Eur J Transl Myol 11555, 2023 doi: 10.4081/ejtm.2023.11555

# Proteomic profiling of the brain from the *wobbler* mouse model of amyotrophic lateral sclerosis reveals elevated levels of the astrogliosis marker glial fibrillary acidic protein

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***Abstract***

The *wobbler* mouse is a widely used model system of amyotrophic lateral sclerosis and exhibits progressive neurodegeneration and neuroinflammation in association with skeletal muscle wasting. This study has used *wobbler* brain preparations for the systematic and mass spectrometric determination of proteome-wide changes. The proteomic characterization of total protein extracts from *wobbler* specimens was carried out with the help of an Orbitrap mass spectrometer and revealed elevated levels of glia cell marker proteins, i.e., glial fibrillary acidic protein and the actin-binding protein coronin. In contrast, the abundance of the actin-binding protein neurabin and the scaffolding protein named piccolo of the presynaptic cytomatrix were shown to be reduced. The increased abundance of glial fibrillary acidic protein, which is frequently used in neuropathological studies as a marker protein of glial scar formation, was confirmed by immunoblotting. In analogy, the proteomic profiling of the brain from another established murine model of motor neuron disease, the *SOD1* mouse, also showed increased levels of this intermediate filament protein. This suggests that neurodegenerative processes are associated with astrogliosis in both the *wobbler* and *SOD1* brain.

**Key Words**: amyotrophic lateral sclerosis; astrogliosis; GFAP; glial fibrillary acidic protein; *SOD1* mouse; *wobbler* mouse.

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Motor neuron diseases are relatively rare neuromuscular diseases of both spontaneous or familial type that belong to the class of heterogeneous neuro-degenerative disorders. The most common adult-onset form of motor neuron disease that is associated with progressive muscular weakness is amyotrophic lateral sclerosis, also referred to as Lou Gehrig's disease, which typically shows degeneration of upper and lower motor neurons.[1] This causes a dysfunctional flow of excitatory signals from the neurons that connect the cortex to the brain stem and spinal cord, as well as an impaired neuronal outflow from the brainstem and the spinal cord towards voluntary muscles. Motor neuron diseases appear to be due to complex interdependent factors, such as genetic susceptibility, environmental effects, lifestyle and aging.[2]

The development of novel treatments to address the complex pathophysiological challenges associated with amyotrophic lateral sclerosis focus mostly on new pharmacological avenues and gene modulation therapy.[3-5] Currently available amyotrophic lateral sclerosis drugs which only exhibit modest benefits on survival include the glutamatergic neuro-transmission inhibitor Riluzole and the antioxidant agent Edaravone.[6] Symptomatic therapies include speech therapy for dysarthria and the application of muscle relaxants to treat spasticity.[7] Of note, moderate intensity exercise to induce skeletal muscle strengthening and select forms of cardiovascular exercise have potentially beneficial effects on patients by reducing the levels of muscular deconditioning and disuse-related muscular atrophy.[8-10] The combined usage of moderate aerobic and isometric exercise regimes

showed promising results to partially counteract the detrimental effects of progressive levels of physical inactivity.[11] Exercise regimes below maximal effort improved skeletal muscle strength in patients afflicted with amyotrophic lateral sclerosis. Positive effects have been observed on energy levels, oxygen consumption and fatigue resistance for the performance of daily activity levels, as well as overall increased motor function and independence.[11]

The clinical symptoms of amyotrophic lateral sclerosis include a broad range of bodily changes and include both severe motor alterations and also extra-motor abnormalities. Some patients suffer from limb muscle weakness and others show bulbar disease with dysarthria and dysphagia.[2,7] Mutations in a large number of genes that encode proteins with a great variety of biological functions have been identified in association with amyotrophic lateral sclerosis.[12] This includes established primary abnormalities in the *SOD1*, *TARDBP*, *FUS*, *VCP*, *OPTN*, *ALS2*, *SETX*, *C9ORF72*, *PFN1*, *VAPB*, and *UBQLN2* genes; plus a large number of new candidate genes.[13]

Of crucial importance is the discovery of new biomarker candidates for the improved diagnosis, prognosis and therapeutic monitoring of amyotrophic lateral sclerosis.[14] In the case of the *SOD1* mutation, it was established that the abnormal aggregation of superoxide dismutase, which normally functions as an essential anti-oxidant enzyme that provides cellular protection against toxic insults via reactive oxygen species, triggers severe oxidative stress causing neuronal cell death.[1,2,12] However, the genetic heterogeneity of neuronal abnormalities in amyotrophic lateral sclerosis has established many additional pathophysiological mechanisms besides oxidative stress, such as defects in cytoskeletal networks, axonal transport dysfunction, dysregulated vesicle transport, the increased release of inflammatory cytokines, mitochondrial dysfunction, impaired ion homeostasis, ion channel and pump dysfunction, impaired glutamate uptake, defective nucleo-cytoplasmic transport, RNA dysregulation and impaired DNA repair, as well as glia cell defects in oligodendrocytes, Schwann cells, astrocytes and microglia.[1,2,7,13]

