analysis of proteomic markers in dystrophin-deficient skeletal muscle extracts. Shown is a silver-stained gel and corresponding immunoblots labelled with antibodies L-9393, ab2413 and ab124684 to the basal lamina component laminin (LAM), the extracellular matrix protein fibronectin (FN) and the membrane repair protein dysferlin (DYSF), respectively. Protein extraction, gel electrophoresis and immunoblotting were carried out by standardized methodology (Murphy et al., 2019a). Lanes 1 to 3 represent 3-month old wild type (wt) versus age-matched mdx and mdx-4cv hind limb muscle extracts, respectively. The position of immuno-labelled protein bands is indicated by arrowheads. Molecular mass standards are marked on the left of panels.

an increase in central nucleation, fibrotic areas and zones of inflammation. These histopathological hallmarks of fibre degeneration agree with the severely impaired force generation in the dystrophic diaphragm (Beastrom et al., 2011). The immunofluorescence labelling of the full-length dystrophin isoform Dp427-M shows mostly a peripheral localization of this protein at the sarcolemma in normal diaphragm versus an almost complete loss in the *mdx-4cv* fibre population. The proteomic profiling of the dystrophic *mdx* and *mdx-4cv* diaphragm muscle has revealed abnormal calcium handling and impaired calcium buffering (Doran et al., 2006a), a drastic increase in molecular chaperones (Doran et al., 2006b), significantly elevated levels of extracellular matrix proteins (Carberry et al., 2012, 2013a; Holland et al., 2015a) and changes in the expression pattern of various metabolic enzymes, cytoskeletal components and sarcomeric proteins (Murphy et al., 2019b).

These proteome-wide changes agree with the concept of (i) impaired excitation-contraction coupling and disturbed calcium homeostasis (Alderton and Steinhardt, 2000; Mareedu et al., 2021), (ii) up-regulation of heat shock proteins and other molecular chaperones as part of a robust cellular stress response (Paepe et al., 2012; Brinkmeier and Ohlendieck, 2014), (iii) reactive myofibrosis and loss of tissue elasticity due to accumulation of collagens, proteoglycans and matricellular components (Klingler et al., 2012; Holland et al., 2016; Ohlendieck and Swandulla, 2017) and progressive fibre degeneration (Duan et al., 2021; Flanigan, 2014) in X-linked muscular dystrophy, as summarized in Fig. 5. The collapse of the sarcolemmal dystrophin-glycoprotein complex appears to trigger the disintegration of plasmalemmal integrity, which causes an increased frequency of membrane micro-rupturing during ongoing excitation-contraction-relaxation cycles that are associated with considerable load bearing and membrane stretching. The fragile and leaky dystrophin-deficient membrane structure facilitates the passive release of muscular content and an abnormal influx of Ca^{2+}-ions (Mareedu et al., 2021), which impairs proper cellular signalling (Allen et al., 2016; Starosta and Konieczny, 2021) and renders myofibres more susceptible to enhanced proteolytic degradation (Alderton and Steinhardt, 2000; Mareedu et al., 2021). As a result of chronic myonecrosis, the innate immune system is activated and infiltrates the dystrophic muscle with macrophages and other immune cells causing an inflammatory phenotype (Tidball et al., 2018). In addition, reactive myofibrosis results in tissue scarring and the gradual loss of fibre elasticity (Ohlendieck and Swandulla, 2017).

