ABSTRACT

Title of document: ALTERATIONS IN HUMAN SKELETAL MUSCLE PROTEINS IN AMYOTROPHIC LATERAL SCLEROSIS

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Amyotrophic lateral sclerosis (ALS) is the most common fatal neurodegenerative disease, resulting in loss of voluntary muscle control, atrophy, paralysis, and eventually death. Although the pathophysiology of ALS is not completely understood, recent research in Dr. Chin's lab has identified alterations in skeletal muscle proteins in ALS mice. The purpose of this study was to investigate alterations in proteins involved in calcium handling (SERCA1 and SERCA2), endoplasmic reticulum (ER) stress (Grp78/BiP, PDI, and CHOP) and protein synthesis (Akt) in human ALS skeletal muscle. The ER chaperone protein Grp78/BiP and Akt, a protein involved in protein synthesis, were higher in ALS compared to CON. The calcium pump SERCA1 was lower in diaphragm compared to quadriceps muscles of ALS cases. These data highlight alterations in skeletal muscle proteins not only between ALS and CON, but also between different muscles in ALS, which are helpful for informing future research study designs.

ALTERATIONS IN HUMAN SKELETAL MUSCLE PROTEINS IN AMYOTROPHIC LATERAL SCLEROSIS

By

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Thesis submitted to the faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
of the requirements for the degree of
Master of Arts
2015

Advisory committee:
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Professor Stephen Roth

Acknowledgements

I would like to thank Drs. Ben Hurley and Steve Roth for their input and willingness to serve on my committee. Additional thanks are extended Dr. Roth for his support and guidance as the Graduate Director. I am especially grateful for my advisor, Dr. Eva Chin, for the time she put into this document and my graduate training as a whole. Her understanding, encouragement, and support for my current and future goals are greatly appreciated.

Thank you to my fellow graduate students for being such a helpful, friendly, intelligent bunch. Being surrounded by an excellent group of people made the past 2.5 years fun and enjoyable. Special thanks to my fellow lab members Dapeng Chen and Davi Mazala for always being willing to help me, and to Dapeng for his frequent guidance in lab techniques. Thanks to all of my friends, family, and loved ones for their support during my graduate studies.

Financial support for this project was provided through the State of Maryland's Technology Development Corporation (TEDCO). Thank you to our collaborators Dr. Justin Kwan (University of Maryland, Baltimore) and Dr. Lyle Ostrow (Johns Hopkins University) for providing insight into this project and muscle autopsy samples. Special appreciation is given to the individuals who donated muscle samples for this project; without you, this research would not have been possible.

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Abbreviations

ALS- Amyotrophic Lateral Sclerosis
UMN- upper motor neuron
LMN- lower motor neuron
fALS- familial ALS
sALS- sporadic ALS
SOD1- superoxide dismutase 1
TARDP- transactive response DNA-binding protein of 43 kilo Daltons
FUS/TLS- fused in sarcolemma/ translated in liposarcoma
C9orf72- chromosome 9 open reading frame 72
Ca^{2+}- Calcium
SR/ER- sarco/ endoplasmic reticulum
ER- endoplasmic reticulum
ROS- reactive oxygen species
UPR- unfolded protein response
Grp78/BiP- 78 kDa glucose-regulated protein/ Binding immunoglobulin protein
CHOP- C/EBP homologous protein
SERCA1- sarco/endoplasmic reticulum Calcium-ATPase 1
SERCA2- sarco/endoplasmic reticulum Calcium-ATPase 2
Akt- protein kinase B
PV- parvalbumin
PDI- protein disulfide isomerase
IRE1α- inositol-requiring enzyme 1- alpha
GAPDH- Glyceraldehyde 3- Phosphate Dehydrogenase
CON- healthy controls
SMA- spinal muscle atrophy (disease controls)
CON-SMA- control samples age matched to SMA samples
BCA- bicinchoninic acid
BSA- bovine serum albumin
PVDF- polyvinyladine fluoride
ECL- electrochemiluminescence
n- number of cases
M- mean
SD- standard deviation
ANOVA- analysis of variance
LSD- least significant difference
SE- standard error
EMG- electromyographic
MRS- magnetic resonance spectroscopy
DTI- diffusion tensor imaging
TMS- transcranial magnetic stimulation
UMN-D- upper motor neuron-dominant ALS
LMN-D- lower motor neuron dominant ALS
PLS- Primary Lateral Sclerosis
PMA- Progressive Muscle atrophy

FTLD- frontotemporal lobar degeneration
FTD- frontal temporal dementia
NPPV- noninvasive positive-pressure ventilation
PEG- percutaneous endoscopic gastrostomy
ALSFRS- ALS Functional Rating Scale
NIV- noninvasive ventilator
STIM1- stromal interaction molecule 1
SOCE- store-operated Ca^{2+} entry

Chapter 1: Introduction

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is the most common fatal neurodegenerative disorder in adults, with approximately 4 cases of ALS per 100,000 persons in the United States (103). ALS involves loss of neurons in both the brain and spinal cord (upper and lower motor neurons) that control voluntary muscle movement. The disease is characterized by skeletal muscle atrophy and paralysis, and leads to death in 100% of cases. Two types of disease onset are seen: limb onset and bulbar onset. Patients with limb onset typically first experience symptoms in their arms and legs, often having difficulty running, frequently tripping or stumbling. In the case of bulbar onset, patients first notice loss of motor control in the mouth and may have difficulty speaking or swallowing. In either case, progressive muscle weakness eventually spreads to the trunk, where patients have difficulty eating and breathing, ultimately needing a ventilator to survive. Most often death occurs within 2-5 years of diagnosis, typically due to respiratory failure (83).

It is estimated that 30,000 Americans have ALS and on average 15 new cases are diagnosed every day (3). The average age at diagnosis is ~59 years (83), and highest prevalence rates are between the ages of 70-79 (103). ALS is approximately 1.56 times more common in males than in females, and it is twice as prevalent in Caucasians than in African Americans (103).

Because there is vast variability of symptoms in the early stages of the disease, time from symptom onset to diagnosis is often long (nearly 1 year on average) and alternative diagnoses are frequently given prior to ALS diagnoses (109, 134). To elicit

an ALS diagnosis, progressive muscle weakness and degeneration must be observed clinically in both the upper motor neurons (UMNs) and lower motor neurons (LMNs). Electromyographic needle examination is typically used to assess denervation in LMNs, but detecting UMN involvement in the early stages of the disease is more difficult. ALS can only be definitively determined at autopsy (23). Clinically, ALS is markedly heterogeneous. Even family members with the same gene mutation show differences in site of onset, the degree of involvement of UMNs and LMNs, rate of progression, and cognitive impairments (131).

