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Understanding the molecular consequences of inherited muscular dystrophies: advancements through proteomic experimentation

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1. Introduction

The muscular dystrophies (MDs) are a superfamily of heritable heterogeneous disorders that exhibit similar clinical and pathological features in those affected [1–6]. It is estimated that MDs affect as many as 1 in 6200 people worldwide and costs exceed $1 billion per year in the United States alone [5]. To date, there are upward of 50 discrete diseases, each of which is defined by a distinct genetic mutation and can be inherited as autosomal dominant, autosomal recessive, X-linked or, in rare cases, may develop sporadically [2,5]. Patients commonly present with progressive weakness in the appendicular, axial, and maxillofacial muscles but the age of onset, severity of disease, and concomitant complications vary dramatically between individuals [1,4–7]. The distribution of muscle weakness often promotes distinction between the particular types of disease [1]. As such, MDs have been categorized into various groups based upon clinical and molecular observations; these include but are not limited to Duchenne muscular dystrophy (DMD), myotonic dystrophy type 1 (DM1), facioscapulohumeral muscular dystrophy (FSHD), limb-girdle muscular dystrophy (LGMD), Emery–Dreifuss muscular dystrophy (EDMD), and collagen VI myopathies.

Recent advances in molecular genetics have promoted further understanding of the mechanisms governing the varied types of MDs. Studies have identified over 30 causative genes [1–3] that are involved in the pathogenesis of these diseases (DMD: 1 gene; FSHD: 2 genes; LGMD: 25 genes; EDMD: 6 genes; collagen VI: 3 genes); most of which appear to be protein coding. Localization studies of MD-related candidates indicate perturbations may occur in the skeletal muscle sarcolemma, nuclear membrane, extracellular matrix, intermediate filament network, and sarcomere [1,3,8]. Despite this, the functions, pathways, and downstream targets of these proteins remain to be elucidated. For efficacious therapeutic targeting of MDs, it is imperative that research focuses on the downstream networks of each particular mutation to assess where intervention may restore cellular homeostasis. Proteomic technologies are well equipped to examine such processes and various laboratories have begun utilizing these techniques for identification of biomarkers and novel remedial candidates in MDs.

In this review, we will outline the relative complexities of studying MDs and how these may be addressed by utilizing modern proteomic approaches. We aim to discuss the current knowledge concerning the most common MD – DMD and some of the less prevalent forms including DM1, FSHD, LGMD, EDMD, and collagen VI myopathies. Here, we summarize proteomic-derived advancements in our understanding of these conditions to date and, where possible and/or appropriate, highlight conserved downstream molecular perturbations which may prove

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useful as novel biomarkers for disease progression and future therapeutic investigations.

2. The dystrophies

2.1. DMD

DMD is the most common dystrophy and (to date) the most thoroughly investigated using proteomic methodologies. DMD is a recessive X-linked disease, characterized by muscle degeneration and premature death, typically by the age of 20–30 years. With an incidence of approximately 11–28/100,000 males, DMD is one of the most common and severe types of MD [8]. The cause of DMD is a mutation in the dystrophin gene, leading to an absence of the cytoskeletal protein, dystrophin [9], and subsequent weakening of the structural integrity of muscle cells. The majority of therapeutic approaches for DMD have focused on restoring dystrophin production by modulation of RNA using antisense oligonucleotides [10]. The development of alternative and/or complementary therapeutic strategies to target modifiers of DMD (reviewed by Vo and McNally, 2015 [11]) or the consequence of downstream pathology [12] appears to be emerging areas of research. For work in this area to progress, however, a detailed understanding of the molecules and pathways involved in DMD is required.

2.2. DM1

DM1 typically manifests in early adulthood and is classified as a multisystemic neuromuscular disease [5,13] It is the second most prevalent dystrophy, but most prevalent adult-onset MD affecting up to 1 in 8000 individuals worldwide [14]. The disease displays an autosomal dominant mode of inheritance and patients often present with highly heterogeneous symptoms including clinical myotonia, progressive muscular weakness, cardiac arrhythmia, visual disturbances, and insulin resistance [5,14]. These diverse phenotypes are caused by a large expansion of the (CTG) n trinucleotide repeat in the 3’ untranslated region of the dystrophia myotonica protein kinase gene on chromosome 19q13.3 [5,13,15]. Patients with substantial amplifications of these CTG repeats demonstrate more severe phenotypes and often present with symptoms at a much earlier age, promoting a diminished life span [14,15]. Broadly speaking, the clinical diversity of DM1 phenotypes appears to stem from the sequestration of mutant RNA transcripts encoded by the CTG expansion [13,15]. These transcripts accumulate within the nuclei of various tissues and promote perturbations in the pathways that regulate alternative splicing programs. Mis-splicing of numerous genes has been experimentally observed in DM1 patient tissues and mutant cell lines suggesting that patient phenotypes may be attributed to the aberrant expression of muscle-specific Cl− channels, cardiac troponin T, insulin receptors, and the sarcoplasmic Ca2+ ATPases [13,15–17]. Although alternative splicing appears to demonstrate some involvement within the development and pathogenesis of DM1, there remains a lack in understanding of how mechanistic pathways could be therapeutically targeted to ameliorate disease progression.

