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Proteomic identification of muscle-associated biomarkers of amyotrophic lateral sclerosis using the wobbler mouse model of primary motor neuronopathy

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ABSTRACT

Motor neuron disease is a major group of inherited or spontaneous disorders that are associated with muscular atrophy. Recently, muscle preparations from the genetic wobbler mouse model of primary motor neuronopathy have been analyzed by mass spectrometry-based proteomics. The progressive degeneration of individual motor neurons was shown to cause complex alterations in the concentration or isoform expression pattern of muscle proteins involved in the excitation-contraction-relaxation cycle, the cytoskeleton, ion handling, cellular signaling, the stress response and energy metabolism. In this article, we compare the panel of potential new muscle-associated biomarkers that have been obtained by two different, but complementary, bioanalytical approaches, i.e. label-free mass spectrometric analysis versus fluorescence two-dimensional difference-in gel electrophoresis. The complex disease-associated changes in the muscle proteome are considerably different to the more unilateral skeletal muscle transitions observed in experimentally denervated fibers or disuse-related muscular atrophy. The apparent subtype-specific vulnerability of neuromuscular synapses and compensatory mechanisms of fiber type shifting in motor neuron disease is discussed, and contrasted to other forms of muscular atrophy.

Keywords: Amyotrophic lateral sclerosis; Biomarker discovery; Motor neuron disease; Muscular atrophy; Wobbler mouse.

Abbreviations

ALS: amyotrophic lateral sclerosis; **DIGE:** difference in-gel electrophoresis; **WR**: wobbler.

1. Introduction

Mass spectrometry-based proteomics plays a central role in biomarker discovery [1]. This includes the systematic screening of both patient samples and animal models of human diseases. Ideally diagnostic biomarkers are released or leaked from pathological tissues [2] and are therefore found at a sufficient high concentration in body fluids for convenient sampling by non-invasive or minimally invasive methods [3]. However, internal tissue-associated proteins also present a crucial source for general biomarker discovery [4], including protein markers of neuromuscular disorders [5]. This important aspect of comparative tissue proteomics as related to biomarker discovery is covered in this article, which focuses on muscle-associated proteins and the proteomic discovery of novel potential markers of muscular atrophy in primary motor neuronopathy. Over the last decade, muscle proteomics has been widely applied to studying myogenesis, fibre adaptations, neuromuscular pathology and the natural aging process of contractile tissues [6-8], using both total muscle protein extracts and distinct subcellular fractions [9]. Global proteomic data supports the idea that skeletal muscle fibres are highly plastic cellular structures that can quickly adapt to changed functional demands [10]. However, if the neuromuscular system is not properly loaded due to prolonged inactivity, immobilization, lack of gravity or denervation, a relatively

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rapid process of muscular atrophy occurs [11-13]. Atrophying muscle fibres are also observed as a consequence of sarcopenia of old age and certain neuromuscular pathologies. The heterogeneous group of motor neuron diseases is a neurodegenerative disorder, which is characterized by a specific form of muscular atrophy and progressive paralysis [14]. In this review, we compare the findings from two recent studies that have analyzed the genetic wobbler (WR) mouse model of motor neuron disease by label-free mass spectrometry [15] versus fluorescence two-dimensional difference in-gel electrophoresis [16]. The systematic identification of muscular atrophy-related biomarker candidates by these two different large-scale separation and bioanalytical approaches is discussed with respect to sensitivity and coverage of proteins with differing physicochemical properties.

2. Skeletal muscle diversity

Since the highly complex isoform expression pattern of the constituents of the actomyosin apparatus are severely altered during episodes of muscular atrophy, the main regulatory and contractile proteins are briefly introduced. The biophysical and metabolic diversity of contractile tissues, as well as the physiological adaptability of whole skeletal muscles to changed functional demands, is based on an extensive variability in contractile proteins and bioenergetic enzyme isoforms [17, 18]. In human muscles, the main fiber types can be categorized as type I (slow), type IIA (fast) and type IID/X (fast), as well as a large number of hybrid fibres. Distinct mixtures of fibre types provide the contractile basis of the varying physiological demands of individual skeletal muscles [19]. The distribution of fiber type-specific myosins and metabolic enzymes is characteristic of fast-twitching fibers with glycolytic metabolism, fast-twitching fibers with an oxidative-glycolytic bioenergetics and slow-twitching fibers with predominantly oxidative metabolism. The contractile apparatus contains a variety of contractile and regulatory protein families with numerous isoforms, which have been extensively studied by proteomics [20]. Actomyosin-associated proteins can be classified by their attachment to the thick filaments located in the A-band and the thin filaments in the I-band and overlapping A-band region of sarcomeres [21]. Myosin heavy chains, which head structures are involved in the molecular coupling between myosin filaments and actin via cross-bridge/swinging-lever-arm mechanisms, represent the principal molecules of the thick filament [22-24]. Besides these main motor molecules, the regulatory and essential myosin light chains are engaged in the movement of phosphorylated myosin cross-bridges away from thick filament structures and the fine tuning of myosin motor function [25]. The actin filament-associated tropomyosin molecule plays a key inhibitory role in the regulation of actomyosin interactions [26] and the troponin complex provides the Ca^{2+}-dependent regulatory adjustment of the contractile status [27]. The troponin TnC subunit represents the central Ca^{2+}-sensor of the contractile apparatus and regulates actomyosin coupling, while the troponin TnT subunit is essential for the linkage between the inhibitory tropomyosin molecule and the troponin complex, and the troponin TnI subunit provides binding between the troponin complex and actin thereby mediating inhibition of actomyosin ATPase activity [28]. Besides myosins, actins, troponins and tropomyosins, a very large number of auxiliary proteins are present in the sarcomere structure [20], including the important class of myosin binding proteins [29]. Myosin-binding protein MBP-C is located to the thick myosin filaments and was shown to play a role in the maintenance and continuous stabilization of myosin-containing filaments [30]. Its additional function involves the modulation of cross-bridge formation between myosin and actin molecules within the complex filamentous system of contractile fibres [31], as demonstrated by electron tomography of the physical mechanism that modulates the relative sliding between thick and thin filaments [32]. Besides the isoform specific distribution of contractile proteins, the distribution of metabolic enzymes is strikingly different between predominantly slow versus fast-twitching muscles [33]. During muscle adaptations or pathological changes in the neuromuscular system, the density and/or isoform expression patterns of proteins associated with the glycolytic pathway [34] and oxidative metabolism in mitochondria [35] are majorly altered. These changes in bioenergetic enzyme profiles can be conveniently assayed by mass spectrometry-based proteomics and used to determine compensatory or disease-related shifts between anaerobic and aerobic muscle metabolism.