Diagnostic value of novel proteomic markers of dystrophinopathy

The potential biomedical usefulness of new proteomic markers to evaluate disease progression in dystrophinopathy and monitor therapeutic efficiency was established for experimental exon-skipping therapy (Doran et al., 2009; Roberts et al., 2015). In general, novel therapeutic ways to treat the various aspects of Duchenne muscular dystrophy include new pharmacological agents (Yao et al., 2021), especially improved corticosteroids (Hoffman, 2020b), and modifying approaches that interfere on the genetic or cellular level, such as somatic genome editing, vector transfer, stop codon read-through therapy, stem cell therapy, myoblast transfer, utrophin substitution and exon-skipping therapy (Mackenzie et al., 2021; Sheikh and Yokota, 2021; Stephenson and Flanigan, 2021). Following treatment with anti-sense molecules, the proteomic and cell biological profiling of the dystrophic diaphragm revealed the partial restoration of dystrophin Dp427-M and its associated glycoprotein complex, as well as the rescue of key Ca^{2+}-regulatory proteins and muscle enzymes (Doran et al. 2009). This clearly showed that proteomic markers of both primary and secondary abnormalities in X-linked muscular dystrophy are suitable for therapy-monitoring at the level of muscle-associated alterations (Roberts et al., 2015).

A large number of proteomic studies of other skeletal muscle types have studied the detailed composition of the core dystrophin complex and its wider network (Turk et al., 2016; Murphy and Ohlendieck, 2017) and confirmed the complexity of proteomic changes in dystrophinopathy (Murphy et al., 2015, 2017; Roberts et al., 2015; van Westering et al., 2020; Goswami et al., 2021; Mournetas et al., 2021). The robustness, specificity and sensitivity of novel biomarker candidates is currently assessed for a variety of new proteins (Guiraud et al., 2017; Capitanio et al., 2020). Interesting applications of the proteomic SILAC method using stable isotope labelling by amino acids were reported by Duguez et al. (2013) and Rayavarapu et al. (2013) and revealed novel lysosomal-associated membrane protein LAMP1 and myosin light chain MLC1/3 associated proteins and disease-specific damage pathways in muscular dystrophy, respectively. Major proteomic changes include an altered abundance and/or isoform shifting of muscle-associated proteins involved in the contraction-relaxation cycle and sarcomere structure (myosin heavy chains, myosin light chains, myosin binding proteins, actins, troponins, tropomyosins, nebulin, titin, M-band proteins, Z-disk components), maintenance of the cytoskeletal network (dystrophin, tubulin, desmin, vimentin), the organization and stabilization of various layers of the extracellular matrix (collagens, proteoglycans, fibronectin, biglycan, dermatopontin, periostin, matrix metalloproteinases), energy metabolism and metabolite transportation (mitochondrial enzymes, glycolytic enzymes, fatty acid binding proteins, transporters), excitation-contraction coupling and calcium handling (calsequestrin, sarcalumenin, regucalcin, parvalbumin, Ca^{2+}-ATPases, Ca^{2+}-release channels, voltage-sensing Ca^{2+}-channels) and the cellular stress response (molecular chaperones, including a wide range of heat shock proteins such as

alphaB-crystallin/HspB5, cvHsp/HspB7, Hsp70/HspA and Hsp90/HspC). This is reflected by considerable concentration alterations of promising skeletal muscle markers in biofluids, such as serum, saliva and urine (Ayoglu et al., 2014; Hathout et al., 2014; Gargan et al., 2020). Changed levels include both previously established markers and novel marker candidates, such as carbonic anhydrase CA3, fatty acid binding protein FABP3, fibronectin, creatine kinase, haptoglobin, matrix metalloproteinases MMP9, titin fragments and a large number of skeletal muscle enzymes (Murphy et al., 2018b). Detailed tables summarizing the findings from proteomic studies of both dystrophic tissue specimens and biofluids have been presented in extensive reviews (Fuller et al., 2016; Hathout et al., 2016). Recently, several comprehensive articles have been published that display updated listings of novel biomarker candidates of dystrophinopathy (Carr et al., 2018; Dowling et al., 2019a; Hathout et al., 2019; Al-Khalili Szigyarto, 2020).