2.3. FSHD

FSHD is the third most prevalent of the MDs and is an autosomal dominant disease with variable penetrance [18,19]. Typically, as the name suggests, patients present with weakness in the maxillofacial muscles and shoulder girdle, which subsequently progresses to affect the pelvis and lower extremities [18–20]. The onset, progression, and severity of FSHD are highly variable between and within families and patients can range from asymptomatic to critical [19]. Unlike other MDs, FSHD usually emerges in adulthood with diagnosis typically occurring in the second or third decade [1,19,20]. However, there are reports of patients presenting with early FSHD symptoms in their 60s and 70s [18–20], highlighting the heterogeneity of the disease.

The clinical variability of FSHD likely stems from the mutation the patient is harboring. The most common form of the disease, FSHD1, is the result of a contraction of microsatellite repeats in the D4Z4 element located on the 4q35 subtelomeric region on chromosome 4 [6,18–21]. Patients typically present with 1–10 D4Z4 repeats whereas the general population demonstrate 11–100 [6,18–21]. Correlations between the repeat size and clinical severity of patients have been reported with those harboring 1–3 copies often more severe than individuals with 8–10 copies [18,19]. Evidence suggests that this reduction in D4Z4 copies induces chromatin remodeling promoting cellular toxicity and degeneration in skeletal muscle [20]. Although there are no obvious mutations in any protein-coding gene, it has been proposed that there may be erroneous activation of the DUX4, FRG1, FRG2, and ANT1 genes that are located centromeric of the D4Z4 array [18,20]. Little is currently known about the molecular cascades that are responsible for the clinical manifestation of FSHD due to the challenging nature of the disease; thus, the identification of therapeutic targets remains in its infancy. Systematic analyses utilizing ‘-omics’ data will be invaluable in the field in order to establish biomarkers of disease and efficacious treatments for FSHD patients.

2.4. LGMDs

LGMDs are a group of inherited diseases characterized by progressive weakness and wasting of shoulder and pelvic girdle muscles. Broadly, there are 2 subcategories of LGMD: those which display a dominant manner of inheritance, termed LGMD1 (upward of 8 subtypes), and those which are recessive in nature, LGMD2 (with approximately 20 subtypes) [22]. The overall frequency of LGMDs is 20–40/100,000 individuals [23] with clinical onset typically occurring during the second decade of life [22]. The most extensively studied subtypes of the disease include LGMD2A, LGMD2B, and LGMD1B and the molecular genetics underpinning these conditions are now beginning to be unraveled. Recent studies have suggested that LGMD2A may be caused by mutations in calpain-3 [24] which promotes the loss of autocalytic function within skeletal muscle, stimulating fiber degeneration and atrophy [25]. Although there are indications that calpain-3 is involved in the pathophysiology of LGMD2A, the function of
the protein is still to be established, providing complexities in experimental design and interpretation.

LGMD2B is also believed to be caused by mutations in a calcium-handling protein [26]. Patients presenting with LGMD2B demonstrate mutations in the dysferlin (DYSF) gene [27], which encodes a membrane-associated protein localized to the sarclemma. Dysferlin has been noted for its capacity to aid in membrane regeneration and impairments in its function appear to stimulate myonecrosis due to increased calcium influx in skeletal muscle. These pathological processes are thought to lead to the characteristic shoulder and pelvic girdle weakness [28] LGMD1B, like subtypes of EDMD, is caused by mutations in the lamin A/C (LMNA) gene [29,30]. Mutations in this gene result in a diverse range of phenotypes often with muscular and/or cardiac involvement; however, it is not clear how LMNA contributes to these clinical manifestations. Studying the molecular pathways involved downstream of LGMD mutations is especially challenging due to the heterogeneity of genetic mutations, complex clinical diagnosis, and availability of human samples.

2.5. EDMD

EDMD affects 1 in 100,000 males [31] and is characterized by scapulohumero-peroneal muscle weakness, joint contractures, and cardiac defects that include arrhythmias and dilated cardiomyopathy [32]. Onset of EDMD is typically seen during childhood or early adolescence [33] and is caused by mutations in various genes that are localized to the nuclear envelope [34]. Commonly, the disease is X-linked recessive and is associated with mutations in the emerin (EMD) gene, which consequently causes the truncation of emerin proteins (in around two-thirds of patients). However, multiple subtypes mediated by a range of genetic mutations in the autosomes also exist (for more information, see Pillers & Bergen [35]). As discussed in Section 2.4, a degree of homology exists between LGMD1B and autosomal dominant EDMD due to both subtypes demonstrating mutations in the LMNA gene. Although it is poorly understood how mutations in lamin A/C contribute to the LGMD phenotype, EDMD is believed to be caused by single amino acid substitutions that result in destabilization of the protein promoting nuclear fragility [36]. Less prevalent autosomal dominant forms of the disease have demonstrated loss of function mutations in the nesprin-1 (SYNE1 gene) and nesprin-2 (SYNE2 gene) proteins (OMIM no. 310300) fostering perturbations in nuclear architecture [37].

2.6. Collagen VI myopathies

Collagen VI is a ubiquitously expressed extracellular matrix protein (ECM) composed of threefolded chains that form dimers and tetramers. In muscle, the collagen VI network surrounds the basement membrane transferring mechanical and biochemical signals from the ECM to the fiber [38]. Mutations in any of these genes can cause dysfunction in the microfibrilar network in the ECM of muscle, skin, and tendons leading to muscle weakness, joint laxity, contractures, and respiratory compromise [39]. Dominant and recessive mutations in collagen VI are often associated with the COL6A1, COL6A2, and COL6S3 genes and lead to two types of MD: Ullrich congenital muscular dystrophy (UCMD) and Bethlem myopathy (BM) [40–42]. These diseases are relatively rare with an estimated prevalence of 0.1 in 100,000 and 0.5 in 100,000, respectively [43]. UCMD is an autosomal recessive disorder, typically presenting at birth, with infants demonstrating hypotonia and congenital hip dislocation. The majority of patients do not reach the major motor milestones and struggle to walk independently. Accompanying the motor symptoms is severe respiratory problems that require intervention during the first or second decade of life [44]. BM is phenotypically milder than UCMD with patients demonstrating a near normal life span [42,45]. Despite this, it is estimated that 50% of individuals require ambulatory assistance after the age of 50 due to the progressive deterioration of muscle and joint integrity [46]. For a comprehensive review, see Lampe and Bushby [43].