3. Motor neuron disease

3.1. *Motor neuron disease*

The heterogeneous group of neurodegenerative syndromes that encapsulates motor neuron diseases is associated with progressive paralysis and includes spastic paraplegia, spinobulbar muscular atrophy, hereditary spastic paralysis, primary lateral sclerosis and amyotrophic lateral sclerosis (ALS) [14]. Figure 1 gives an overview of muscle fibre type shifting due to physiological adaptations or pathological insults, including motor neuron disease and its association with muscular atrophy [36]. In general, enhanced neuromuscular activity results in fast-to-slow transitions, while neuromuscular unloading is usually characterized by slow-to-fast transformation processes. Prolonged muscle disuse, the lack of gravity, immobilization or prolonged bed rest triggers the establishment of a faster muscle phenotype. Nerve crush, experimental denervation or the effect of natural muscle aging involves progressive muscular atrophy. However, motor neuron disease was shown not to be linked to a clear slow-to-fast transformation process, but is characterized by more complex proteome-wide changes as discussed below in detail. ALS represents the most common form of motor neuron disease [37]. The adult-onset loss of lower and upper motor neurons causes a highly progressive form of paralysis, which

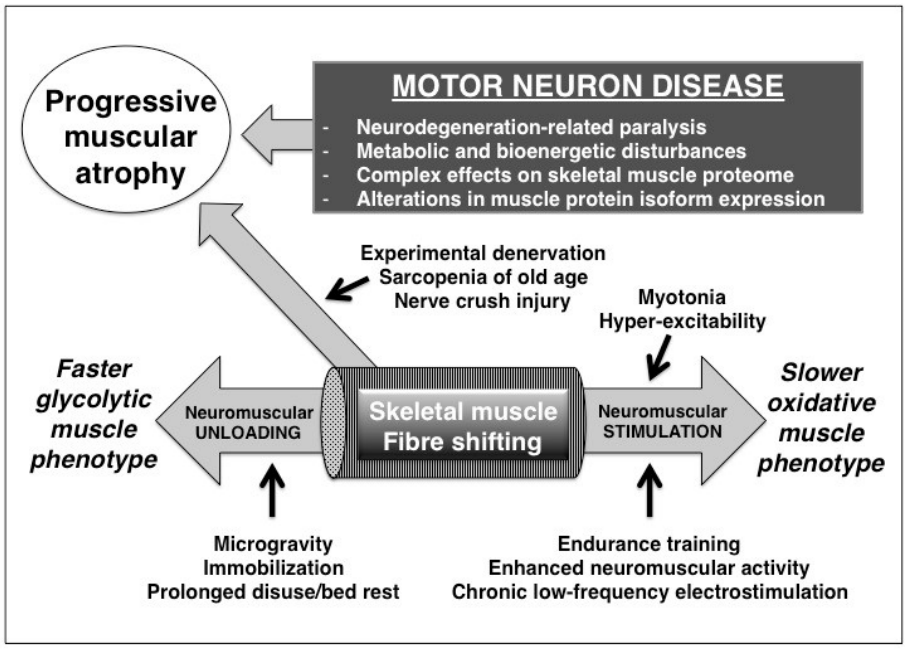

Figure 1. *Overview of skeletal muscle plasticity and fibre transitions due to physiological adaptations or pathological insults to the neuromuscular system.* Motor neuron disease is characterized by progressive muscular atrophy and complex changes in the skeletal muscle proteome.

can be sporadic or of genetic origin [38]. Neurodegeneration -related muscular atrophy results in debilitating limb and bulbar muscle weakness, as well as respiratory insufficiency [39]. A very large number of mutated genes were shown to be involved in familial or sporadic forms of ALS [40-43], including SOD1, Alsin, SETX, SPG11, FUS/TLS, VAPB, ANG, TARDBP, FIG4, OPTN, VCP, UBQLN2, SigMAR1, PFN1, ERBB4, C9orf72, CHMP2B, DAO, DCTN1, SQSTM1, hnRNPA1, Erlin2, UNC13A, NEFH, PRPH, TAF15, GRN, EWSR1 and ATXN2 [44]. Although most transcriptomic analyses of motor neuron disease have focused on spinal cord preparations and shown changed patterns of lipid metabolism, inflammation, cell adhesion and the immune response [45-47], recently alterations in early gene expression were analysed by microarray screening of the *gastrocnemius* muscle from the SOD1 mouse model of ALS [48]. Motor neuron disease appears to be a multisystem disorder with a defective muscle metabolism, which is linked to differential gene activation levels in epithelial-mesenchymal transitions and the Wnt/PI3-K signaling pathways in pre-symptomatic skeletal muscle. The inhibition of cell death, the promotion of cell proliferation and the repair of atrophying fibres seems to be impaired in the *gastrocnemius* muscle of SOD1-G93A transgenic mice. Importantly, neuromuscular impairments seem to precede motor neuron death at pre-symptomatic periods of motor neuron disease [48]. This agrees with the gene expression analysis of skeletal muscle biopsies from ALS patients that demonstrated severe alterations in mRNA levels of major muscle proteins. Drastically lower mRNA levels were described for the fast isoform of myosin binding protein MPB-C and actinin alpha-3, while the mRNAs encoding collagen, actin, myosin-8 and annexin were shown to be elevated [49].