ALS has no cure. There is one FDA-approved drug for treating ALS, Riluzole, which increases lifespan by approximately 3 months (104). Over 100 compounds have been tested for the treatment of ALS, many of which were successful in mice, but none of which were effective in humans (64). Stem cell therapies for ALS are currently being investigated. Although this research is still in the early stages, findings from mouse models appear promising and Phase I and II clinical trials are currently underway (101).

Exercise as a therapeutic intervention has been studied extensively in ALS mice and a limited number of studies have been conducted in humans. Although results are mixed, it appears that moderate intensity exercise has a positive impact on symptom onset (140), progression (26), and longevity in mice (76), while high intensity exercise may be harmful (98). Light muscle exercise in individuals with ALS resulted in slight improvements in function and quality of life (19, 39, 47). Nevertheless, physicians are often hesitant to recommend exercise for individuals with ALS, possibly due to the mixed results in the murine literature and a potential link between competitive sports and development of ALS (31, 54, 121). Current treatments for ALS patients focus on

maintaining independence, increasing quality of life and prolonging survival. Multidisciplinary care (133) and ventilatory support (22) have both been shown to increase survival of ALS patients.

Healthcare costs for ALS patients are high and increase significantly as the disease progresses. Due to the relentlessly fast progression of the disease, ALS patients are often unable to work within a rather short timeframe of being diagnosed and require fulltime caregivers. Insurance companies may cover some costs, but coverage is not consistent. Sources of costs include medications to help manage symptoms and reduce pain, non-invasive ventilators and mechanical ventilators, and frequent emergency room and office visits (105).

About 5-10% of ALS cases are inherited, frequently as an autosomal dominant trait (familial ALS; fALS), however, most cases are sporadic (sporadic ALS; sALS) and the cause is unknown. Genetic predispositions, exposure to toxins, and trauma may play a role in some cases of ALS. Early epidemiological studies showed a relationship between previous participation in competitive sports and ALS incidence (54). These findings were corroborated by more recent studies, in which incidence of motor neuron disease was 1.7 times higher in patients who were varsity athletes compared to those who were not (121), and increased incidence of ALS among Italian soccer players (31). However, other studies failed to show an association between competitive sports or sports related traumas and development of ALS (135), hence, the relationship between competitive sports and ALS development remains unclear.

Nearly 25 years ago, researchers identified a close genetic linkage between fALS and a gene that encodes a copper zinc-binding superoxide dismutase (*SOD1*). The discovery that ~20% of individuals with fALS have a mutation on the *SOD1* gene (118) led to the development of an ALS mouse model in which the mutant human *SOD1* gene is overexpressed (69), the most common of which is the G93A SOD1 mouse model. These mice mimic many symptoms seen in ALS, including motor neuron degeneration, hind limb weakness and atrophy, which eventually progresses to complete paralysis. Since the discovery of *SOD1*, many other genes associated with ALS have been identified. The most common ALS-linked genes include: transactive response DNA-binding protein of 43 kilo Daltons (*TARDP*) (6), fused in sarcolemma/ translated in sarcoma (*FUS/TLS*) (85), and chromosome 9 open reading frame 72 (*C9orf72*) (43). Many of these proteins are primary constituents of neuronal cytoplasmic protein aggregates, characteristic of dying neurons in individuals with ALS.

ALS pathophysiology is complicated, and involves multiple organelles. Theories proposed to explain the underlying pathophysiology include oxidative stress, impaired calcium (Ca^{2+}) signaling, mitochondrial dysfunction, protein misfolding and glutamate toxicity (20, 132). The sarco/endoplasmic reticulum (SR/ER) and mitochondria have recently been receiving growing attention in ALS pathophysiology. The endoplasmic reticulum (ER) is a site of Ca^{2+} storage and release, and is involved in protein synthesis and facilitation of protein folding (15). During times of cellular stress—such as when there is an accumulation of reactive oxygen species (ROS)— proteins may be improperly folded in the ER, leading to an unfolded protein response (UPR) (77). Misfolded proteins can accumulate in the ER, inducing ER stress-signaling pathways that result in alterations

in protein expression. Proteins that enhance cell survival may be up-regulated in an attempt to regain homeostasis (e.g. 78 kDa glucose- regulated protein/ binding immunoglobulin protein [Grp78/BiP]), or in cases of severe stress proteins involved in apoptotic pathways (e.g. C/EBP homologous protein [CHOP]) may be up-regulated. The accumulation of misfolded proteins can also cause misregulated Ca^{2+} release or uptake in the ER (15). Impaired Ca^{2+} regulation, ROS, and accumulation of misfolded proteins are all involved in ALS pathology. G93A SOD1 ALS mice show increased intracellular Ca^{2+} in single muscle fibers, potentially due to decreased Ca^{2+} clearance proteins, sarco/endoplasmic reticulum-ATPases (SERCA1 and SERCA2) (28). The SOD1 mutation results in increased production of the hydroxyl radical, which has been shown to inhibit SERCA protein function (149). Given the role of SERCA1 in Ca^{2+} regulation, it is not surprising that ROS production is also linked to altered Ca^{2+} regulation (15). Movement of Ca^{2+} from the SR/ER lumen into the mitochondria also stimulates ROS production and can induce apoptosis. Conversely, mitochondrial ROS leaks into the ER and affects Ca^{2+} release channels, creating bidirectional transmission of information between the ER and the mitochondria (15).

Although protein alterations have been studied extensively in human ALS neurons, few studies have investigated altered proteins in human ALS skeletal muscle. Studies that have been conducted in human skeletal muscle show alterations between ALS and control muscle in proteins involved in protein synthesis and atrophy (90, 150), trophic factors (96), and the Ca^{2+} regulatory protein STIM1 (66).

Recent work in Dr. Chin's lab identified differences between ALS and control samples in Ca^{2+} handling and ER stress proteins in G93A*SOD1 ALS mice and between

human ALS and disease control skeletal muscle autopsy samples (see Appendix A). Specifically, SERCA1, a protein responsible for pumping Ca^{2+} back into the ER lumen after muscle contraction, was significantly decreased in SOD1 mice and in an individual with an SOD1 mutation compared to disease control (post-polio) and individuals with a C9orf72 mutation. The ER chaperone protein Grp78/BiP was significantly up-regulated in ALS mice compared to control mice, and also tended to be higher in individuals with a C9orf72 mutation and sALS compared to individuals with a SOD1 mutation and disease control.