3. Unraveling downstream dystrophic cascades through proteomic investigations

As highlighted in Sections 2.1–2.6, there are numerous MD variants aside from the most well-known DMD, caused by a wide range of associated genetic mutations. There are currently no treatments that ameliorate the neuromuscular phenotype and molecular pathology of any of these diseases [2]. Although several clinical trials for novel therapeutics are in progress, there remains a lack of understanding of the basic molecular biology underpinning these diseases. To identify efficacious pharmacologic targets, it is imperative that the field utilizes modern ‘omic’ technologies to examine the pathways and processes that are perturbed and how these may regulate downstream pathology. This will facilitate a broader understanding of the molecular mechanisms governing muscle development, stability, and pathogenesis and will ultimately enable data-driven interventions that will benefit patients significantly.

As many as 50 discrete diseases fall under the umbrella of the dystrophies but at the time of writing this manuscript, there are only around 26 published proteomic investigations carried out on dystrophy patient or model systems. Of these, 19 studies are focused on the most prevalent and widely known DMD (Supplementary Table 1 [15,18,20,21,39,81–83]) whilst the remaining 8 surround the other less pervasive forms described above (DM1, FSHD, LGMD, EDMD, and collagen VI myopathies; Supplementary Table 3).

3.1. The search for differentially expressed proteins in DMD versus control tissues

To date, approximately 19 separate publications from several different research groups have utilized unbiased quantitative proteomics technologies to identify differentially expressed proteins in models of DMD compared to control subjects. All but one of these studies were conducted in vivo, with diaphragm, cardiac, and various skeletal muscles being the most popular tissue source. Whilst the vast majority of these studies have utilized the mdx mouse model of DMD, material from
DMD patients [47,48] as well as the spontaneous golden retriever muscular dystrophy model [49] have also been investigated (summarized in Supplementary Table 1 [47–49,54–56,71–74,91–98]). Though these experiments have generated a wealth of information, there are several potential issues that may hamper the translation of findings when the data sets are considered in isolation. For these reasons, the focus of discussion in the following section will be on proteins that were consistently changed in expression across three or more of the separate proteomic comparisons listed in Supplementary Table 1. This approach also limits the pool of usable studies. For example, although Ge et al. may have carried out the first of these proteomic studies in 2003, we are unable to include their results in our comparative analysis as the data sets are not freely available [50]. Review of these data sets revealed 34 proteins that met these criteria and are summarized in Supplementary Table 2 [49–50,54–56,71–74,92–95,97–100].

### 3.1.1. Increased expression of structural proteins in DMD
The type III intermediate filament proteins, desmin and vimentin, were consistently increased across 8 and 15 comparisons of DMD tissues, respectively. Considered as a hallmark of developing myotubes [51], the high expression of both proteins has previously been documented in regenerating muscle fibers from different neuromuscular diseases, including DMD [52,53]. Other structural proteins were also consistently increased in DMD tissue across multiple proteomic comparisons, including beta-tubulin, lamin A/C, lamin B1, and spectrin alpha chain, as well as proteins associated with protein assembly (e.g., elongation protein, protein disulfide-isomerase A3) (Supplementary Table 2). One possible explanation for this apparent structural reorganization is that it may represent an attempted compensatory response to stabilize the weakened cytoskeleton [54]. It is interesting to note that increased levels of desmin were also detected in a proteomics study of the mildly affected (and thus, non-regenerating) extracellular muscle (EOM) from the mdx mouse [55], lending support to this notion. The possibility, however, that the changes in structural proteins may merely depict the ongoing process of cellular degeneration and/or fibrosis must also be considered [56].

### 3.1.2. Cellular stress responses in DMD
Several proteins associated with a cell stress response were elevated in multiple proteomic studies of DMD tissue (Supplementary Table 2), including the heat shock proteins 90, 70, 71, and 78 kDa glucose protein (also known as heat shock 70 kDa protein 5). Thought to represent a molecular response to cell stress, the increased expression of heat shock proteins correlates well with their known involvement in dystrophin-deficient muscles [57].

Increased expression of oxidative stress markers including hemopexin and glutathione-S-transferase (GST) was detected across several different proteomic studies of DMD (Supplementary Table 2). Glutathione metabolism is clearly dysregulated in dystrophic muscle [58,59], but the cause and functional consequences of this are unclear. While one report has proposed a model in which altered glutathione metabolism represents an adaptive and attempted compensatory response to oxidative stress [58], others argue that the dysregulation of this pathway may actually be the cause of increased oxidative stress in DMD [59,60]. Reports of GST activity levels in DMD are also contradictory. While one report demonstrated a marked reduction of GST activity in muscle from DMD patients [59], a study of the chicken model of DMD demonstrated a reduction of activity [61].