3.2. *Wobbler mouse model of motor neuron disease*

Since comprehensive biochemical studies and subcellular fractionation procedures usually require relatively large amounts of starting material and because patient biopsy samples are scarce and show considerable inter-individual variability, genetic animal models of human diseases are often used to accumulate sufficient tissue for detailed proteomic analyses [50]. In the case of ALS research, various rodent models of motor neuron disease are used for routine pathobiochemical studies and to evaluate new pharmacological approaches [51]. One of the most established mouse models of hereditary motor neuron disease with progressive denervation is the wobbler mutant (genotype *wr/wr*, phenotype WR) [52]. A recent review by Moser et al. [53] has focused on the research progress made utilizing this animal model of ALS for studying the molecular pathogenesis of motor neuron disease. The underlying genetic abnormality in the WR mouse has been shown to be a missense mutation in the ubiquitously expressed gene *Vps54* [54]. The protein product of the affected gene is the vesicular protein sorting factor VPS54 and the mutation causes a leucine-to-glutamine replacement (L967Q) within the C-terminal domain of VPS54. The hydrophobic-to-hydrophilic amino acid exchange in the primary sequence of this crucial subunit of the hetero-trimeric Golgi-associated retrograde protein (GARP) complex results in the destabilization of its tertiary protein structure causing a reduction in the VPS54 protein

[55, 56]. Despite the fact that the WR mutation has not yet been identified in humans suffering from motor neuron disease [57], the progressive WR mouse pathology resembles many aspects of ALS [53]. The progressive neurodegenerative and neuroinflammatory processes in the WR mouse are associated with astrocyte overgrowth in the brain stem and spinal cord, hippocampal hyper-excitability, a reduced number of interneurons and muscular atrophy [58-60]. Various experimental drug treatments have been tested with the WR mouse [61-63], emphasizing the international acceptance of this ALS model mimicking the major pathophysiological and histopathological aspects of motor neuron disease [53].

4. Proteomics of muscular atrophy and motor neuron disease

Large-scale screening studies at the level of the genome, transcriptome, proteome and metabolome have been initiated to determine global changes in patient biopsy specimens and animal models of motor neuron disease [64, 65]. Besides studies on spinal muscular atrophy [66, 67], a variety of proteomic investigations have focused on ALS biomarker discovery and the pathobiochemical consequences of progressive neurodegeneration on biofluid proteomes [68, 69]. Proteome-wide profiling studies have included the comprehensive analysis of cerebrospinal fluid, cervical spinal cord specimens, lumbar spinal cord preparations and skeletal muscles [70-75]. The WR mouse model of ALS has been studied by proteomics, focusing on abnormal protein expression in skeletal muscle [15, 16] and defective spermatogenesis [76].

4.1. *Proteomic profiling of muscular atrophy*

Muscular atrophy is associated with a variety of changes in neuromuscular activity levels, such as immobilization, zero gravity, extended periods of bed rest, prolonged muscular disuse, natural aging and traumatic denervation. Skeletal muscle atrophy has a profound effect on muscle fibre type distribution with an overall tendency of a slow-to-fast transition process [13, 77]. In contrast, endurance exercise, hyperexcitability or chronic low-frequency stimulation of muscle triggers the opposite changes in fibre type specification, i.e. fast-to-slow muscle transformation, as clearly confirmed by proteomics [7, 78-80]. The fact that different loading of the neuromuscular system is reflected by distinct changes in the isoform expression pattern of contractile proteins has long been established by gel electrophoretic methodology [81] and the biochemical complexity of this process has more recently been confirmed by proteomics [20].

Proteome-wide changes during different degrees of muscular unloading were evaluated by a variety of experimental systems, including hind limb suspension, joint fixation-induced immobilization, long-term bed rest or complete denervation via sciatic nerve transection [82-96]. The general tendency of atrophying muscle to undergo slow-to-fast transitions was confirmed by changes in the abundance of a variety of metabolic enzymes, such as lactate dehydrogenase, enolase, triosephosphate isomerase and isocitrate dehydrogenase, which was accompanied by considerable alterations in contractile, structural and stress proteins [82-96]. Alterations in contractile proteins included specific isoforms of myosin heavy chains, myosin light chains, troponins and tropomyosin [89, 92, 94, 95]. Although individual studies have shown that the degree of muscular atrophy differs considerably following denervation, immobilization or prolonged disuse [85-88] and that transient alterations are initially associated with a massive decrease in contractile proteins [82-84], at a more advanced stage of fibre atrophy a metabolic oxidative-to-glycolytic shift is observed [89, 94-96]. Table 1 lists major changes in key protein families due to muscular atrophy following denervation, immobilization or prolonged disuse.