Proteins involved in protein synthesis, such as protein kinase B (Akt), have been linked to survival in individuals with ALS (151). Preliminary data from Dr. Chin's lab support the notion that Akt and the protein synthesis pathway is altered in ALS. Western blot analysis showed reduced Akt protein level in ALS with a C9orf72 mutation but not in ALS with a SOD1 mutation (see Appendix B). Thus, preliminary data suggested that protein markers in ALS muscle tissue differ not only between ALS and control cases, but also between subgroups of individuals with ALS, depending on the cause of the disease.

Research Focus and Hypotheses

This research study investigated whether proteins involved in SR/ER stress, Ca^{2+} handling, and protein synthesis were altered in human ALS skeletal muscle. Specifically, alterations in proteins involved in Ca^{2+} regulation (SERCA1, SERCA2, parvalbumin [PV]), ER stress (protein disulfide isomerase [PDI], Grp78/BiP, CHOP, inositol-requiring enzyme 1- alpha [IRE1α]), and protein synthesis (Akt) were assessed between ALS and healthy and diseased control human skeletal muscle. It also investigated Glyceraldehyde

3- Phosphate Dehydrogenase (GAPDH), α-actin and β-actin as loading controls. Results of the β-actin loading control experiments led to this protein being investigated as a target protein instead of a loading control. In addition, an exploratory analysis of differences within ALS subgroups was conducted (e.g. differing sites of onset, presence/ absence of gene mutations). To this author's knowledge, no studies have investigated differences in expression of these proteins in human ALS skeletal muscle, with the exception of Akt. Previously, Dr. Chin's lab identified alterations in SERCA1, SERCA2, PV, PDI, Grp78/BiP, CHOP, IRE1α, and Akt in the common mouse model of ALS. Based on these findings, it was hypothesized that SERCA1, SERCA2, and PV levels would be lower in ALS and PDI, Grp78/BiP, CHOP, IRE1α, and Akt levels would be higher in ALS compared to healthy controls.

Specific Aims

Specific Aim 1: to optimize the protein quantification assay (western blot technique) for assessment of SERCA1, SERCA2, PV, PDI, Grp78/BiP, CHOP, IRE1α, Akt, and β-actin in human skeletal muscle samples.

Specific Aim 2: to quantify differences in muscle protein levels of SERCA1, SERCA2, PV, PDI, Grp78/BiP, CHOP, IRE1α, Akt and β-actin in skeletal muscle samples from ALS cases compared to disease and healthy controls.

Specific Aim 3: to conduct exploratory analyses of differences in muscle protein levels of SERCA1, SERCA2, PV, PDI, Grp78/BiP, CHOP, IRE1α, Akt, and β-actin between subsets of ALS cases (i.e. limb onset vs. bulbar onset; sALS vs. fALS).

Chapter 2: Methods

Autopsy skeletal muscle samples from ALS, healthy controls, and disease controls were analyzed via western blot. ALS samples included sALS, fALS, samples with a C9orf72 mutation, and one sample with a SOD1 mutation. Additionally, muscle samples were from individuals with limb and bulbar onset, and taken from several different muscles. Samples were probed for SERCA1, SERCA2, PV, PDI, Grp78/BiP, CHOP, IRE1α, Akt, and β-actin and protein levels quantified by chemiluminescence. Differences in protein levels of each target protein were analyzed between ALS and controls. Exploratory analyses were conducted between ALS subgroups.

Muscle Samples

Sixty-three muscle autopsy samples from 47 cases, including ALS of varying subtypes (sALS, fALS, C9orf72), healthy controls (CON), and disease controls were analyzed. Banked skeletal muscle samples from autopsy were obtained from Dr. Lyle Ostrow and Dr. Justin Kwan. Dr. Chin has an IRB Exemption category #4 letter (Project #404343-1) for the "Analysis of human skeletal muscle biopsy samples for ALS biomarkers" which covers this project (Appendix O). Disease controls were spinal muscle atrophy (SMA) cases; because these were not age-matched to ALS and healthy control cases, a control SMA (CON-SMA) group that was age matched to the SMA samples was also included.

Study blinding

The researcher conducting analyses was blind to sample identities during immunoblotting and quantification of target proteins (see Appendix B). After quantification was completed, Dr. Chin un-blinded the data for analyses.

Tissue Homogenization

Muscle samples were stored at -80°C prior to use. During the homogenization process samples were stored in liquid nitrogen until just before homogenization took place. Samples were transferred from liquid nitrogen directly to a tube that contained chilled RIPA buffer (0.15M NaCl, 0.01M Tris-HCl pH 8, 0.005M EDTA, 0.5% Sodium Deoxycholate, 0.1% SDS, 1% Triton-X100, and Roche protease inhibitor cocktail tablet REF# 11836170001) on ice. Dr. Chin's lab previously conducted an experiment comparing buffers for homogenization of human skeletal muscle, and determined the RIPA buffer produced the best results with human muscle tissue. The muscle sample/ RIPA buffer combination remained on ice throughout the entire homogenization process. The muscle tissue was minced with sterile lab scissors and further homogenized with a polytron for 3 x 10sec (~ 30 seconds total). Homogenates were then spun at 4^{o}C for 10 minutes at 14000 rpm (20000 RCF). Soluble protein (i.e., the supernatant) was extracted with a micropipette.

Determination of Protein Concentration

Protein concentration of lysates was determined by the bicinchoninic acid (BCA) assay (Thermo Scientific BCA Assay Protocol). Briefly, a serial dilution of bovine serum albumin (BSA) was loaded in triplicates into a 96-well plate, along with triplicates of each sample lysate and a triplicate of diluted RIPA buffer (25µl of each/well). BCA reagents were combined (1 part reagent B: 50 parts reagent A) and 200µl of the combined reagents was added to each well utilizing a multi-tip pipette. The 96-well plate was incubated at 37°C for 30 minutes on a rotary platform then analyzed with a

spectrophotometer. Sample lysates of unknown protein concentrations were compared to the BSA standard (known protein concentration) and protein concentrations of the sample lysates were extrapolated.

Sample Preparation

To prepare samples for electrophoresis, equal amounts of total protein (as determined by BCA assay) of each sample was combined with Bio-Rad Laemmli 2x Loading Buffer and boiled at 100°C for 5 minutes. Samples were then chilled on ice and stored at 4°C until ready for use.

Electrophoresis

Equal amounts of total protein (approximately 14µg) of each sample was loaded into Bio-Rad Mini-Protean TGX precast gels along with a molecular weight marker (Bio-Rad Precision Plus Dual Xtra Protein Standard).