Though the biochemical studies above detail the differential expression of several key players in the glutathione metabolism pathway, as well as the activity of GST, the actual protein expression levels of GST have not yet been verified at the biochemical level. Given that elevated levels of GST were detected in four separate proteomic studies of DMD (Supplementary Table 2), it would seem there is an opportunity to examine this further and to determine whether it is possible to alter the capacity of DMD cells to respond to oxidative stress by manipulating GST expression and activity.

### 3.1.3. Increased membrane permeability in DMD
Increased membrane permeability is a hallmark of DMD and though theories exist about what may cause this (reviewed by Allen and Whitehead [62]), including contraction-induced tears due to fragility of the already weakened membrane, oxidative damage to membranes, or altered regulation of calcium ion channels, the precise mechanisms remain elusive. An increased level of serum albumin was detected across 10 separate proteomic comparisons of DMD versus control tissues (Supplementary Table 2) and likely reflects the increased membrane permeability of the target tissue [56]. Indeed, damage-induced disruption of muscle fiber membranes is commonly associated with an influx of extracellular components, containing albumin, into the muscle [63] and has previously been detected at the histological level in DMD muscles too [64,65].

Parvalbumin, on the other hand, was reduced across eight separate proteomics studies and was one of only two proteins showing a consistent decrease across the multiple proteomic comparisons of DMD and control tissues (Supplementary Table 2). In contrast, a separate proteomics-based biomarker discovery project detected increased parvalbumin levels in mdx mouse sera [66], possibly indicating that the reduction of parvalbumin in DMD tissues may be a result of parvalbumin leaking out into the extracellular space rather than an intracellular-controlled mechanism. Reduced levels of parvalbumin in DMD muscle have also been reported previously from biochemical studies [67,68] and have been implicated in the ‘Ca$^{2+}$ overload theory,’ proposed as a leading mechanism of cellular degeneration in DMD (reviewed by Vallejo-Illarramendi et al. [69]). Potential consequences of Ca$^{2+}$ overload were also detected in multiple proteomic comparisons, including an increased expression of the Ca$^{2+}$-binding protein troponin C (three comparisons) and increased expression of the Ca$^{2+}$-effector proteins, annexin 2 (eight comparisons), and annexin 5 (six comparisons) (Supplementary Table 2). Does the influx of albumin, another Ca$^{2+}$-binding protein, also contribute to Ca$^{2+}$ overload mechanisms in DMD tissues?

### 3.1.4. Immune cells may contribute to the proteome of DMD tissues
A prominent feature of DMD muscle is the presence of an obvious immune response, though the functional consequences of this are still a matter of debate [70]. Several
types of immune cells have been shown to infiltrate mouse and human DMD muscle, including macrophages, eosinophils, natural killer T cells, CD4^+ T cells and CD8^+ T cells [70]. Whilst proteomics comparisons may have revealed insights into the molecular response to this influx (e.g. increased levels of leukocyte elastase inhibitor A (Supplementary Table 2)), it is important to consider that each of these immune cell types could potentially contribute a unique repertoire of proteins – quite different from the muscle itself – and would thus skew the proteomic profile of the sample. Western blot verification of differential protein expression from total protein extracts appears to have been common practice but few studies of this nature have also provided histological analysis of the same proteins, meaning that any changes in protein expression that are related to tissue heterogeneity would likely be unattributed.

### 3.1.5. Proteomic insights into the differential vulnerability of muscles in DMD

Of note is that there are a large number of proteins (50+) detected across the studies of the proteomics investigations (summarized in Supplementary Table 1) that showed contradictory patterns of expression in different comparisons (i.e. increased in expression in one or more proteomic comparisons but decreased in others). Examples of such proteins are GAPDH, various myosin chains, creatine kinase, glycogen phosphorylase, myoglobin, and adenylate kinase (also identified in [71]). As alluded to previously, changes in the levels of some of these proteins could be ‘false positives,’ arising from variations in disease models, tissue heterogeneity, or tissue sampling techniques. There is also the possibility, however, that some of these differences may be useful for determining which constitutive and/or adaptive molecular pathways contribute to the differential vulnerability of different muscles in DMD.

Two of the proteomics studies listed in Supplementary Table 1 specifically aimed to shed light on the molecular pathways that determine how vulnerable a particular muscle type is to an absence of dystrophin. A 2-dimensional difference in gel electrophoresis-based quantitative proteomics comparison of the mildly affected EOM from mdx and control mice revealed differential expression of just seven proteins [55]. The authors highlight how these results are a stark contrast to previous 2D-gel-based comparisons of the severely affected diaphragm muscle, where between 20 and 35 differentially expressed proteins were detected [72, 73]. This suggests that there is a minimal perturbation of molecular pathways in the EOM muscle and perhaps also implies that adaptive molecular pathways may not extend far beyond a straightforward upregulation of the dystrophin homolog, utrophin [55].

A later study from the same group – in which the proteome of the soleus (SOL), extensor digitorum longus (EDL), flexor digitorum brevis (FDB), and interosseus (INT) muscles from the mdx mouse was quantitatively compared with control mice – also found differences between the number of differentially expressed proteins across different muscle types [74] (see Supplementary Table 1). The histological analysis revealed a higher degree of hypertrophy and central nucleation (a hallmark of muscle fiber regeneration) in the SOL and EDL muscles compared to INT and FDB. This clearly does not consistently correlate with the degree of differential protein expression, however, since just 5 proteins were differentially expressed in the INT but 19 were differentially expressed in FDB (i.e. more than the number detected in the EDL muscle; Supplementary Table 1). Notwithstanding the potential limitations of 2D-gel-based quantitative proteomic comparisons, these findings serve to highlight the importance of considering results from multiple tissue types before drawing generalizable mechanistic conclusions about DMD.