4.2. *Gel electrophoresis-based analysis of atrophying muscle in motor neuron disease*

During the early stages of establishing proteomics as a new discipline within the field of protein biochemistry, gel electrophoretic methodology has been widely employed for the efficient separating of complex protein mixtures prior to in-gel trypsinisation for the swift identification of proteins of interest [97-99]. Although the application of gel-free methods using advanced liquid chromatography coupled with tandem mass spectrometry is now extensively used in proteomic screening studies [100], fluorescence two-dimensional in-gel electrophoresis (DIGE) is still widely employed for analyzing urea-soluble muscle protein populations [101]. In muscle proteomics, a considerable proportion of proteins can be separated by routine high-resolution two-dimensional gel electrophoresis, including myosins, actins, troponins, tropomyosins and various auxiliary proteins of the contractile apparatus, as well as mitochondrial proteins, glycolytic enzymes, molecular chaperones and cytoskeletal proteins [102]. Since two-dimensional gels usually do not represent integral membrane proteins and high-molecular-mass proteins sufficiently, large one-dimensional gradient gels can be used in a complementary way to separate very large proteins prior to on-membrane digestion and subsequent mass spectrometric identification [103-105]. The flowchart in Figure 2 outlines the usage of fluorescence 2D-DIGE versus label-free LC-MS/MS analysis for the screening of pathological muscle specimens. The routine verification of novel biomarker candidates of motor neuron disease by immunoblot analysis is shown, illustrating an equal distribution of the extracellular matrix protein laminin as a loading control, the drastic decrease of the fast MBP-C isoform of myosin binding protein and increase of mitochondrial prohibitin, as previously shown by proteomics of WR muscle [15, 16].

2D-DIGE using fluorescent Cy2, Cy3 or Cy5 dyes has been originally developed by Minden et al. [106-108] and is now an established and reliable biomarker discovery method in

Proteomic study	Methods	Changed proteins	References
Analysis of total extracts from denervated rat *soleus* muscle	2D-GE, CBB, MALDI-ToF MS	Complex transient alterations during post-denervation days 1 to 10 with initial increases in AS, ENO, CA3 and fast MLC and decreases in FABP, HSP20, TnT and slow MLC	Isfort et al. [82]
Analysis of total extracts from rat *soleus* muscle following hindlimb suspension	2D-GE, CBB, MALDI-ToF MS	Transient increases of AS, ENO and CA3, as well as decreased levels of HSP20 and slow MHC	Isfort et al. [83]
Analysis of total extracts from rat *soleus* and *tibialis anterior* muscle following hindlimb immobilization by the pin-heel method	2D-GE, CBB, MALDI-ToF MS, LC-MS/MS	Complex transient alterations during post-immobilization days 1 to 10, including AS, CA3, PGM, DES, MLC, HSP60, αBC, HSP20	Isfort et al. [84]
Subproteomic analysis of cytosolic fraction from mouse *tibialis anterior* muscle following hindlimb immobilization	ICAT labeling and MS/MS analysis	Increased levels of various GLY enzymes, CA3, ICDH, HSPs and decreases in ALD, ACO, GT, MLC	Toigo et al. [85]
Analysis of total extracts from denervated rat laryngeal muscle	2D-GE, CBB, MALDI-ToF MS	Increases in CK, MLC1, MLC2, MHCIIB, TUB and PVA, as well as decreases in LDH, ENO, various OXPHOS enzymes	Li et al. [86]
Analysis of total extracts from denervated rat *gastrocnemius* muscle	2D-GE, CBB, MALDI-ToF MS	Transient changes with initial increases in ENO, αBC and HSP20, and decreases in CK, TM, ACT, MHCIIx and MHCIIB	Sun et al. [87]
Analysis of total extracts from rat *soleus* muscle following hindlimb suspension	2D-GE, CBB, MALDI-ToF MS	Muscle unloading caused a decrease in TM, ACT, MLC1, αBC and HSP20, and increases in CK, GLY and OXPHOS enzymes	Seo et al. [88]
Analysis of total extracts from rat *soleus* muscle following hindlimb suspension	2D-DIGE, MALDI-ToF MS, IB	Tail suspension triggered increases in various GLY enzymes, MYO, CA3 and ALB, and decreases in OXPHOS enzymes, ACT, TnT and a variety of MHCs and MLCs	Moriggi et al. [89]
Subproteomic analysis of rat *soleus* muscle following hindlimb immobilization	1D-GE, MALDI-ToF MS, IB	Focused study revealed preferential oxidization of CA3 and four-and-a-half LIM protein during muscle unloading	Chen et al. [90]
Analysis of proteolysis in rat *gastrocnemius* muscle following hindlimb suspension	2D-GE, CBB, MALDI-ToF/ToF MS, IB	One week of muscle unloading was associated with coordinated and time-dependent activation of proteolysis of contractile proteins; increase in GLY enzymes	Ferreira et al. [91]
Subproteomic analysis of sarcoplasmic and myofibrillar fractions from denervated rat *soleus* muscle	2D-GE, Flamingo gel stain, MALDI-ToF MS, IB	Denervation resulted in a decrease in slow MLC1 and an increase in fast MLC1; differential effects on HSPs, GLY and OXPHOS enzymes	Sato et al. [92]
Subproteomic analysis of membrane proteins from rat *soleus* muscle following hindlimb unloading	Native Blue 2D-GE, silver staining, MALDI -ToF MS, IB	Complex changes including an increase in AS and the AQP4 water channel, as well as differential effects on dystrophin-glycoprotein complex	Basco et al. [93]
Analysis of total extracts from human *vastus lateralis* and *soleus* muscle following long-term bed rest	2D-DIGE, MALDI-ToF MS, LC-MS/MS, IB	Long-term disuse caused increased MHCI, GT, various HSPs, MYO and GLY enzymes, and decreased MHCIIA, ACT and OXPHOS enzymes	Moriggi et al. [94]
Analysis of total extracts from denervated rat *tibialis anterior* muscle	iTRAQ labeling, LC-MS/MS, IB	Labeling analysis revealed large numbers of differential changes in metabolic enzymes, molecular chaperones and contractile proteins, including a decrease in many MHCs, MLCs, TM, TUB and OXPHOS enzymes, and increases in HSP70, HSP90, αBC and GT	Sun et al. [95]
Analysis of total extracts from denervated rat *tibialis anterior* muscle	iTRAQ labeling, LC-MS/MS, IB	Switch of α-isoform and β-isoform of ENO during slow-to-fast transitions, as well as differential changes of OXPHOS enzymes	Sun et al. [96]

Table 1. *Proteomic profiling of muscular atrophy*. Listed are major studies that have used mass spectrometry-based proteomics to investigate muscular atrophy due to immobilization, denervation or prolonged disuse.