Gels were electrophoresed at 175V for various lengths of time, depending on the molecular weight of the target protein. After electrophoresis, proteins were transferred to a polyvinyladine fluoride (PVDF) membrane using Bio-Rad Trans Blot ® Turbo Transfer packs and the Bio-Rad Trans Blot ® Turbo Transfer system. Membranes were then blocked in 3% BSA for 1 hour at room temperature on a rotary platform.

Immunoblotting and Target Protein Detection

Membranes were incubated in primary antibodies against target proteins (SERCA1, SERCA2, PV, PDI, Grp78/BiP, CHOP, IRE1α, Akt, and β-actin) overnight at 4°C on a rotary platform. See Table 1 for antibody vendors and dilutions. The following day,

membranes were washed in 1x TBST 3 times for 10 minutes each at room temperature. Membranes were then incubated in the appropriate secondary antibody (see Table 1) for 1 hour at room temperature, followed by 3 x 10 minute 1x TBST washes at room temperature. Membranes were incubated in Bio-Rad Clarity Electrochemiluminescence (ECL) substrate for 4 minutes, and then exposed using the Bio-Rad Chemidoc MP equipment. If weak or no signal was detected from target bands, the membrane was immediately incubated in Thermo Scientific SuperSignal ® West Dura Extended Duration Substrate and re-imaged.

Table 1. *Antibody vendors and dilutions*

Antibody	**Vendor**	**Catalog Number**	**Dilution**	**Secondary**
SERCA1	CST	12293s	1:1000	Rabbit
SERCA2	CST	9580s	1:1000	Rabbit
PV	Swant	pvg-214	1:1000	Goat
PDI	CST	2446s	1:1000	Rabbit
Grp78/BiP	CST	3177p	1:1000	Rabbit
CHOP	CST	2895s	1:1000	Mouse
IRE1α	CST	3294	1:1000	Rabbit
Akt	CST	4685s	1:1000	Rabbit
β-actin	Santa Cruz	47778	1:1000	Mouse
GAPDH	Thermo Scientific	MA5-15738-BTLN	1:1000	Mouse
α-actin (Acta1)	Protein Tech Group	C7521	1:1000	Rabbit
Anti-goat	Novus	NB710-H	1:1000	N/A
Anti-mouse	CST	7076s	1:1000	N/A
Anti-rabbit	CST	7074s	1:1000	N/A

In some cases, membranes were stripped and re-probed for a different target protein. In these cases, membranes were incubated in strip buffer (0.1M BME, 2% SDS, and .06M Tris-HCl pH 6.8) for 30 minutes at 60°C, washed in 1x TBST (2 x 15 minutes each followed by 2 x 5 minutes each), and incubated in a different primary antibody (overnight at 4°C, followed by the same procedure previously detailed).

Target Protein Identification and Quantification

Target proteins were identified by comparison to a protein standard with known molecular weight markers run on the gel (Bio-Rad Precision Plus Protein Dual Xtra Protein Standard). Using Bio-Rad Image Lab 5.0 software, images of the molecular weight marker were taken under the Alexa setting and overlaid with chemiluminescent images (Appendix C). Target chemiluminescent bands were identified according to molecular weight. Target proteins were then quantified for volume intensity with Bio-Rad quantitation analysis tools (Image Lab Software 5.0).

Loading Control

Total protein was selected as a loading control (see loading control experiments under Condition Optimization Experiments section below). Membranes were stained for total protein with Thermo Scientific Memcode PVDF stain (according to Thermo Scientific protocol). Membranes were then imaged with the Bio-Rad Chemidoc MP equipment and total protein signal quantified for volume intensity with Bio-Rad Image Lab software.

Condition Optimization Experiments

The lab recently acquired a new system (Bio-Rad Trans-Blot ® Turbo Transfer System) for transferring protein to PVDF membranes. Hence, it was necessary to optimize Western Blot conditions to the new system. Several preliminary experiments were run to determine optimal loading volumes of proteins, SDS-PAGE gel percentages, transfer times, and a reliable loading control.

Transfer times. To assess the optimal length of time for transferring proteins from SDS-PAGE gels to PVDF membranes, different transfer times were tested, and two PVDF membranes were placed back to back to determine if any proteins were being over-transferred. After transfer, gels and membranes were visualized to determine the amount of protein that was transferred to the PVDF membrane, if any protein still remained on the gel, and if any protein was over-transferred through the first PVDF membrane onto the second membrane.

Percentage of SDS-PAGE gel. Determination of the optimal percentage of SDS-PAGE gel to use was primarily trial and error. Initial western blots utilized the percentage gel recommended by Bio-Rad according to the molecular weight of the target protein. This percentage was adjusted depending on quality of the western blot results.

"Housekeeping gene" loading control determination. Multiple loading controls that are frequently used in the literature were tested, including GAPDH, β-actin, α-actin, and total protein. In many cases, the same membrane was probed for different loading controls, and quantification of bands from the same membrane were compared. Bio-Rad stain-free gels were tested, with the hope of using total protein on the PVDF membrane visualized with the stain free technique as a loading control. With the stain free technique, gels contain trihalo compounds that react with tryptophan residues in proteins and can be visualized on gels or PVDF membranes, yielding an image of the total protein. Total protein was also visualized using Memcode Stain for PVDF membranes (Thermo Scientific).

Linearity of chemiluminescent signal. To determine if chemiluminescent signal increased linearly with increasing protein amount, western blots were conducted in which a range of total protein was loaded across a gel, and chemiluminescent signal in each lane was quantified. Specifically, 6-20μg of protein of the same sample was loaded across lanes and western blot was carried out as usual. Signal of the target bands were then quantified and graphed to determine if chemiluminescent signal increased linearly with increasing protein. This was done for four different samples: one that previously showed high signal for Grp78/BiP and one that showed low signal for Grp78/BiP, as well as two others that showed high/ low signal for SERCA1. Linear regression analyses were completed to look at linearity of chemiluminescent response.

Statistical Analysis

One-way between subjects analysis of variance (ANOVA) with least significant difference (LSD) post hoc analyses (when appropriate) was used to compare protein levels of each target protein between groups. In some instances, t-tests were used to assess the difference in protein levels between two groups. Except when indicated otherwise, results reported are one-way between-subjects ANOVA (number of cases [n], mean [M], standard deviation [SD]). Additionally, because a shift from type II to type I muscle fibers has been observed in ALS, the ratio of SERCA2 to SERCA1 protein levels was also compared between groups. A p value of < 0.05 was considered significant. Group comparisons included:

- Different disease groups (all samples): ALS, healthy controls (CON), SMA, and controls age matched to SMA (CON-SMA)

    - Different disease groups using only psoas muscles
- Cases with confirmed C9orf72 mutation present vs. absent
- Cases with known familial history of ALS (fALS) vs. those known to lack a familial history (sALS)
- Limb vs. bulbar onset
- Analyses of samples from specific muscles:
    - Quadriceps muscle vs. biceps muscle from the same individual with ALS

Chapter 3: Results

Method for Identifying Target Proteins

Utilizing Bio-Rad Image Lab software, chemiluminescent images showing antibody – protein signals and Alexa images showing protein marker (Bio-Rad Dual Xtra) with bands of known molecular weight were superimposed (see Appendix C). Target bands were then identified according to expected molecular weight and the molecular weight standard.