Conclusions and perspectives

Neuromuscular disorders are complex diseases that may severely affect whole-body physiology due to the high abundance of myofibres and organ crosstalk in the organism. The most frequently inherited muscle disease, Duchenne muscular dystrophy, is primarily characterized by distinct histopathological changes in voluntary

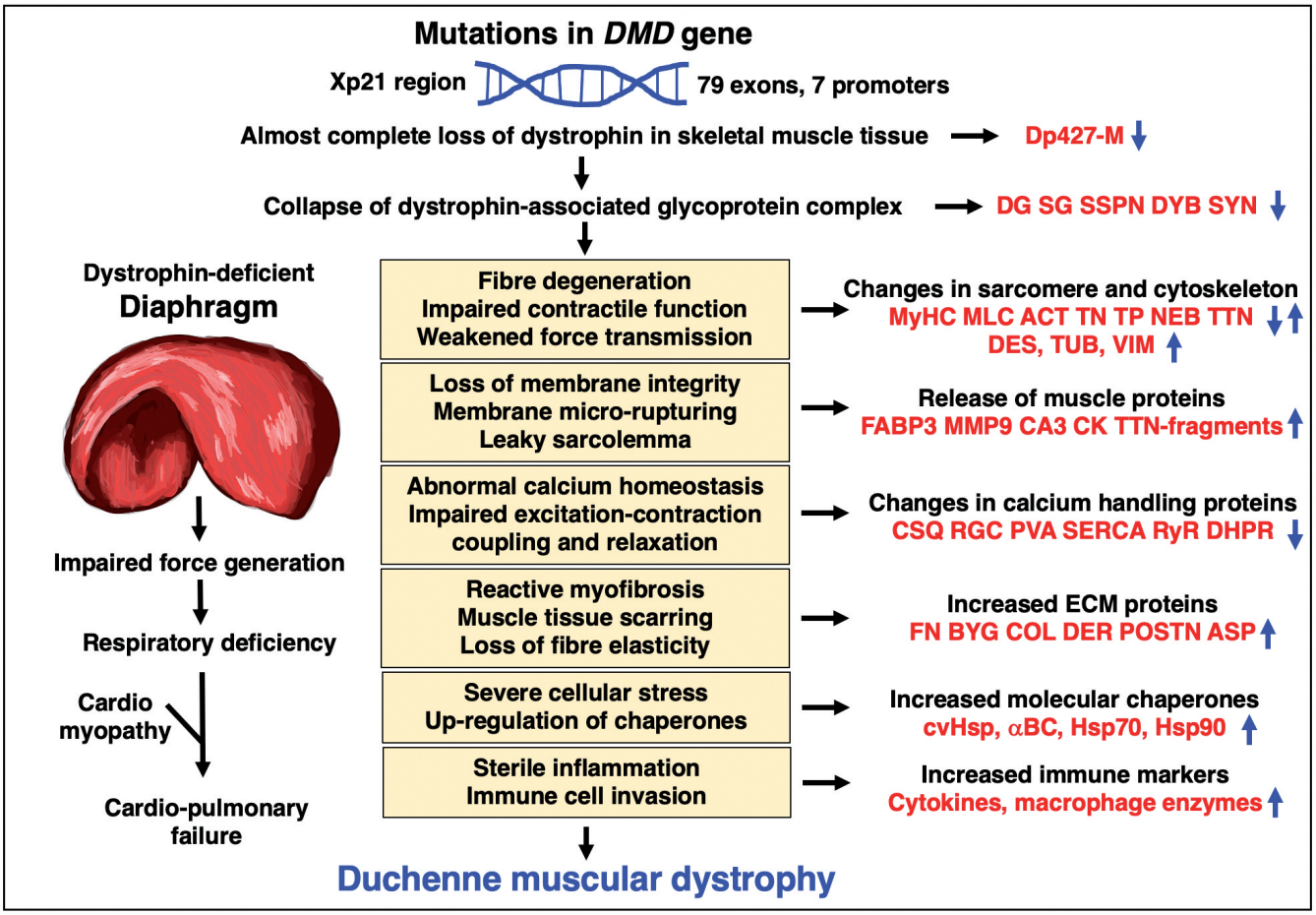

Fig. 5. Overview of the correlation between major molecular and cellular changes and proteomic markers of muscular dystrophy. Listed are major biochemical, physiological and cellular abnormalities found in dystrophic skeletal muscle tissue that are associated with the almost complete loss of the membrane cytoskeletal protein dystrophin and accompanying reduction in dystrophin-associated glycoproteins. On the right side of the diagram are shown select proteomic biomarker candidates of individual pathophysiological steps that were identified by mass spectrometry-based proteomics of the dystrophic and fibrotic diaphragm muscle. Abbreviations used: αBC, alphaB-crystallin; ACT, actin; ASP, asporin; BYG, biglycan; CA3, carbonic anhydrase 3; CK, creatine kinase; COL, collagen; CSQ, calsequestrin; cvHsp, cardiovascular heat shock protein; DER, dermatopontin; DES, desmin; DG, dystroglycan; DHPR, dihydropyridine receptor L-type Ca^{2+}-channel; Dp427-M, muscle-specific dystrophin isoform of 427 kDa; DYB, dystrobrevin; FABP3, fatty acid binding protein 3; FN, fibronectin; Hsp70, heat shock protein of 70 kDa; Hsp90, heat shock protein of 90 kDa; MLC, myosin light chain; MMP9, matrix metalloproteinase 9; MyHC, myosin heavy chain; NEB, nebulin; POSTN, periostin; PVA, parvalbumin; RGC, regucalcin; RyR, ryanodine receptor Ca^{2+}-release channel; SERCA, sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPase; SG, sarcoglycan; SSPN, sarcospan; SYN, syntrophin; TN, troponin; TP, tropomyosin; TTN, titin; TUB, tubulin; and VIM, vimentin.

contractile fibres. This includes a greater variety in fibre diameters as compared to normal muscles, as well as a more round fibre shape, central nucleation, myonecrosis, chronic inflammation, reactive myofibrosis and fat substitution. These types of pathological hallmarks can be routinely assessed by standard histological and histochemical staining techniques, as well as muscle imaging