Abbreviations used: αBC, alphaB-crystallin; ACO, aconitase; ACT, actin; ALB, albumin; ALD, aldolase; AS, ATP synthase; CBB, Coomassie Brilliant Blue; CK, creatine kinase; DES, desmin; DIGE, difference in-gel electrophoresis; GE, gel electrophoresis; GLY, glycolytic enzymes; GT, Glutathione transferase; ENO, enolase; FABP, fatty acid binding protein; HSP, heat shock protein; ICDH, isocitrate dehydrogenase; iTRAQ, isobaric tags for relative and absolute quantification; LC, liquid chromatography; ICAT, isotope-coded affinity tag; LDH, lactate dehydrogenase; MALDI, matrix assisted laser desorption ionization; MHC, myosin heavy chain; MLC, myosin light chain; MS, mass spectrometry; MYO, myoglobin; OXPHOS, enzymes of oxidative phosphorylation; PGM, phophoglucomutase; PVA, parvalbumin; TnC, TnI, TnT; troponin subunits; TM, tropomyosin; ToF, time of flight; TUB, tubulin.

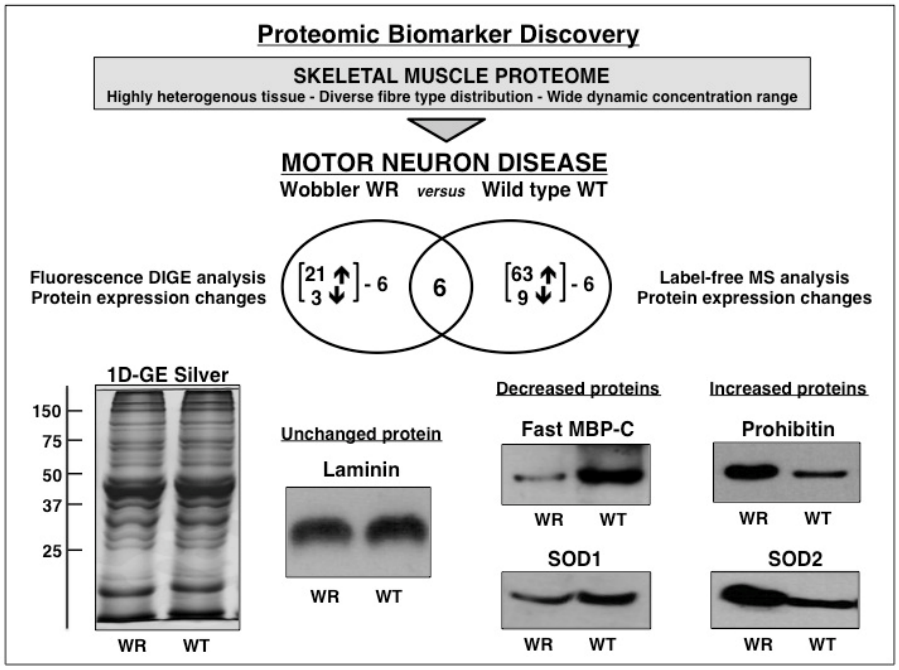

Figure 2. *Proteomic identification of novel biomarker candidates of motor neuron disease.* Contrasted are the proteomic findings of fluorescence 2D-DIGE analysis versus label-free LC-MS/MS analysis of the WR model of ALS. In the lower panel are shown a representative silver-stained gel of wobbler (WR) versus wild type (WT) muscle preparations and corresponding immunoblots labeled with antibodies to laminin, fast myosin binding protein MBP-C and prohibitin, as well as the SOD1 and SOD2 isoforms of superoxide dismutase. Gel electrophoresis and immunoblot analysis was carried out as previously described in detail [15, 16].

skeletal muscle pathology [109]. Fluorescent 2-CyDye or 3-CyDye systems are used to establish differential and pre-electrophoretic tagging of more than one proteome, thereby decisively reducing gel-to-gel variations during comparative protein separation [110]. In conjunction with optimized 2D software analysis tools [111], the advanced DIGE technique represents an unparalleled analytical tool for the establishment of distinct differences in protein expression patterns between normal and diseased tissue specimens [112]. Changes in skeletal muscles from the SOD mouse [75] and the WR mouse [16] model of motor neuron disease have been recently studied by 2D-DIGE analysis. Table 2 lists key findings from these proteomic studies of motor neuron disease and Figure 3A shows graphically the identified protein species with a differing abundance in atrophying WR muscle. Characteristic signs of denervation have previously been shown to