The complexity of molecular and cellular abnormalities is at least partially reflected in murine models of motor neuron disease. The *wobbler* mouse is a well-established model system of adult-onset amyotrophic lateral sclerosis[15] which is due to a partial loss-of-function mutation in the *Vps54* gene that encodes the vacuolar protein sorting-associated protein VPS54 protein of the multi-subunit Golgi-associated retrograde protein (GARP) complex.[16] The GARP complex is located within the trans-Golgi network and mediates the crucial tethering of retrograde transport vesicles. The *wobbler* mouse is characterized by progressive neuro-degeneration and neuroinflammation in association with progressive skeletal muscle wasting.[17-19] Based on previous proteomic surveys of changes in the skeletal musculature and impaired spermiogenesis of the *wobbler* mouse,[20-22] this report has focused on the *wobbler* brain using mass spectrometry. Proteomic findings were compared to the *SOD1* brain model of amyotrophic lateral sclerosis, which also exhibits severe alterations in skeletal muscles.[23]

## Materials and Methods

For the proteomic profiling of the *wobbler* and *SOD1* mouse brains, general materials and chemicals were obtained from Bio-Rad Laboratories (Hemel-Hempstead, Hertfordshire, UK), GE Healthcare (Little Chalfont, Buckinghamshire, UK) and Sigma Chemical Company (Dorset, UK). Protease inhibitors were purchased from Roche Diagnostics (Mannheim, Germany). For protein digestion, trypsin and Lys-C were obtained from Promega (Madison, WI, USA). Primary antibodies were purchased from Abcam, Cambridge, UK (ab7260 to glial fibrillary acidic protein GFAP; and ab16048 to lamin-B1). Peroxidase-conjugated secondary antibodies were from Chemicon International (Temecula, CA, USA). Chemiluminescence substrate was obtained from Roche Diagnostics (Mannheim, Germany). Protein concentration was determined with the Pierce 660-nm Protein Assay (ThermoFisher Scientific, Dublin, Ireland).

### *Ethical approval, animal license and animal maintenance*

Wild type C57/BL6 mice and murine models of amyotrophic lateral sclerosis, the *wobbler* mouse (C57BL/6-Vps54$^{wr}$) and the *SOD1* mouse (SOD1-G93A), were obtained from the Bioresource Unit of the University of Bielefeld.[22] Animals were kept at a constant room temperature of 22°C on a 12:12-h dark-light cycle with *ad libitum* access to food and water. All protocols and experiments were performed under the terms of the German animal protection law and were permitted by the local authorities. Mice were sacrificed by cervical dislocation and all biochemical and proteomic studies were carried out with *post-mortem* brain specimens. For brain dissection, protein extraction and proteomic analyses, 2-months old mice were used. Freshly dissected brain specimens were quick-frozen in liquid nitrogen and transported to Maynooth University in accordance with the Department of Agriculture (animal by-product register number 2016/16 to the Department of Biology, National University of Ireland, Maynooth) on dry ice and stored at -80ºC prior to proteomic analysis.[22]

### *Preparation of mouse brain tissue extracts for proteomic analysis*

Whole brain specimens from wild type mice (n=4) versus murine models of amyotrophic lateral sclerosis (n=4) were homogenized in lysis solution containing 50 mM Tris-HCl pH 8.0, 8 M urea and 1 mM EDTA. The lysis buffer was supplemented with a freshly prepared protease inhibitor cocktail.[24] The brain extract was then

incubated for 1.5 hours at 4°C. Brain tissue extracts were processed for mass spectrometry following centrifugation at 16,000x*g* for 5 minutes.[24] Protein samples were treated with the Ready Prep 2D clean up kit from Bio-Rad Laboratories (Hemel-Hempstead, Hertfordshire, UK) and the subsequent protein pellets were resuspended in 6 M urea, 2 M thiourea, 10 mM Tris-HCl, pH 8.0. Samples were reduced, alkylated, and digested overnight with trypsin as described previously.[24]