### 3.1.6. Temporal proteomic studies of DMD

Several proteomic studies aimed to identify temporal changes during disease progression in DMD mouse models. These studies provide insights into the longer term secondary molecular changes that occur during disease progression in mdx mouse hindlimb muscle [71], cardiac muscle [75], tibialis anterior [76], and diaphragm [77].

Some potentially interesting differences were detected in aged hearts from mdx mice, including a reduction of lamin A/C, vimentin, and annexin [75], that were both undetected in the other aging studies and contrast with findings from various DMD versus control comparisons (Supplementary Table 2). Though the authors were unable to verify the reduction of lamin A/C and vimentin by Western blot, reduced levels of annexin were confirmed. In addition, while the expression level of the developmentally regulated protein, myosin light chain 2, was consistently increased in mdx mouse hindlimb muscle at 1, 3, and 6 months of age compared to age-matched controls [71], the levels in aged mdx mouse hearts appear reduced compared to controls [75]. It is clearly not possible to draw direct comparisons between the various studies because of differences in the age of the tissue being compared, but it would be interesting in the future to determine whether the differential expression of such candidates offers insights into mechanisms underlying the differential vulnerability of muscles in DMD.

### 3.2. Proteomic insights into other MDs

In recent years, several studies utilizing proteomics have appeared in the literature surrounding these less prevalent MDs with the aim of enhancing our understanding of the molecular mechanisms underlying the downstream effects of the causative mutations. The majority of these investigations have employed human patient muscle tissue for comparative characterization of protein expression versus controls, in an attempt to distinguish groups of dysregulated proteins in various MD subtypes. This has allowed the generation of lists of differentially expressed proteins, providing indications of the biological functions and pathways involved in the pathogenesis of various MDs. However, there is a requirement for larger numbers of well-executed studies to dissect cause/consequence relationships and determine which alterations may reflect conserved responses in the range of diseases.

The complexity of the genetic background of each disease as well as the limited availability of human donors provides challenges. Currently, there are only seven proteomic-based studies focusing on the diseases discussed in Section 2 above,
most of which have utilized 2D gels, followed by mass spectrometry (MS) analysis. Selected reports discussed here have attempted to discern the commonalities and differences in protein expression between genetic variants of particular MDs as well as between diseases using these techniques (see Supplementary Tables 3 and 4 for a summary).

3.2.1. DM1

There is only one readily available publication employing proteomic techniques in an attempt to identify the molecular cascades which are perturbed downstream of the causative genetic insult in DM1. Hernández-Hernández and colleagues [15] utilized a 2D-gel-based proteomic analysis on DM1 transgenic mice with 45 kb of human genomic DNA originally cloned from a patient with DM1 [78,79]. Here, they identify potential alterations in post-synapsin I (SYN1) translational modifications and elements of RAB3a and its downstream cascades. Various RAB alterations have been associated with other neurodegenerative conditions including retinopathies [80], suggesting the possibility of conserved mechanistic cascades across multiple apparently unrelated neurodegenerative conditions. However, this study is limited by its choice of controls, depth of coverage granted by the use of 2D gels and lacks clarity in terms of identification of sample type used for the experiments.

3.2.2. FSHD

At present, there are three proteomic studies attempting to address distinct molecular alterations that may be specifically associated with FSHD. An early study performed by Celegato et al. [18] utilized 2 dimensional electrophoresis (2-DE), high performance liquid chromatography–MS, and transcriptomic methods to characterize deltoideus muscle protein expression in groups of patients (aged 8–69 years) with varying D4Z4 repeat lengths. The group identified a common profile of proteins associated with FSHD, independent of repeat size, suggesting proteins associated with glycolysis, the tricarboxylic acid cycle, and protein synthesis (particularly, elongation factor Tu) are upregulated in patient versus control samples; conversely, detoxification and degradation proteins (SOD, PRDX2) and actin isoforms are downregulated in FSHD samples; conversely, detoxification and degradation proteins (SOD, PRDX2) and actin isoforms are downregulated in FSHD patients. Proteins involved in muscle differentiation also appeared to demonstrate differential expression between FSHD patient groups and controls: these included COP9, HSP27, alpha-crystallin B, phosphoglycerate mutase, creatine kinase, and myosin heavy-chain proteins. Upon further analysis, the study identified a conserved upstream regulator – MyoD, levels of which were shown to be consistently reduced in patients. The authors hypothesized that defects in MyoD signaling promoted the failure of regeneration of fast glycolytic muscle fibers after episodes of mechanical stress, leading to a progressive increase in slow oxidative fibers, promoting weakness and dystrophy in FSHD patient muscle.

In a similar study by Laoudj-Chenivesse et al. [21], 2-DE proteomics coupled with MS also identified alterations in the detoxification and oxidative stress machinery in FSHD patient muscle biopsies. The specimens were obtained from the deltoideus and quadriceps muscles and included a range of individuals (aged 17–66 years), all demonstrating various D4Z4 repeat lengths. Although there appear to be overlaps in the pathways detected between the Laoudj-Chenivesse et al. [21] study and the work performed by Celegato et al. [18], the directionality of the protein expression alterations contrasts. For instance, Celegato et al. [18] report the down-regulation of proteins associated with detoxification processes, whereas Laoudj-Chenivesse et al. [21] demonstrate a significant upregulation of these cascades (proteins include SOD1 and GST). Due to the reported upregulation of oxidative stress markers, the Laoudj-Chenivesse et al. [21] study focused on the potential impact of mitochondrial dysfunction on muscle fiber integrity that was hypothesized to be regulated by increased ANT1 expression – a gene neighboring the D4Z4 repeat locus.