Proteomic study	Methods	Changed proteins	References
Analysis of total extracts from hindlimb muscle of wobbler mouse model of motor neuron disease	2D-DIGE, LC-MS/MS, IB	Disease-associated muscular atrophy with preferential effects on specific synapse types is associated with a drastic decrease in fast MBP-C, as well as increases in MLC1, ACT, TnC, GLY enzymes, MYO, CK and DES	Staunton et al. [16]
Analysis of total extracts from hindlimb muscle of SOD mouse model of motor neuron disease	2D-DIGE, MALDI-ToF MS, LC-MS/MS, IB	Disease-associated muscular atrophy with preferential effects on specific synapse types is associated with complex changes in proteins involved in metabolism, contraction and cellular stress response, such as GLY enzymes, OXPHOS enzymes, TUB, HSP90 and αBC	Capitanio et al. [75]
Analysis of total extracts from hindlimb muscle of wobbler mouse model of motor neuron disease	Label-free MS analysis, IB	Disease-associated muscular atrophy with preferential effects on specific synapse types is associated with drastic decreases in fast MBP-C, titin and SERCA1, and increases in DES, various HSPs, AS, ALB, MLC1/3, TnI, TnC and TnT	Holland et al. [15]

Table 2. *Proteomic profiling of motor neuron disease.* Listed are recent studies that have used mass spectrometry-based proteomics to investigate muscular atrophy due to motor neuron disease.

Abbreviations used: αBC, alphaB-crystallin; ACT, actin; ALB, albumin; AS, ATP synthase; CK, creatine kinase; DES, desmin; DIGE, difference in-gel electrophoresis; GLY, glycolytic enzymes; HSP, heat shock protein; LC, liquid chromatography; MBP, myosin binding protein; MLC, myosin light chain; MS, mass spectrometry; MYO, myoglobin; OXPHOS, enzymes of oxidative phosphorylation; TnC, TnI, TnT; troponin subunits; TUB, tubulin; SERCA, sarcoplasmic or endoplasmic reticulum calcium ATPase.

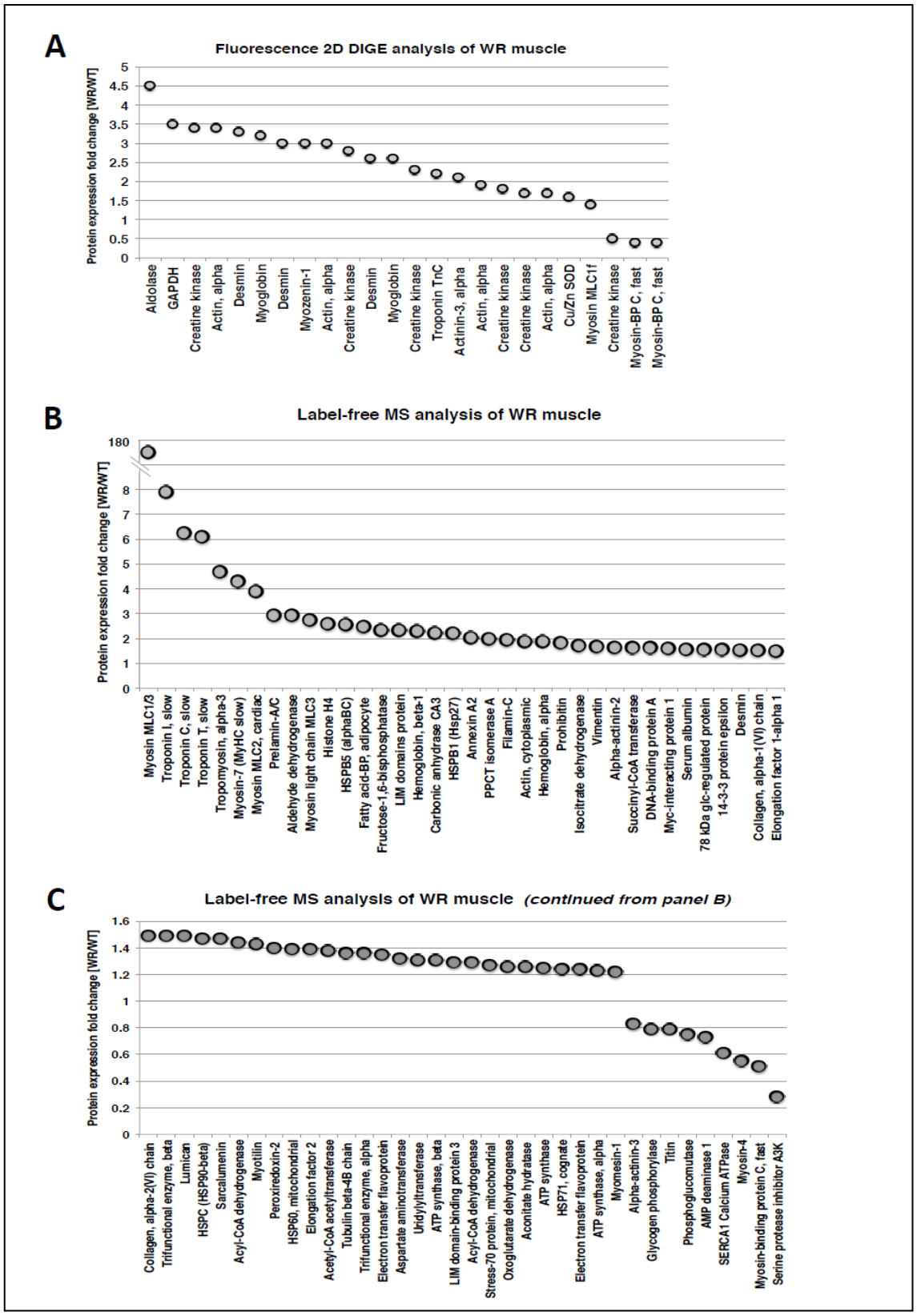

Figure 3. *Comparison of proteomic markers of motor neuron disease as determined by fluorescence difference in-gel electrophoresis (DIGE) (A) versus label-free mass spectrometric (MS) analysis (B, C) using skeletal muscle from the wobbler (WR) mouse model [15, 16].* The fold-change of individual muscle proteins is graphically presented and clearly shows that the application of label-free mass spectrometry has resulted in a larger number of identified protein species with a wider range of concentration changes as compared to gel-based proteomics.

occur in WR skeletal muscles, including an increase of mRNA levels encoding the principal α-subunit of the nicotinic acetylcholine receptor at the junctional folds [113] and a drastic shift to a high proportion of the fast-glycolytic MHCIIB isoform of myosin heavy chain [81].