### *Mass spectrometric analysis of brain extracts and proteomic data analysis*

Brain protein identification and the comparative analysis of tissue extracts from wild type versus murine models of amyotrophic lateral sclerosis was performed by an optimized label-free liquid chromatography mass spectrometry procedure.[24] All preparative steps of the proteomic analysis pipeline, as well as analytical procedures using data-dependent acquisition and bioinformatic data handling, have been described in detail.[25-27] A Q-Exactive mass spectrometer and an Orbitrap Fusion Tribrid mass spectrometer were employed for the analysis of brain specimens. A Thermo UltiMate 3000 nano system was used for reverse-phased nano-flow high-pressure liquid chromatography and directly coupled in-line with a Thermo Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The qualitative data analysis of mass spectrometric files was carried out with the help of the UniProtKB-SwissProt *Mus musculus* database with Proteome Discoverer 2.2 using Sequest HT (Thermo Fisher Scientific) and Percolator.[26] For protein identification, the following crucial search parameters were employed: (i) peptide mass tolerance set to 20 ppm, (ii) MS/MS mass tolerance set to 0.6 Da, (iii) an allowance of up to two missed cleavages, (iv) carbamidomethylation set as a fixed modification and (v) methionine oxidation set as a variable modification.[27] Peptides were filtered using a minimum Xcorr score of 1.9 for 1, 2.2 for 2, and 3.75 for 3 charge states, with peptide probability set to high confidence. Quantitative label-free data analysis was performed using Progenesis

***Table 1.*** *List of identified brain proteins with changed concentration levels in the wobbler mouse model of amyotrophic lateral sclerosis as determined by label-free liquid chromatography tandem mass spectrometry.*

| Accession number | Protein name | Peptide counts | Confidence score | Anova (p) | Fold change |
|---|---|---|---|---|---|
| Q99PU5 | Long-chain-fatty-acid--CoA ligase ACSBG1 | 2 | 132.34 | 0.01572 | 4.14 |
| Q9WUM4 | Coronin-1C | 3 | 144.85 | 0.00605 | 3.82 |
| Q05920 | Pyruvate carboxylase, mitochondrial | 2 | 162.42 | 0.03267 | 3.61 |
| Q00612 | Glucose-6-phosphate 1-dehydrogenase X | 2 | 79.96 | 0.01139 | 3.47 |
| Q9WUB3 | Glycogen phosphorylase | 2 | 97.04 | 0.02155 | 2.55 |
| Q8R0Y6 | Cytosolic 10-formyltetrahydrofolate dehydrogenase | 2 | 108.71 | 0.00524 | 2.44 |
| P22752 | Histone H2A type 1 | 2 | 139.57 | 0.00241 | 2.21 |
| P03995 | Glial fibrillary acidic protein | 9 | 710.27 | 0.01580 | 2.21 |
| Q9D0F9 | Phosphoglucomutase-1 | 2 | 147.84 | 0.02593 | 2.14 |
| Q8BMS1 | Trifunctional enzyme subunit alpha, mitochondrial | 4 | 283.71 | 0.00190 | 2.13 |
| P51174 | Long-chain specific acyl-CoA dehydrogenase, mitochondrial | 2 | 117.66 | 0.00501 | 2.01 |
| Q9QYX7 | Protein piccolo | 3 | 153.35 | 0.01498 | -2.00 |
| Q3THE2 | Myosin regulatory light chain 12B | 2 | 98.6 | 0.03079 | -2.02 |
| Q9D8Y0 | EF-hand domain-containing protein D2 | 5 | 296.75 | 0.01890 | -2.09 |
| Q6R891 | Neurabin-2 | 2 | 80.86 | 0.01875 | -2.21 |
| P23927 | Alpha-crystallin B chain | 4 | 228.52 | 0.00570 | -2.32 |
| O35685 | Nuclear migration protein nudC | 2 | 74.37 | 0.01409 | -2.94 |

QI for Proteomics (version 2.0; Nonlinear Dynamics, a Waters company, Newcastle upon Tyne, UK). Peptide and protein identification were achieved with Proteome Discoverer 1.4 using Sequest HT (Thermo Fisher Scientific) and Percolator, and were then imported into Progenesis QI software for further analysis.[26] Protein identifications were reviewed, and only those which passed the following criteria were considered differentially expressed between experimental groups with high confidence and statistical significance: (i) an ANOVA p-value of ≤0.05 between experimental groups; (ii) proteins with ≥2 unique peptides contributing to the