The final study concerning FSHD has provided another perspective on the molecular pathogenesis of the disease. Tassin et al. [20] utilized patient-derived myoblasts (n = 2) and gel-free shotgun proteomics (2DLC–MS/MS) to characterize atrophic and disorganized FSHD myotubes versus control cells. In total, 336 proteins were quantified from the quadriceps-derived myoblasts with the study illustrating that myosin heavy and light chain (MYH8, MYH3, MYH7; MYL1, MYL6B) and caveolar proteins appeared dysregulated in primary FSHD cells. The authors highlighted caveolin-3 (CAV3) and its associated networks as potentially perturbed in FSHD promoting the reduction of muscle fiber integrity that was hypothesized to be regulated by increased ANT1 expression – a gene neighboring the D4Z4 repeat locus.

3.2.3. Molecular overlaps across multiple dystrophies

There is a requirement for further comparative studies in order to elucidate how these membrane micro-domains may play a role in pathogenesis. One such study by De La Torre et al. [81] similarly documented an impairment in myotube differentiation in LGMD2B patient muscle biopsies. This comparative investigation focused on differentially expressed proteins between LGMD2A, LGMD2B, FSHD, and control triceps and quadriceps muscle using 2-DE and MALDI–TOF MS. The authors provided details on 17 conserved proteins that appear altered in all the neuromuscular diseases characterized versus the control samples. These proteins displayed involvement in energy metabolism, the myofibril, and muscle development and repair, agreeing with the previously discussed manuscripts studying FSHD. Much like the Celegato et al. investigation [18], the group shows alterations in the muscle fiber distribution with a significant increase in slow-twitch fibers. These remodeling events appear to be occurring in numerous neuromuscular diseases and track with disease progression. The authors also elaborated to include proteins that demonstrated alterations specifically in LGMD2B patients. These 14 candidates exhibited similar functional categories to those 17 that were conserved through the neuromuscular diseases examined. Although in the De La Torre et al. study [81] these proteins appeared to demonstrate unique alterations in LGMD2B patients, upon further inspection of the literature, there are indications that several of these candidates have been discussed in a range of neuromuscular diseases including collagen VI.
myopathies (De Palma et al. [39]), FSHD (Celegato et al. [18]), and Duchenne MD (see Supplementary Table 4).

Considerable overlaps exist between investigations examining FSHD and LGMD subtypes, which may be due to the upstream regulators of disease or the conserved downstream processes of muscle degeneration. Magagnotti et al. [82] also noted similar pathways may be disrupted in EDMD with patients harboring mutations in the LMNA gene, namely LGMD1B. 2-DE proteomics, MALDI–TOF MS, and in silico analyses of patient fibroblasts suggested that proteins regulating cytoskeletal/structural organization were less abundant in individuals with a diagnosed laminopathy. Conversely, and in keeping with the Laoudji-Chenivesse et al. FSHD study [21], oxidative stress markers appeared enriched in patient cells versus controls. Despite Magagnotti et al. [82] utilizing groups of patients with general myopathies as an internal control to assess specific protein alterations in laminopathies, it is clear from examination of multiple published data sets that several of these candidates have been reported to be differentially expressed in other neuromuscular diseases (see Supplementary Table 4 [18,20-21,39,49,54-56,72-74,81-83,92-94,99]).

Suggestions of skeletal muscle remodeling in MDs are frequently referenced in the literature due to the dynamic alterations in proteins involved in myofibrillar architecture and cytoskeletal integrity (Celegato et al. [18], Tassin et al. [20], De La Torre et al. [81], Magagnotti et al. [82] – see Supplementary Tables 2 and 4). De Palma et al. [83] also proposed that in LGMD2B patients, there was a redistribution of muscle fiber type as proteins involved in oxidative phosphorylation were increased and those associated with anaerobic metabolism decreased versus control samples. In accordance with the alterations in expression of bioenergetic candidates, myosin light-chain isoforms were also differentially expressed in the LGMD2B patients’ quadriceps muscle, suggesting functional impairments in contractile velocity and force may be due to increased numbers of slow-twitch fibers.

A further study conducted by the same authors (De Palma et al. [39]) focused on the collagen VI myopathies: UCMD and BM (see Section 2.6). Human quadriceps muscle biopsies from BM (n = 8), UCMD (n = 4), and control (n = 2) patients revealed bioenergetics pathways were altered in both BM and UCMD individuals versus healthy samples. The downregulation of the hexosamine biosynthetic pathway (HBP) was highlighted as a key driver of BM and UCMD progression due to associations with protein homeostasis in the endoplasmic reticulum and unfolded protein response systems. Although this reduction in the HBP and glycosylation appeared conserved between the collagen VI MDs, the downstream biochemical alterations in UCMD and BM displayed unique properties, likely reflecting the differences in disease severity. In BM patient samples, the authors suggest that the muscle protein quality control system is sustained by metabolic adaptation. This allows the cells’ energy requirements to be met and the catastrophic consequences of the ER protein misfolding response to diminish. In contrast, UCMD patients demonstrate disruption in this pathway and the compensatory-layered mechanism, likely leading to lipotoxicity and cellular apoptosis. Interestingly, other MDs such as spinal muscular atrophy (SMA) demonstrate perturbations in proteostasis with ubiquitin homeostasis defects influencing neuromuscular pathology [84] (see Section 2.1).