However, the DIGE analysis of the WR muscle proteome has demonstrated that global changes in motor neuron disease are highly complex [16] and differ from the mostly unilateral shifts in isoform expression patterns observed following experimental denervation or prolonged muscle disuse [89, 92, 94]. Increased expression levels in WR muscle were shown for the glycolytic enzymes aldolase and glyceraldehyde 3-phosphate dehydrogenase, the contractile and cytoskeletal proteins actin, desmin, myozenin, troponin TnC, actinin and fast myosin light chain MLC1f, as well as myoglobin [16]. The M-type isoform of creatine kinase exhibited differential changes in a variety of 2D spots, which suggested the presence of differently phosphorylated isoforms following gel electrophoretic separation. An interesting finding was the elevated level of Cu/Zn superoxide dismutase, an enzyme of crucial importance for the antioxidant defence of skeletal muscle fibres, which is intrinsically involved in various forms of ALS [114]. As illustrated in the immunoblot analysis of Figure 2, the abundance of the mostly cytosolic SOD1 isoform and the mitochondrial SOD2 isoform of superoxide dismutase is differently affected in WR skeletal muscle. The 2D-DIGE analysis of the WR model also revealed the drastic reduction in the density of protein spots representing the fast isoform of myosin binding protein MBP-C [16], which has been clearly confirmed by immunoblotting (Figure 2). This makes the muscular atrophy-related modulations in MBP-C, in conjunction with concentration changes in myosin light chains, myosin heavy chains, actin, troponin, actinin and myozenin, an interesting finding and suggests MBP-C as a novel muscle-associated biomarker candidate of motor neuron disease. Interestingly, the cardiac equivalent of this regulatory protein of the contractile apparatus, cMBP -C [115], was shown to be cleaved during ischemic injury to the heart in a phosphorylation-dependent manner [116] and the release of an N-terminal fragment into the circulation may be useful as a new biomarker of diagnosing myocardial infarction [117].

4.3. *Label-free mass spectrometric analysis of atrophying muscle in motor neuron disease*

In contrast to gel-based methods, liquid chromatography in combination with advanced mass spectrometry can routinely identify low copy numbers of integral membrane proteins, high-molecular mass proteins and components with extreme isoelectric points and/or post-translational modifications. Figure 2 shows the comparison of total numbers of identified proteins using label-free MS analysis versus the DIGE method. While the fluorescence 2D-DIGE analysis identified 21 decreased protein spots and 3 decreased protein spots in WR muscle samples [16], the label-free mass spectrometric analysis of the same tissue preparations revealed 63 increased proteins and 9 decreased proteins in mutant muscle, including integral membrane proteins such as the Ca^{2+}-ATPase of the sarcoplasmic reticulum [15]. The mass spectrometric identification of the SERCA1 isoform of the luminal Ca^{2+}-pump demonstrates the advantageous and complementary nature of the gel-free LC-MS/MS method for detecting changes in a highly hydrophobic muscle protein. The considerably larger number of proteins identified by label-free mass spectrometry is graphically presented in Figure 3B,C. In analogy to the findings from 2D-DIGE studies of motor neuron disease [16, 75], label-free LC-MS/MS analysis confirmed the pathobiochemical complexity of changes in the WR muscle proteome due to progressive neurodegeneration [15]. Proteome-wide alterations included components associated with energy metabolism, metabolite transportation, muscle contraction, ion homeostasis, structural integrity and the cellular stress response [15].

A bioinformatic STRING analysis of the proteomic data from the label-free mass spectrometric analysis of WR leg muscle was carried out [15]. In order to determine potential protein-protein interactions of the mass spectrometrically identified proteins with an altered concentration in atrophying WR muscle, the publically available STRING (http://string-db.org/; version 9.1) database was used, which contains known and predicted protein interactions including direct physical and indirect functional protein associations [118]. The resulting interaction map revealed how complex the protein interactions patterns are between the affected protein species in WR muscle (not shown). This is especially striking with respect to interaction nodes containing contractile elements, cytoskeletal proteins, metabolic enzymes and molecular chaperones. A significantly increased abundance was shown for myosin light chains, the troponin subunits TnI, TnC and TnT, and the regulatory element tropomyosin, as well as aldehyde dehydrogenase, histone protein H4, the nuclear envelope protein lamin, fatty acid binding protein, fructose-1,6-bisphosphatase, four-and-a-half LIM domains protein 1, carbonic anhydrase CA3 and annexin. Higher levels of heat shock protein beta-1, peptidyl-prolyl cis -trans isomerase A and the small heat shock protein αB-crystallin suggested an elevated cellular stress response in WR muscle [15]. The previous immunoblot analysis of essential Ca^{2+}-binding proteins demonstrated that the luminal Ca^{2+}-shuttling protein sarcalumenin of the longitudinal tubules and the highly abundant Ca^{2+}-buffering protein calsequestrin of the terminal cisternae are increased in WR muscle [15]. In contrast, the cytosolic Ca^{2+}-binding protein parvalbumin is decreased in atrophying fibres [15, 16]. These complex proteome-wide alterations suggest that distinct differences exist between disease-related muscular atrophy and the above outlined changes due to muscular disuse or experimental denervation. Differential changes in proteins associated with glycolytic or oxidative mitochondrial metabolism indicate that WR muscle does not undergo a unidirectional transition towards a particular bioenergetic pheno-

type. In motor neuron disease, the metabolic weighting of bioenergetic pathways and fibre type specification appears to be influenced by at least two main mechanisms. Firstly, a differing degree of a subtype-specific vulnerability of neuromuscular synapses and secondarily, highly complex patterns of compensatory mechanisms of fibre type shifting. Thus, in contrast to an overall slow-to-fast transformation process during muscular disuse or denervation, motor neuron disease seems to be related to changes in both slow and fast isoforms of muscle marker proteins.