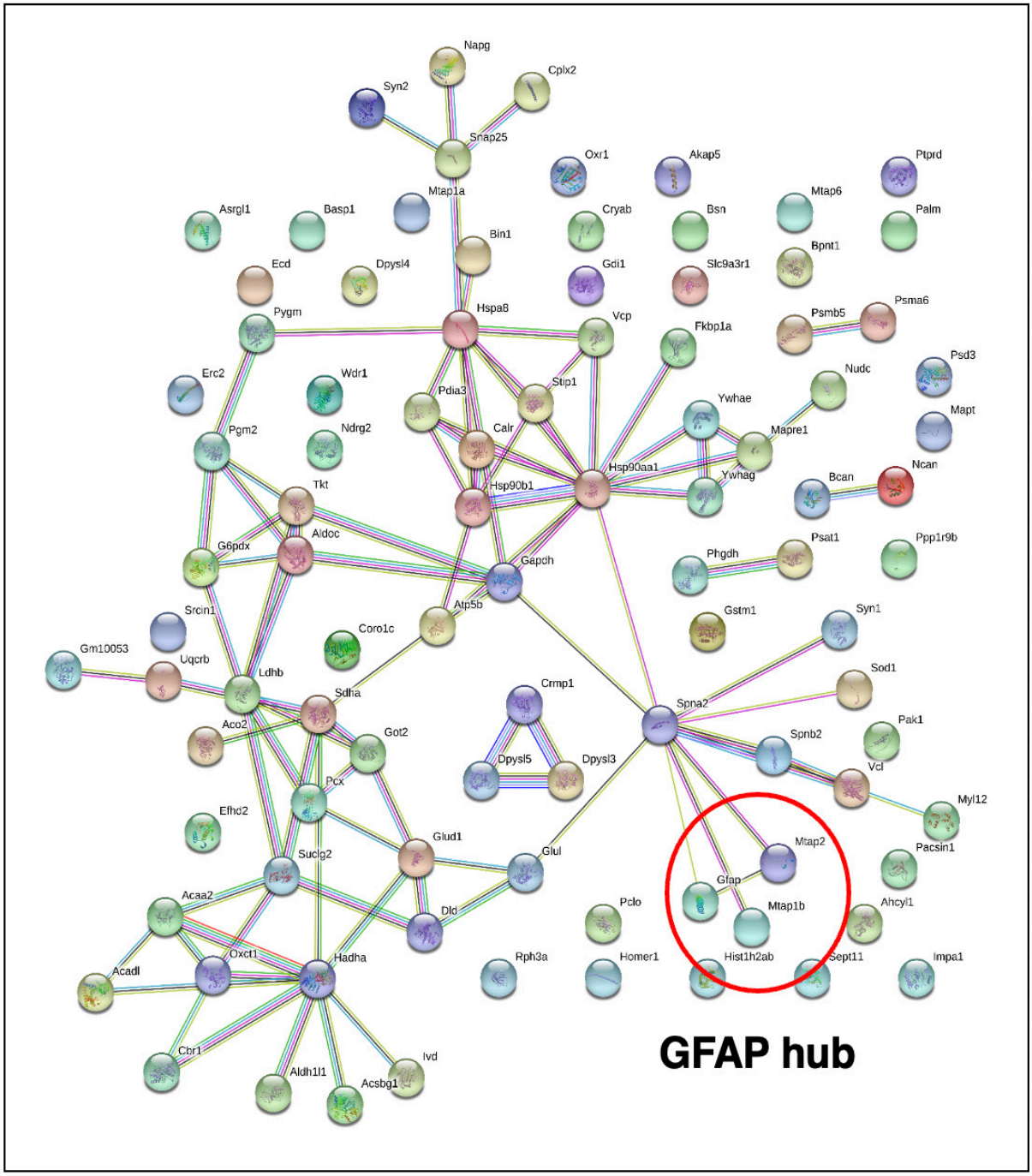


***Fig 1.*** *Bioinformatic analysis of changes in protein-protein interaction patterns in the wobbler brain model of amyotrophic lateral sclerosis. Potential protein interactions were determined with the help of the bioinformatics software programme STRING (https://string-db.org).[28] The position of the protein hub containing the glial fibrillary acidic protein (GFAP) is marked by a red circle.*

identification.[25-27] To calculate the maximum fold change for a protein, Progenesis QI calculates the mean abundance for that protein in each experimental condition. These mean values are then placed in a condition-vs-condition matrix to find the maximum fold change between any two condition's mean protein abundances.[26] The bioinformatic analysis of proteins with an altered expression in *wobbler* brain samples for the determination of potential protein-protein interaction patterns was carried out with the freely available software package STRING (https://string-db.org).[28]

### *Immunoblot analysis of glial fibrillary acidic protein*

The comparative immunoblot analysis of wild type brain versus *wobbler* brain extracts was carried out by an optimized method.[24] Electrophoretic separation of brain proteins was performed with 10% polyacrylamide slab gels. Control gels were silver-stained for the visualization of protein band patterns. Unstained proteins were transferred at 100V and 4°C for 70 min to nitrocellulose sheets in a Transblot Cell from Bio-Rad Laboratories (Hemel-Hempstead, Hertfordshire, UK). The increased abundance of glial fibrillary acidic protein, as detected by mass spectrometry, was confirmed by immuno-decoration using primary antibody ab7260 and a secondary peroxidase-conjugated secondary antibody. The evaluation of equal protein loading was carried out by immuno-decoration with primary antibody ab16048 to lamin-B1. The visualization of immuno-decorated protein bands was achieved with the enhanced chemiluminescence method as per manufacturer's guidelines. Densitometric scanning and statistical analysis of immunoblots was performed with a HP PSC-2355 scanner and ImageJ software (NIH, USA), in conjunction with GraphPad Prism software (San Diego, CA, USA), in which a p value <0.05 was deemed to be statistically significant.