3.2.4. In silico analysis suggests proteomic studies are highlighting downstream consequences of degenerative cascades

Although the appearance of common themes in the MD field may permit enhanced understanding of the molecular pathology of the various diseases, it may also be a great hindrance. The proteomic studies discussed examined groups of heterogeneous conditions caused by numerous discrete genetic mutations that all encode for different proteins, pathways, and processes (see Section 2). Thus, the emergence of these conserved changes in cytoskeletal and bioenergetic families throughout various neuromuscular disorders suggests that these alterations are likely a downstream consequence of causal upstream perturbations. Many alterations are likely an adaptive response to ongoing myofibrillar degeneration – a process occurring in all MDs discussed. In fact, with in silico analysis of the proteins identified in Supplementary Table 4, there are clear indications that a substantial number of these candidates may be involved in downstream degenerative cascades occurring in a wide range of tissues – notably myofibrils (Figure 1(a)). Alterations in expression of upward of 15 of these proteins have been associated with Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and motor neuron disease in published manuscripts (Figure 1(b)), illustrating likely late-stage consequences of cellular dysregulation. Thus, it is probable that these candidates do not represent viable therapeutic targets or biomarkers for MDs. Despite this, vimentin is consistently identified as upregulated throughout the different MD studies. Interestingly, vimentin also appears to lie upstream of the majority of the candidates identified by the investigations reviewed here (Figure 1(c)). These observations may warrant further analyses into what lies further upstream of vimentin and how this may be potentially promoting dysregulation selectively within the myofibrillar architecture. However, in order to successfully elucidate the upstream regulators of various MDs, there are several considerations that require attention before experimentation. Fortunately, the field is now in the position to successfully draw on lessons learned from other fields (such as SMA), where considered applications of proteomic techniques have yielded tangible gains (Figure 2).

4. Future directions in dystrophy research through proteomic investigations

The data sets reviewed in Section 3 provide novel and valuable insights into the molecular pathways that may be disrupted in MDs. The search for conserved and unique molecular alterations in MD variants has begun to shed light on the downstream pathways affected by these discrete mutations. Despite this, the current studies demonstrate some shortcomings in proteomic experimental design and data analysis that should be addressed.

4.1. Tissue selection and characterization

The investigations discussed in this review appear to focus on static stages of disease in a variety of animal model or pooled patient samples. This is a fundamental flaw whilst attempting to
elucidate biomarkers of disease progression because this approach does not account for the numerous variables that may have influenced the data acquired. Primarily, there appears to be an oversimplification of proteomic investigations, leading to the loss of potentially relevant information that may indicate how MDs are regulated. Commonly, the studies report two-way comparisons of pooled samples: disease versus control patients; however, the patient cohorts vary significantly. Within the MD pooled patient (and animal model) samples, authors include tissues from a variety of muscles, ages, clinical severities, and genetic mutations—promoting substantial heterogeneity and preventing the possibility of also running a variable-matched control sample. It is now well established that different tissues and even various anatomical regions of the same tissue sample (e.g. proximal versus distal) do not display uniformity in protein expression [85]. Thus, pooling or comparing numerous biopsies from a wide selection of skeletal muscles and cellular populations will only hamper the identification of proteins regulating pathogenesis.

In contrast to the majority of studies conducted on other MDs, the vast majority of proteomic investigations into DMD have utilized tissue from the mdx mouse and whilst two proteomics studies of DMD patients have been conducted, one utilized serum [47] and the other analyzed urine [48]. These sources can certainly be useful for identifying easily accessible biomarkers of disease as demonstrated by Coenen-Stass and colleagues' [86] innovative identification of peripherally accessible biomarkers which demonstrate response to therapeutic attempts in mdx mice. However, such peripherally accessible samples do not necessarily offer easily translatable mechanistic insights into disease pathology. For example, while one protein may appear elevated in the serum of DMD patients, its expression in the primary tissue (i.e. muscle) could be entirely the opposite. In order to determine that alterations in protein expression are due to the presence of disease, the same ages, sexes, and clinical severities of patients, as well as muscle, and, ideally, the same portion of muscle, must be utilized in the pooled MD and control samples. Furthermore, in order to understand disease mechanisms, it is imperative that protein expression in individuals without the condition is characterized so analogous alterations can be eliminated as pathogenic.

4.1.1. Characterization of protein expression profiles throughout disease progression

The degenerative process displays complex and dynamic spatiotemporal molecular profiles, which demonstrate variability.
throughout disease progression dependent upon the upstream genetic mutation. Fluctuations in protein expression throughout the disease course indicate tissue-specific cascades, with differing biochemical alterations often occurring in neighboring populations of cells [87]. These varying protein expression profiles often reflect the vulnerability status of particular cellular clusters that display an enhanced response to insult. In order to determine how alterations in protein expression may modulate cellular and tissue vulnerability, it is important to track candidate alterations through the time course of MDs – from early presymptomatic time points to end-stage disease. There is abundant evidence to suggest that alterations in causative upstream molecular cascades begin long before the onset of detectable pathology [84]. From our own studies, we have observed significant up/downregulation of numerous proteins during the early stages of disease but at later time points, protein expression is quite the contrary [87]. Therefore, focusing on the early stages of disease may provide an enhanced understanding of the molecular mechanisms governing muscle degeneration and offer a viable data source for the identification of novel drug targets. Additionally, a comparison of multiple disease variants (i.e. Duchenne vs. Becker-Kiener dystrophinopathy) with differing severity may also offer more tenable insights into potential upstream moderating and/or regulating molecular cascades.