Condition Optimization

Timing of PVDF transfer. The Bio-Rad preset transfer setting "turbo" was ideal for most target proteins (see Appendix D). Under this setting, the majority of proteins below 100 kDa were transferred from the SDS-PAGE gel to the PVDF membrane, and over-transfer was minimal. For higher molecular weight proteins such as SERCA1 and SERCA1, the "high molecular weight" preset setting was used.

Percentage of SDS-PAGE gel. Both 10% and 12% Bio-Rad Mini-Protean TGX pre-stained, precast gel produced quality results (see Appendix E). In some instances, 7.5% gels appeared to produce diffuse target bands, thus only 10% and 12% gels were used.

"Housekeeping gene" loading control determination. Different endpoints were investigated as a loading control (see Appendix F). GAPDH, β-actin and α-actin (specifically, Acta1) showed much variability across lanes, and were thus determined to be unreliable as loading controls. After observing β-actin variability, the decision was

made to include β-actin as a target protein to determine if there were certain groups (or muscles) that tended to show higher or lower protein levels.

Bio-Rad stain free gels were tested to determine total protein, however, results showed very weak signal and high background. Total protein visualized with Memcode PVDF stain provided the clearest visualization of total protein and was determined to be the best loading control option. Total protein signal for each entire lane was quantified with Image Lab Software and the coefficient of variation for each membrane was determined. Only those membranes with coefficients of variation less than 20% were considered acceptable.

Linearity of chemiluminescent signal and quantity of protein loaded per lane. After the optimizations above were completed, the linearity of response of the chemiluminescent signal with increased total protein load was evaluated for linearity as a way to determine whether differences between samples and between subject groups fell within the linear range of this assay. Chemiluminescent signal increased linearly with increasing amount of protein at both the lower range of signal and higher range of signal for both SERCA1 and Grp78/BiP. It is possible that signal may drop off at extremely high protein values (see Figure 1A and 1B). Alternatively, the observed results could have been due to less than the desired amount of protein (20μg) loaded into these lanes due to the small size of the wells (i.e., some of the sample may have "spilled over" and not stayed within the well). These results were also utilized to determine the optimal amount of protein to load into the gels. Due to the decrease in signal observed at 20μg, it was determined that less than this amount was optimal. Because signal appeared linear between 6-18μg of protein, ~14μg of protein per lane was chosen—an amount that was

thought to balance between effects of pipetting error (which would be more prominent at very low amounts of protein) and conservation of samples (loading higher amounts of protein would require more of the muscle tissue, perhaps unnecessarily).

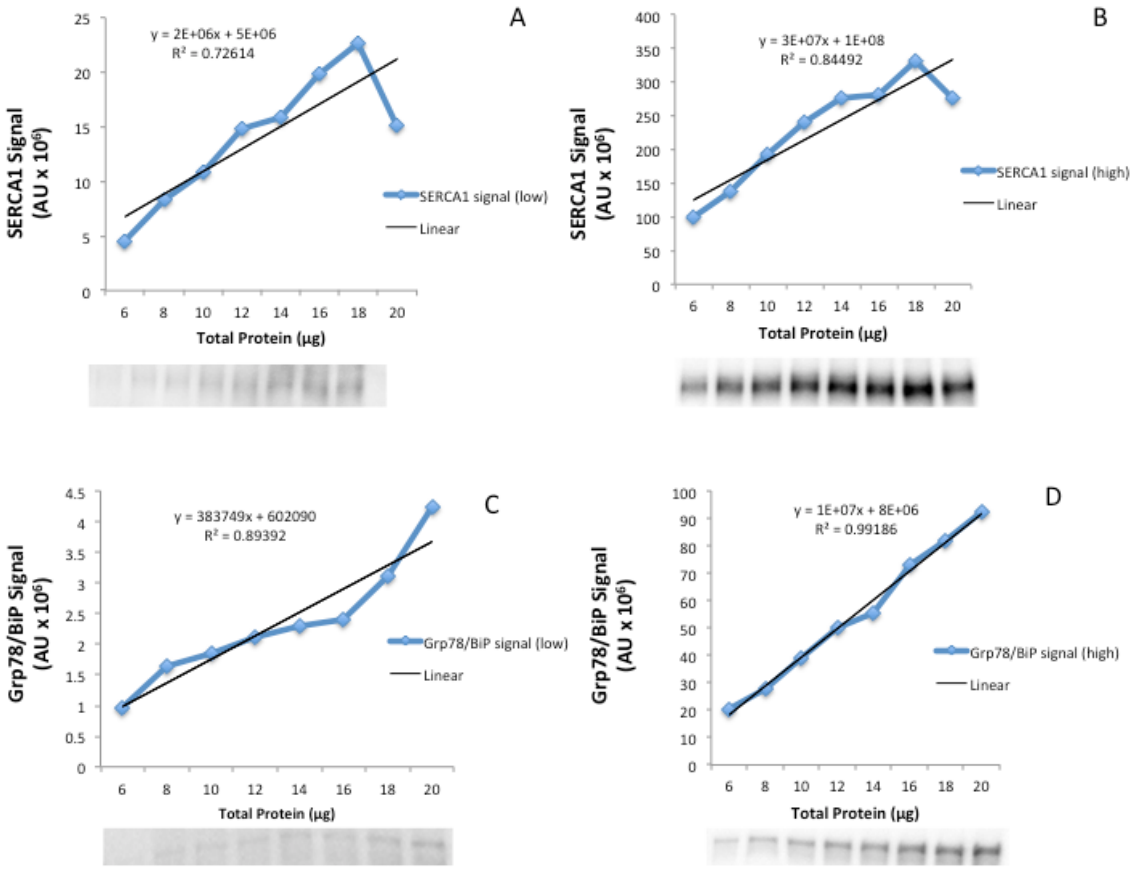

Figure 1. Graphs of chemiluminescent signal for SERCA1 (A and B) and Grp78/BiP (C and D) for a range of total protein concentrations, with raw chemiluminescent image depicted below each graph. Samples with very low signal for the target protein are shown on the left (A and C), while samples with high signal for the target protein are shown on the right (B and D). X-axis values are µg of total protein loaded in each lane; y-axis values are protein signal (AU x 10^6). Blue lines indicate signal of target protein; black lines indicate a linear regression. Equations for regression lines and R^2 are shown above the lines.