## Results

### *Proteomic identification of altered proteins in the wobbler mouse model*

The main underlying objective of this investigation was to identify proteome-wide changes in mouse models of amyotrophic lateral sclerosis employing crude brain protein extracts from small tissue specimens. The mass spectrometry-based proteomic surveys described here used a minimum of preparative steps for a streamlined biochemical approach that eliminates excessive bioanalytical artefacts for the efficient detection of brain proteoforms. Whole brain preparations, rather than specific neuroanatomical structures, were chosen as starting material in order to elucidate global alterations in the entire brain proteome. The systematic evaluation of changes in specific protein species is based on the availability of established proteomic maps of the wild type mouse brain.[29-34] This is crucial, since the proper interpretation of new findings from comparative

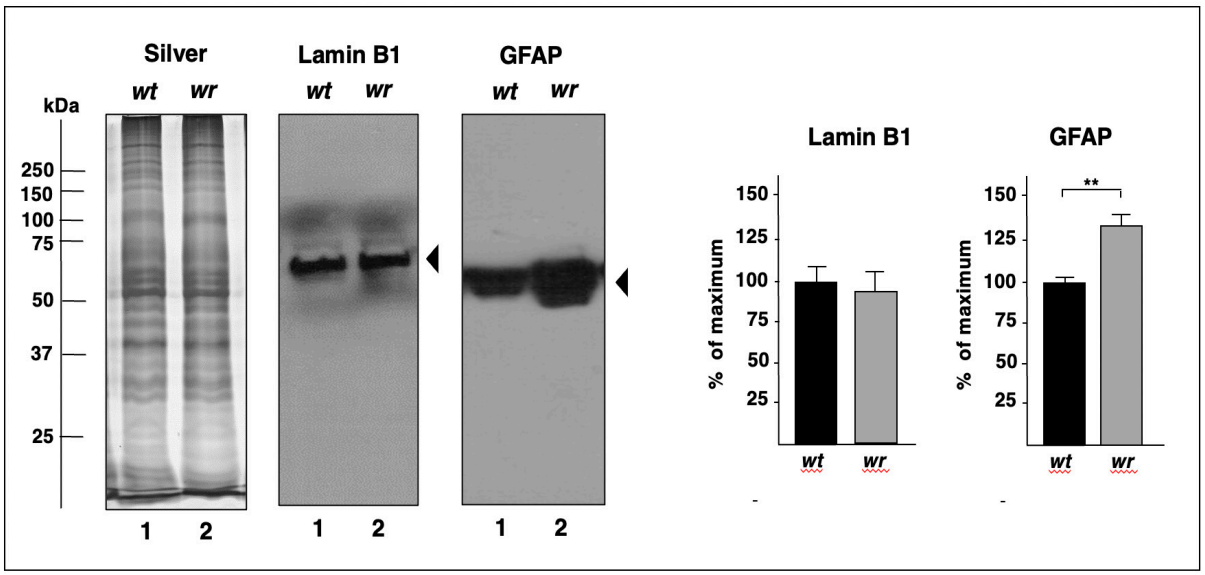


***Fig 2.*** *Comparative immunoblot analysis of glial fibrillary acidic protein in brain extracts from wild type mice versus the wobbler mouse model of amyotrophic lateral sclerosis. Shown is a silver-stained protein gel using sodium dodecyl sulphate polyacrylamide slab gel electrophoresis for the separation of wild type (wt) brain (lane 1) and wobbler (wr) brain (lane 2) samples, as well as identical nitrocellulose replicas used for immunoblot analysis. Immunoblots were labelled with antibodies to lamin-B1 and glial fibrillary acidic protein (GFAP). In the adjacent panels are shown the statistical analysis of immunoblotting (Student's t-test; n=4; **p<0.01). The value of molecular mass standards (x10$^{-3}$ kDa) is marked on the left side of the gel.*

proteomic studies heavily depends on the availability of established proteomic data banks of normal tissues.[34]

The comparative proteomic analysis of wild type versus *wobbler* brain extracts identified 149 increased and 142 decreased protein species. Table 1 lists only those proteomic hits that were recognized by at least 2 peptides and exhibited a 2-fold change in abundance. A higher abundance in the *wobbler* mouse model of amyotrophic lateral sclerosis was shown for long-chain-fatty-acid-CoA ligase, coronin, pyruvate carboxylase, glucose-6-phosphate-1-dehydrogenase, glycogen phosphorylase, formyltetrahydrofolate dehydrogenase. Histone H2A, glial fibrillary acidic protein GFAP, phospho-glucomutase-1, trifunctional enzyme and long-chain specific acyl-CoA dehydrogenase.