4.1.2. Proteomic techniques

The availability of modern proteomic techniques is beginning to direct the field away from 2D gels. Tools such as label-free proteomics as well as labeled approaches including isobaric tags for relative and/or absolute quantitation (iTRAQ) or tandem mass tagging enable a more comprehensive characterization of the molecular alterations occurring throughout disease progression. Label-free techniques enable comparative analyses of multiple samples with low concentrations of protein extracts [87,88], which may be beneficial when working with precious resources such as human patient samples. These techniques enable analysis with as little as 3–5 µg of material for injection into an orbitrap. There are of course limitations with the existing tools such as limited dynamic range, compression of ratios calculated for tagged samples, and even something as basic as coverage of the proteome when compared to more established transcriptomics. Whilst transcriptomics may be ahead of proteomics in coverage and usability, it is protein and not RNA which are the ultimate effector molecules and the two do not necessarily correlate well [89]. Therefore, continued developments for the field of proteomics in software (such as Progenesis) that allow the processing and analysis of complex timecourse profiles and/or comparisons, facilitate improved methodologies in the MD field.

4.1.3. Data analysis

Filtering and refining of proteomic data is absolutely essential. There remains a requirement for laboratories to follow standardized criteria in order to provide more reliable and comparable analyses in publications. For example, posttranslational modifications (PTM) and distinct isoforms should be reported (if known), as they may be a source of contradictions
in the data shown. If the technique/software/database for the identification of protein used is not able to distinguish between protein isoform/PTM, strict filtering should be applied to avoid low-quality identifications. The re-reporting of published data sets without reanalysis has the potential to propagate erroneous conclusions throughout published literature within the field. Investigators should also utilize available software for in silico analyses. These tools enable unbiased comprehension of the pathways and processes that may be altered within the samples analyzed. It has become increasingly clear that discrepancies exist in the data reported by investigators and this affects the outputs from independent pathway analyses (see Supplementary Tables 2 and 4 and Figure 1).

5. Five-year perspective on advancements in MD research

The relatively recent advances in proteomic tools and techniques (as discussed in Section 4), coupled with the broad range of disparate mutations leading to multiple forms of dystrophy with varying prevalence, have (to date) severely impeded a coherent approach to the molecular characterization of the downstream molecular cascades regulating the vulnerability of distinct muscle populations and the progression of individual disease variants. We have outlined various experimental obstacles in Section 4, which require attention before conducting proteomic experiments (Figure 2).

There is clearly scope for future work in this area, using modern approaches such as iTRAQ or label-free MS, to quantitatively compare the proteome of MD muscles that show differential vulnerability. Indeed, recent publications examining other neurodegenerative conditions such as the childhood motor neuron disease SMA have taken a concerted proteomic molecular genetic approach to identify regulators pathways and therapeutic targets [84]. Here, the authors applied iTRAQ proteomics to vulnerable neuronal populations in a SMA murine model to identify differentially expressed proteins. Selected candidates were examined for their ability to regulate neuronal stability in multiple small animal model systems including Drosophila and zebrafish before scaling back up to murine systems providing a complete rescue of the neuromuscular system. As a result, the authors have published the most effective non-survival motor neuron replacement therapy to date. The samples and models exist within the dystrophy field to allow the replication of such proven target-rich workflows to be implemented to inform novel (non-replacement-based) therapeutic interventions for the dystrophies.

6. Expert commentary

Current proteomic studies regarding inherited MDs are unraveling common-specific disrupted pathways in terms of the molecular constituents altered, but these candidates are not necessarily altered in a consistent manner due to the nature of the investigations/experimental design employed. Though they provide a window for a better understanding of the process of degeneration, some issues should be addressed in future work. The development of novel proteomics, such as label-free techniques, facilitates a more complex experimental design where factors such as tissue-specific vulnerability and disease stages may be taken into account. This will allow researchers to distinguish between early and late responses of the specific mutations causing MDs and a more accurate mapping of the dynamic processes taking place in the muscle. Moreover, the production of animal and cellular models that faithfully recapitulate the disease phenotype seen in patients will also help for a more comprehensive characterization of the molecular changes taking place throughout disease progression than can later be correlated to human disease. As there are currently no effective therapeutics for the dystrophies, the field is reminiscent of where the SMA field was 10 years ago, i.e. gene replacement therapy is on the extreme horizon, but the tools and techniques are available to make some tangible headway into our understanding of the disease processes underpinning the condition leading to the identification of novel potential non-gene replacement therapeutics along the way [84,87].

Key issues

- Proteomics is a powerful tool for the identification of biomarkers and therapeutic targets.
- Investigators must endeavour to utilise strict and standardised methodologies for comparison of control and disease tissues.
- If identified candidates are enzymes or have a role in metabolic processes, ex vivo biochemical or in vivo reporter assays (in model organisms i.e. Drosophila) should be performed to determine if detection of altered abundance correlates with altered activity/function.
- Studies should utilise western blotting and immunohistochemical analysis, as well as multiple model organisms for validation of candidate relevance to human physiological alterations and to assess their ability to moderate disease processes in vivo.
- Such candidates should be assessed for their ability to moderate disease processes in vivo and in multiple organisms (i.e. Drosophila/Zebrafish/Rodents) in order to confirm relevance in a species/model independent manner.

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