Western Blot Results

Target bands for PV and IRE1α could not be confidently identified. Membranes for these proteins contained significant amounts of non-specific binding, and no clear bands at the target molecular weight of these proteins could be identified (Appendix G shows results for IRE1α). Hence, these two proteins were excluded from the study.

Differences in protein levels between disease and control groups.

Levels of Akt, Grp78/BiP, CHOP, and β-actin, were significantly different between disease groups, while SERCA1, SERCA2, and PDI levels were not significantly different between groups (see Appendix H).

Akt. One-way between-subjects ANOVA conducted between ALS, CON, SMA, and CON-SMA revealed a significant effect of disease group on Akt protein level ($p = 0.02$). LSD post hoc comparisons indicated that mean protein level for the ALS group ($n = 38$, $M = 94{,}261{,}937$, $SD = 62{,}180{,}996$) was significantly higher than mean protein level for the CON group ($n = 13$, $M = 43{,}353{,}982$, $SD = 47{,}917{,}747$; $p = 0.007$). Mean protein level for the SMA group ($n = 7$, $M = 117{,}595{,}063$, $SD = 36{,}943{,}467$) was also significantly higher than the control group ($p = 0.007$; Figure 2). The CON-SMA group ($n = 3$) was not significantly different from any other groups.

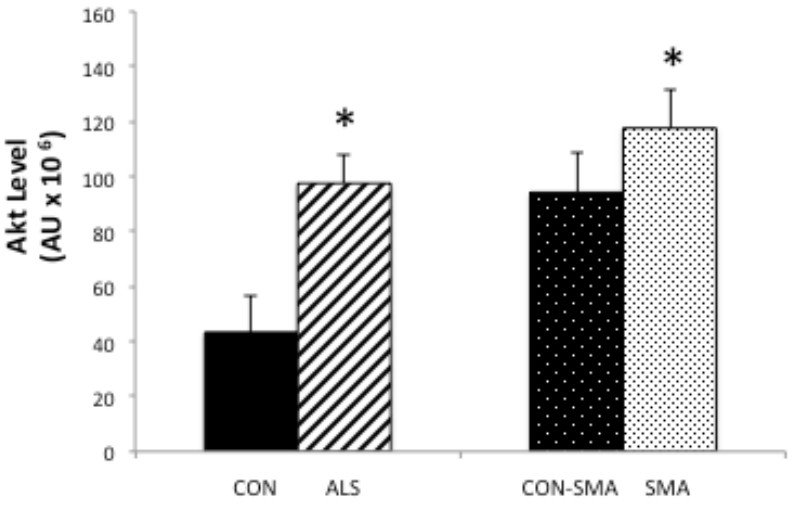

Figure 2. Average Akt levels from western blot analyses for the 4 disease groups. Akt protein level was significantly higher in ALS and SMA skeletal muscle compared to CON. Data shown are mean protein levels (AU x 10^6) + standard error (SE). *Indicates significant difference from CON, $p < 0.05$.

Grp78/BiP. Analyses also revealed a significant effect of disease group on Grp78/BiP protein level between the four groups ($p = 0.034$). Post hoc comparisons indicated that mean protein level for the ALS group ($n = 39$, $M = 51{,}634{,}409$, $SD = 41{,}020{,}106$) was significantly higher than mean protein for the CON group ($n = 13$, $M = 14{,}483{,}527$, $SD = 19{,}883{,}948$; $p = 0.005$; Figure 3). The CON-SMA group ($n = 3$) and SMA group ($n = 5$) were not significantly different from any other groups.

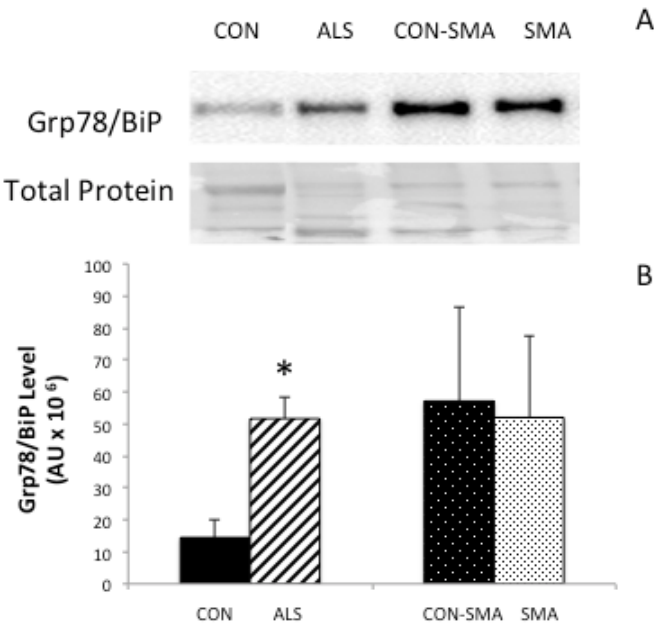

Figure 3. Grp78/BiP western blot results showing protein levels for different disease groups. Grp78/BiP chemiluminescent image is shown with total protein below demonstrating equal loading across lanes (A). Bar graph (B) shows mean protein levels (AU x 10^6) + SE for each group. *Indicates significant difference from CON, $p < 0.05$.

CHOP. Significant differences were seen for CHOP protein levels between groups ($p = 0.029$). ALS was not different from CON, but ALS had significantly higher protein levels ($n = 38$, $M = 95,146,570$, $SD = 62,225,605$) than SMA ($n = 6$, $M = 41,525,999$, $SD = 41,799,636$; $p = 0.036$) and CON-SMA ($n = 3$, $M = 13,152,370$, $SD = 17,349,400$; $p = 0.019$; Figure 4). The CON group ($n = 12$) was not significantly different from any other groups.