Interesting findings in the context of gliosis are the increased levels of the astrocyte marker glial fibrillary acidic protein GFAP[35] and the filamentous-actin binding protein coronin-1C of the microglia.[36] Decreased brain proteins in the *wobbler* mouse included the scaffolding protein named piccolo, myosin regulatory light chain, EF-hand domain-containing protein, the actin-binding protein neurabin-2, the molecular chaperone alphaB-crystallin and the nuclear migration protein nudC (Table 1). Of note is the reduced expression of protein piccolo, which has a key role in the organization of the presynaptic cytomatrix. In Figure 1 is shown the bioinformatic STRING analysis of potential protein-protein interaction patterns in the altered *wobbler* brain proteome. One of the apparent protein hubs includes the glial fibrillary acidic protein.

### Immunoblot analysis of the glial fibrillary acidic protein in the *wobbler* brain

To confirm the increased abundance of the gliosis marker glial fibrillary acidic protein GFAP in the *wobbler* mouse model of amyotrophic lateral sclerosis, as determined by mass spectrometry (Table 1), comparative immunoblotting was carried out with brain extracts from wild type mice versus *wobbler* mice. Figure 2 clearly illustrates a significant increase of glial fibrillary acidic protein in the *wobbler* brain. Previous immunoblot analyses of glial fibrillary acidic protein have unambigiously established the restricted presence of this astrocyte marker in brain tissue and established a relatively broad banding pattern which probably reflects

***Table 2.*** *List of identified brain proteins with changed concentration levels in the SOD1 mouse model of amyotrophic lateral sclerosis as determined by label-free liquid chromatography tandem mass spectrometry.*

| Accession number | Protein name | Peptide count | Confidence score | Anova (p) | Fold change |
|---|---|---|---|---|---|
| Q91XV3 | Brain acid soluble protein 1 | 3 | 181.93 | 0.02500 | 3.67 |
| Q60864 | Stress-induced-phosphoprotein 1 | 2 | 111.61 | 0.00666 | 3.33 |
| Q8CDN6 | Thioredoxin-like protein 1 | 2 | 58.54 | 0.00584 | 2.78 |
| O08756 | 3-hydroxyacyl-CoA dehydrogenase type-2 | 2 | 85.72 | 0.00811 | 2.74 |
| P62259 | 14-3-3 protein epsilon | 2 | 61.05 | 0.03096 | 2.64 |
| P58771 | Tropomyosin alpha-1 chain | 2 | 88.89 | 0.03876 | 2.60 |
| P03995 | Glial fibrillary acidic protein | 2 | 105.13 | 0.03623 | 2.44 |
| Q8QZT1 | Acetyl-CoA acetyltransferase, mitochondrial | 4 | 168.61 | 0.00588 | 2.44 |
| Q7TSJ2 | Microtubule-associated protein 6 | 6 | 352.07 | 0.02964 | 2.30 |
| P27773 | Protein disulfide-isomerase A3 | 3 | 97.33 | 0.01248 | 2.24 |
| P45376 | Aldose reductase | 2 | 72.22 | 0.00035 | 2.18 |
| Q810U4 | Neuronal cell adhesion molecule | 2 | 137.62 | 0.00765 | 2.15 |
| P63101 | 14-3-3 protein zeta/delta | 2 | 130.27 | 0.03162 | 2.04 |
| Q8BFR5 | Elongation factor Tu, mitochondrial | 2 | 157.46 | 0.00139 | 2.02 |
| O08599 | Syntaxin-binding protein 1 | 4 | 242.76 | 0.02934 | -3.89 |
| P17183 | Gamma-enolase | 2 | 65.20 | 0.01066 | -10.28 |
| P62737 | Actin, aortic smooth muscle | 2 | 60.41 | 0.00010 | -21.19 |

isoforms with slightly differing electrophoretic mobility.[24] Silver-staining of protein gels showed no major change in the overall protein banding pattern between wild type and *wobbler* preparations. The immuno-decoration of the nuclear protein lamin-B2 was used as a loading control.

### *Proteomic identification of altered proteins in the SOD1 mouse brain*

The increased levels of glial fibrillary acidic protein GFAP in the *wobbler* brain, as determined by mass spectrometry (Table 1) and verified by comparative immunoblotting (Figure 2), was also shown to occur in another murine model of amyotrophic lateral sclerosis, the *SOD1* mouse.[23]

The comparative proteomic analysis of wild type versus *SOD1* brain extracts identified 150 increased and 51 decreased protein species. Table 2 lists only those proteomic hits that were recognized by at least 2 peptides and exhibited a 2-fold change in abundance. Increased brain proteins included brain acid soluble protein, stress-induced-phosphoprotein, thio-redoxin-like protein, 3-hydroxyacyl-CoA dehydrogenase, 14-3-3 protein epsilon, tropomyosin alpha-1, glial fibrillary acidic protein GFAP, acetyl-CoA acetyl-transferase, microtubule-associated protein, protein disulfide-isomerase, aldose reductase, neuronal cell adhesion molecule and 14-3-3 protein zeta/delta. Drastically decreased *SOD1* brain-associated proteins were identified as elongation factor Tu, syntaxin-binding protein 1 and gamma-enolase (Table 2).