technology. In addition, subcellular changes can be determined with the help of immunofluorescence microscopy and electron microscopical methodologies. Following the establishment of mass spectrometry-based proteomics as a distinct subdiscipline of protein biochemistry over the last two decades, these cell biological analyses of muscular dystrophy could be extended to the detailed identification of proteome-wide alterations in dystrophinopathy.

Comparative proteomics was instrumental to establish distinct changes in the dystrophin-deficient skeletal muscle proteome at the level of the sarcolemma and associated membrane systems, the intracellular cytoskeletal networks, metabolic pathways, the contractile apparatus and the extracellular matrix. Ongoing research initiatives attempt to correlate proteomic changes to the distinct cell biological and histological alterations in dystrophic muscles in order to establish superior biomarker signatures of Duchenne muscular dystrophy. Novel proteomic markers of crucial histopathological hallmarks should ideally be highly useful to improve diagnostic procedures, as well as optimize the monitoring of disease progression and the clinical evaluation of new therapeutic approaches, such as stem cell or gene therapy. Especially the future application of microdissection and single fibre isolation approaches in combination with optimised protein separation techniques, advanced mass spectrometric methods and systems bioinformatic analysis promise an even deeper understanding of the complexity of dystrophic changes at the level of the skeletal muscle proteome.

If proteomic changes within the affected muscle tissues are properly reflected by biofluid-associated changes in muscle marker concentration, then new testing regimes of disease indicators can be developed that are minimally invasive or even completely non-invasive. Ideally, these kinds of diagnostic molecules should not be majorly influenced by modifying factors, such as age, gender, ethnicity, lifestyle, exercise, co-morbidities and/or circadian rhythms. In combination with genetic testing, muscle imaging and histological tissue biopsy procedures, these new proteomic biomarkers can then be utilized for repeat sampling procedures and optimum disease monitoring.

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