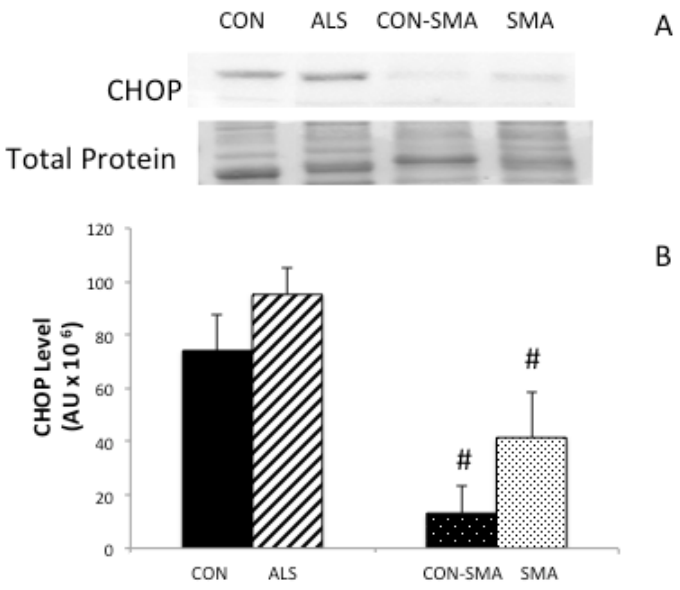

Figure 4. CHOP western blot results showing protein levels for different disease groups. CHOP chemiluminescent image is shown with total protein below demonstrating equal loading across lanes (A). Bar graph (B) shows mean protein levels (AU x 10^6) + SE for each group. # Indicates significant difference from ALS, $p < 0.05$.

β-actin. There was also a significant effect of disease group on β-actin protein levels (Figure 5) between the four groups. Specifically, protein levels in the CON group (n = 13, M = 28,556,806, SD = 42,686,415) and ALS group (n = 38, M = 50,856,808, SD = 63,639,054) were significantly lower than protein levels in the SMA (n = 7, M = 111,434,993, SD = 64,084,267; p = 0.005 vs. CON; p = 0.018 vs. ALS) and CON-SMA (n = 3, M = 125,095,703, SD = 75,905,545; p = 0.015 vs. CON; p = 0.045 vs. ALS) groups.

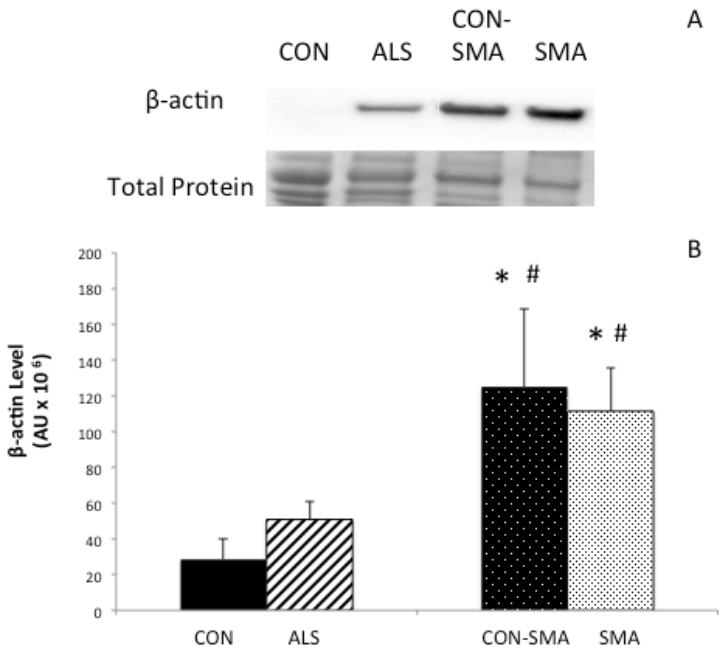

Figure 5. β-actin western blot results showing protein levels for different disease groups. β-actin chemiluminescent image is shown with total protein below demonstrating equal loading across lanes (A). Bar graph (B) shows mean protein levels (AU x 10^6) + SE for each group. *Indicates significant difference from CON, $p < 0.05$. # Indicates significant difference from ALS, $p < 0.05$.

SERCA2, SERCA1 and PDI. ANOVA indicated there was not a significant effect of disease group on SERCA2 (Figure 6A), SERCA1 (Figure 6B), or PDI (Figure 6C) protein levels between the four groups.

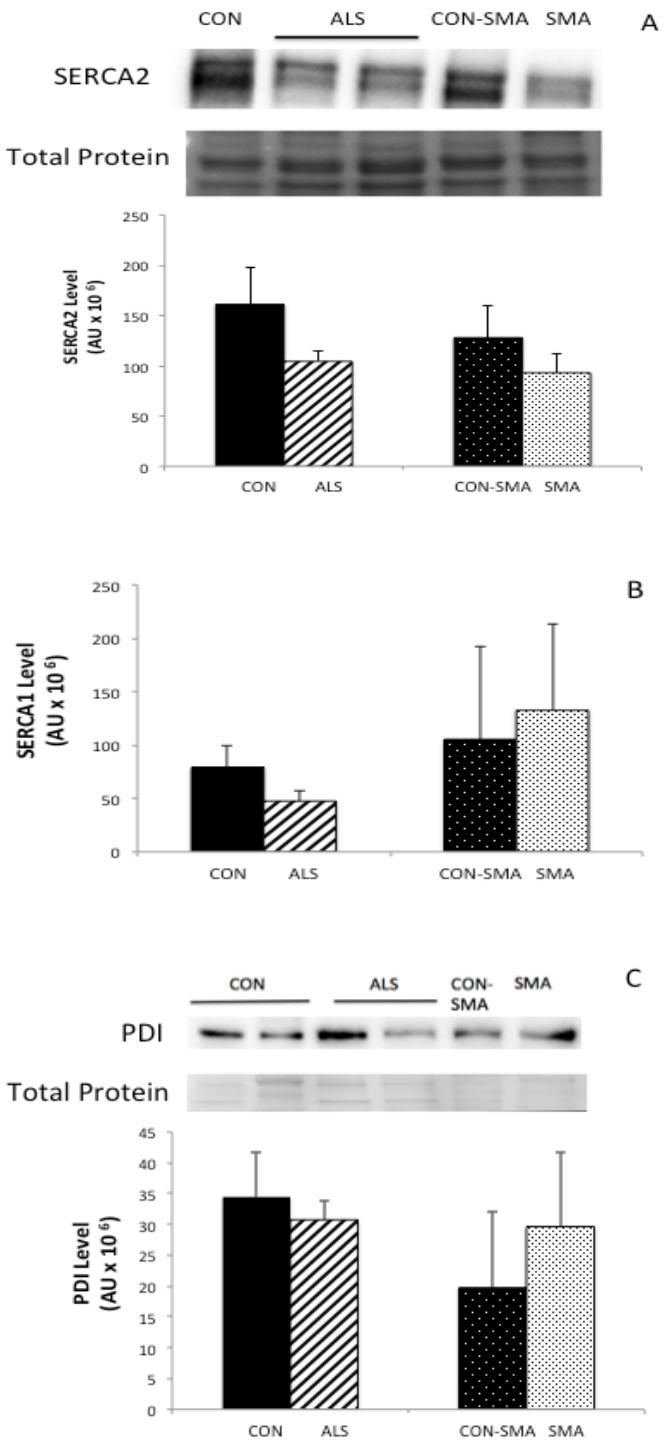

Figure 6. Western blot results for SERCA2 (A), SERCA1 (B) and PDI (C) protein levels in different disease groups. Chemiluminescent images for SERCA2 (A) and PDI (C) are shown

with total protein below demonstrating equal loading across lanes. Bar graphs for each target protein show mean protein levels (AU x 10^6) + SE for each group.