## Discussion

The mass spectrometric analysis of the *wobbler* mouse model of amyotrophic lateral sclerosis[15] presented in this report has established drastic changes in the brain proteome due to the primary abnormality in the *Vps54* gene.[16] The loss of function of the VPS54 protein of the multi-subunit GARP complex causes primarily impaired vesicular trafficking and protein mis-sorting,[18,37] which is associated with an excitatory-inhibitory imbalance, spatio-temporal pattern of cerebellar degeneration, hippocampal hyperexcitability and reduced numbers of interneurons.[38-40] The observed neurodegeneration and neuroinflammation in the *wobbler* mouse[20,21] are typical features that are also present in the central nervous system of patients afflicted with amyotrophic lateral sclerosis.[1,2,12,13] The progressive degeneration of upper and lower motor neurons causes distinct patterns of muscular atrophy. Mass spectrometry-based proteomics has been instrumental in the characterization of the skeletal musculature[20,22] and testis[21] of this widely employed mouse model of motor neuron disease.[17] This report has characterized both the *wobbler* and *SOD1* mouse brain, two established models of motor neuron disease.

A striking finding of the proteomic survey of the *wobbler* brain is the increased abundance of two well-established glia cell markers, *i.e.* glial fibrillary acidic protein GFAP, a marker of astrocytes,[35] and coronin-1C, a marker of microglia.[36] The higher levels of glial fibrillary acidic protein were confirmed by immunoblotting and the same proteomic result was also found in the *SOD1* model[23] of primary motor neuronopathy. This strongly suggests the occurrence of elevated astrocyte and microglia cell populations in motor neuron disease. The increased appearance of glial fibrillary acidic protein is frequently used in neuropathological studies as a marker protein of glial scar formation.[41] The glial fibrillary acidic protein is a major element of the intermediate filament system of astrocytes.[35] Thus, the approximately 2-fold increase of this protein agrees with the pathobiochemical concept of neuro-degenerative processes that are associated with astrogliosis in the *wobbler* and *SOD1* brain.

Neuronal abnormalities may result in a non-specific form of reactive gliosis.[41] The increased levels of coronin-1C are also of pathophysiological importance. Coronins are filamentous-actin binding proteins with a homo-trimeric configuration. Specific isoforms have been shown to locate to microglia.[36] Coronin-1C (CORO1C), also named Coronin-3, is highly expressed in the mature brain and is involved in cellular migration, neurite outgrowth and neuron morphogenesis. Coronins probably mediate branching within the actin filament network of the membrane cytoskeleton. High levels of coronin-3 expression have previously been described to occur in diffuse gliomas.[42] Hence, the microglia cell population appears to be elevated in the *wobbler* mouse brain agreeing with the concept of reactive gliosis. The formation of glial scars is therefore probably associated with impaired motor neuron functions at the level of the central nervous system. The observed proteomic findings of elevated levels of gliosis markers in murine models of amyotrophic lateral sclerosis agree with the cellular pathogenesis of patients suffering from adult-onset motor neuron disease.[1,2,12,13]

The proliferation of glial cells, specifically astrocytes, plays at least partially a role in the cellular pathogenesis of neurodegeneration.[43] Astrocytes represent a crucial cell type in the central nervous system that are involved in the maintenance of neuronal metabolism, synaptic function and the blood-brain barrier.[44] Pathological disturbances cause astrocyte activation, differentiation and morphological remodeling. Glial cell dysfunction, mainly involving microglia and astrocytes, was clearly established to occur in the nervous system of patients afflicted with amyotrophic lateral sclerosis.[45] This agrees with the pathoproteomic findings presented in this report and the elevated levels of both microglia and astrocyte marker proteins in *wobbler* and *SOD1* brain preparations. Interestingly, the removal of activating factors from reactive astrocytes was demonstrated to trigger a slowing of disease progression in the *SOD1* mouse model of motor neuron disease.[46] Thus, the prevention or reversal of reactive gliosis and reduction of activated astrocytes may present a potential therapeutic target to address a

serious histopathological complication of amyotrophic lateral sclerosis.