5. Concluding Remarks

Pathobiochemical insights from label-free mass spectrometry and fluorescence 2D-DIGE analysis are of considerable interest to the field of muscle pathology, but are also crucial for furthering the discovery of specific protein biomarkers of ALS. Figure 4 summaries the main groups of muscle-associated proteins changed in the WR model of motor neuron disease as recently determined by proteomics. Interesting new findings are the identification of increased levels of certain mitochondrial proteins, suggesting that muscular atrophy in WR muscle is not directly linked to slow-to-fast fibre type shifting, but probably initially involves a preferential loss of neuromuscular synapses that function under normal conditions within a fast type of innervation process. The increased concentration of a variety of heat shock proteins agrees with the idea of extensive cellular stress in ALS and the continuous requirement to remove or re-fold large numbers of affected proteins. A promising new muscle-associated biomarker of motor neuron disease is the fast isoform of MBP-C. A decrease in this major auxiliary protein of the contractile apparatus was demonstrated by the fluorescence 2D-DIGE technique [16], LC-MS/MS analysis [15] and the transcriptomic screening of ALS muscle biopsies [49], making this filament-associated component a suitable biomarker candidate of ALS. The future screening of large numbers of ALS patient samples will be needed to fully establish the newly identified proteomic markers as reliable indicators of disease-related muscular atrophy [119]. Hopefully a few of these proteins will be exploitable to design superior prognostic and therapy monitoring assay systems consisting of a meaningful and diagnostically conclusive biomarker signature. In the future, it can be expected that biomarkers of ALS will play a prominent role for a more accurate diagnosis, the proper monitoring of muscular atrophy and the determination of clinical outcome measures.

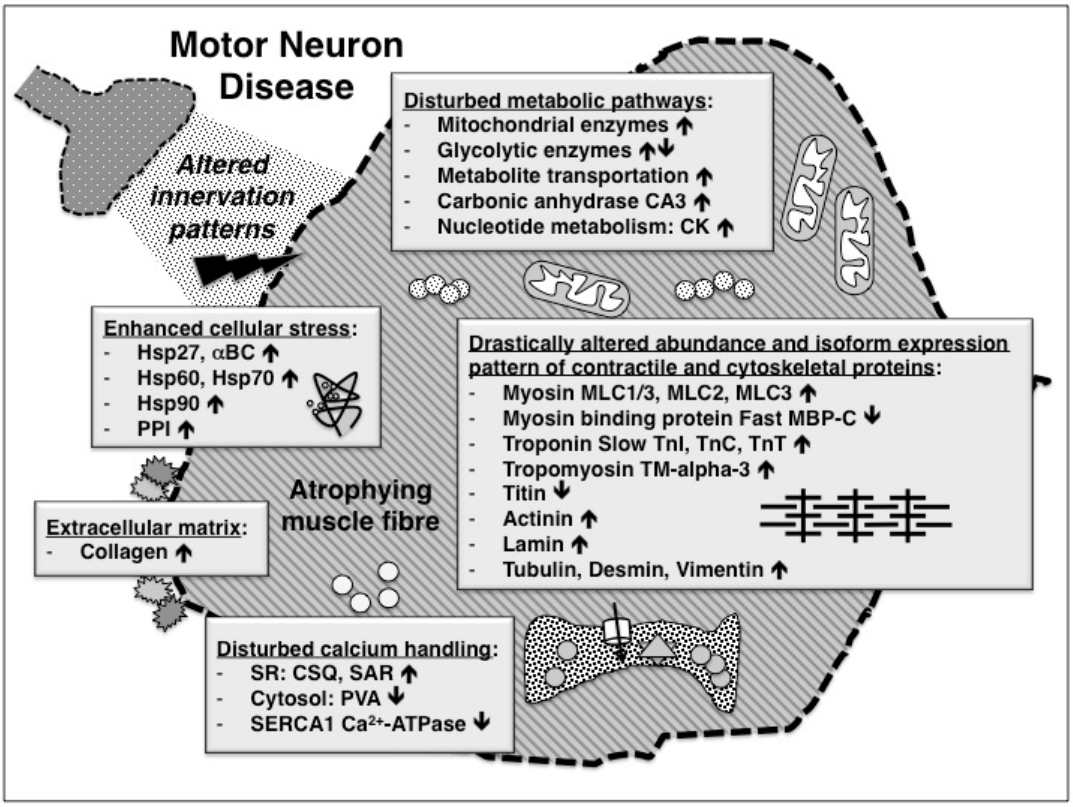

Figure 4. *Overview of major pathobiochemical changes in motor neuron disease as revealed by mass spectrometry-based proteomics.* Listed are subcellular regions, metabolic pathways, cellular processes and protein families that are majorly altered in skeletal muscles due to progressive neurodegeneration. Various newly identified muscle-associated proteins with a changed abundance may be useful as novel biomarker candidates for the design of improved diagnostic, prognostic or therapy monitoring assay systems.

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