Besides glia cell proteins, other proteomic changes were established to occur in proteoforms that are involved in the maintenance of cytoskeletal networks, energy metabolism and the cellular stress response. These complex alterations in the brain agree with the down-stream changes in the skeletal musculature. The proteomic profiling of mouse models of primary motor neuronopathy has shown that the progressive degeneration of distinct motor neurons alters the density and/or fibre-type specific isoform expression patter of a large number of diverse proteins that are involved in muscle contraction, energy metabolism, metabolite transportation, regulation of ion homeostasis, the cellular stress response, structural maintenance and the cytoskeleton.[20,22,23]

The results from systematic proteomic profiling studies of both the brain and skeletal musculature can now be used to establish new biomarker candidates to improve diagnostic and prognostic approaches[14] and identify novel therapeutic targets to treat the highly complex neuromuscular pathogenesis of amyotrophic lateral sclerosis.[47] Besides evaluating tissue-related changes in motor neuron disease, an important aspect of biomarker research is the identification of biofluid markers in serum or cerebrospinal fluid.[14,48] Biofluid markers of clinical interest include GFAP and neurofilament light chain.[49-53] The evaluation of a panel of serum biomarker candidates for the improved diagnosis and prognosis of amyotrophic lateral sclerosis, which took into account the critical correlation between the altered abundance of biomarkers and several clinical parameters, was recently carried out by Falzone et al.[54] Interestingly, this analysis of specimens from a large cohort of patients afflicted with motor neuron disease revealed that serum GFAP levels were elevated in amyotrophic lateral sclerosis patients that suffer from cognitive-behavioral impairments, as compared to patients with normal cognition.[54]

Thus, altered GFAP concentration represents a promising diagnostic indicator that reflects extra-motor involvement in amyotrophic lateral sclerosis, i.e. complications due to frontotemporal dementia or cognitive impairmen.[53,54] In addition, neurofilament light chain (NfL), one of the polypeptide chains of the neurofilament triplet that structurally supports the neuronal cytoskeleton of axons, was shown to be a suitable diagnostic marker and strongest predictor of patient survival.[51,52,54] Ubiquitin C-terminal hydrolase UCHL1 was established as a robust prognostic indicator for stratifying slow disease progression and survival rates in patients with low levels of neurofilament light chain.[54] These findings demonstrate the clinical importance of established novel biomarkers for the improved diagnosis, prognosis and therapeutic monitoring of amyotrophic lateral sclerosis.[55] In addition to changes in minimally invasive biofluid markers and invasive tissue-associated disease indicators, another important category of amyotrophic lateral sclerosis markers are non-invasive imaging biomarkers of neurodegenerative processes. An early recognition pattern seen in magnetic resonance imaging (MRI) is the motor band sign (MBS), which can be helpful in the early differential diagnosis of motor neuron diseases as a marker for upper motor neuron involvement.[56-58] Thus, the combined usage of diagnostic and prognostic biomarkers in serum, cerebrospinal fluid and suitable tissue biopsies, in combination with advanced imaging technology, can be valuable for the improved evaluation of amyotrophic lateral sclerosis.

Motor neuron disease, which can be both spontaneous or familial, is a heterogeneous neurodegenerative disorder. Amyotrophic lateral sclerosis represents the most common adult-onset form with progressive muscular weakness being one of the clinical hallmarks of this type of motor neuron disease. The mass spectrometry-based biochemical profiling of brain extracts from the *wobbler* mouse model of amyotrophic lateral sclerosis has revealed complex changes in its proteome, affecting especially the expression of glia cell markers. Elevated levels of the astrocyte marker glial fibrillary acidic protein GFAP and the microglia marker coronin-1C in the *wobbler* brain strongly indicate that reactive gliosis plays a central role in motor neuron disease. Thus, abnormal functioning of the trans-Golgi network and impaired tethering of retrograde transport vesicles due to loss of VPS54 function has severe down-stream effects in the brain.

In conclusion, progressive astrogliosis is clearly evident in the *wobbler* brain and this could be a crucial pathophysiological factor during progressive neurodegeneration in amyotrophic lateral sclerosis.

## List of acronyms

GARP - Golgi-associated retrograde protein
GFAP - glial fibrillary acidic protein
SOD1 - superoxide dismutase 1
VPS54 - vacuolar protein sorting-associated protein 54

## Contributions of Authors

SM, TSJ, PD, DS, and KO were involved in the conceptualization and initiation of this project, as well as the design of the research strategy. SM and PD performed the biochemical experiments and analysed the data. SM and TSJ were involved in the preparation of tissue samples. MH and PM performed the mass spectrometric and bioinformatic analysis. All authors were involved in the writing and final editing of the manuscript. All authors approved the final version of this paper.

## Acknowledgments

None.

## Funding

This work was supported by the Kathleen Lonsdale Institute for Human Health Research at Maynooth

University. The Q-Exactive mass spectrometer and the Orbitrap Fusion Tribrid mass spectrometer were funded under Science Foundation Ireland Infrastructure Awards SFI-12/RI/2346/3 to Maynooth University and SFI-16/RI/3701 to Dublin City University, respectively.

## Conflict of Interest

Sandra Murphy is an employee of Charles River Laboratories. All other authors declare no competing interests.

## Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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Submission: June 29, 2023
Revision received: August 01, 2023
Accepted for publication: August 01, 2023