SERCA2/SERCA1 ratio. Although no significant differences were found between ALS and CON in SERCA1 or SERCA2 levels, t-test revealed the SERCA2/SERCA1 ratio was significantly higher in ALS (n = 38, M = 13.87, SD = 21.46) compared to CON (n = 13, M = 3.89, SD = 2.79; p = 0.008; Figure 7B). Levene's test for equality of variances between ALS and CON for SERCA2/SERCA1 ratio was significant (p = 0.012; Appendix I), indicating a significant difference in variance of SERCA2/SERCA1 ratio between ALS and CON (see Figure 8 for scatterplot of SERCA2/SERCA1 ratios for ALS and CON). Hence, the t-test results for equal variances not assumed were considered (and were significant). Additional t-tests were conducted in an effort to identify a potential source of the greater variability seen in the SERCA2/SERCA1 ratio in ALS. T-tests revealed the SERCA2/SERCA1 ratio was significantly higher in diaphragm muscles (n = 3, M = 12.55, SD = 1.27) compared to quadriceps muscles (n = 7, M = 1.48, SD = 1.43; p < 0.001; Figure 13A) in ALS. No significant differences in SERCA2/SERCA1 ratio were found between sites of disease onset, or congruence/ incongruence between site of onset and muscle (e.g. limb onset and psoas muscle considered congruent, bulbar onset and psoas muscle considered incongruent).

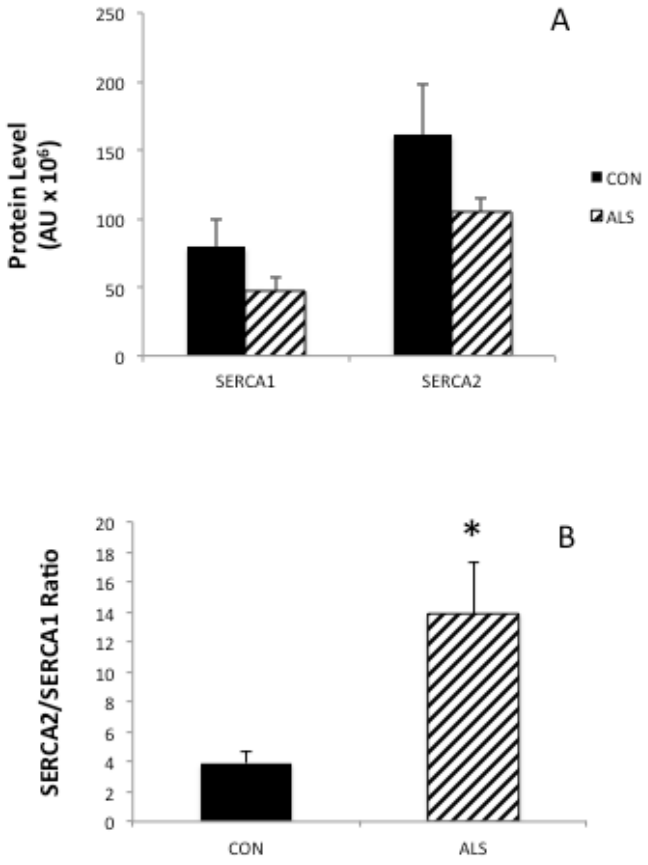

Figure 7. Bar graph showing SERCA1 and SERCA2 levels in ALS and CON (A) and SERCA2/ SERCA1 ratio for ALS and CON (B). Values shown in A are mean protein levels (AU x 10^6) + SE. *Indicates significant difference from CON, $p < 0.05$.

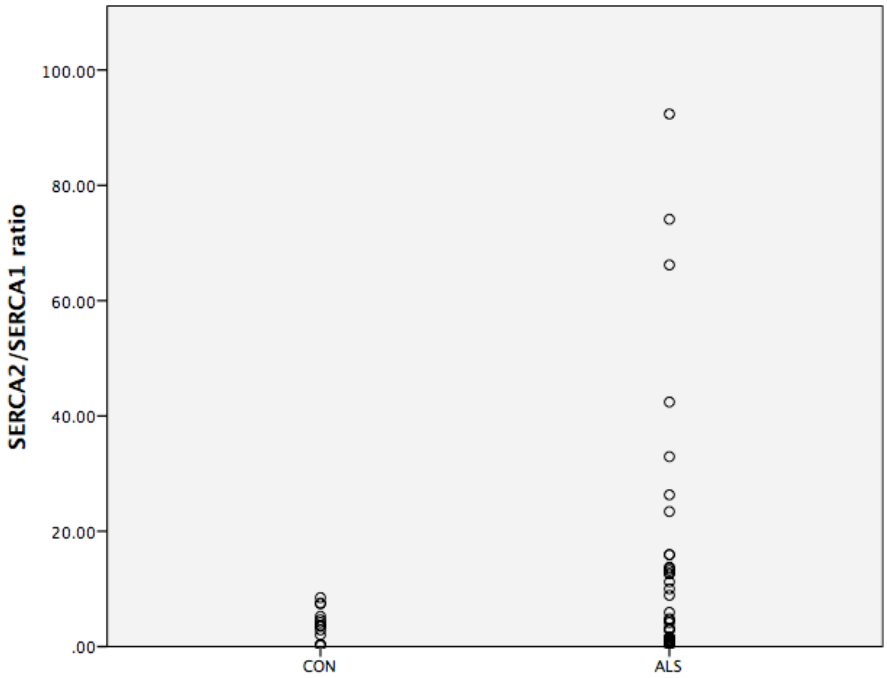

Figure 8. Scatterplot of SERCA2/SERCA1 ratio for CON and ALS cases.

Analyses of differences in protein levels between disease groups for psoas muscles only. Due to the different muscles obtained for the CON and ALS cases and the concern that there could have been differences in protein levels between the different muscles, separate analyses were completed on a sub-set of cases where psoas muscle was available (Appendix K). T-tests conducted between CON and ALS for psoas muscles showed that Grp78/BiP was significantly higher in ALS (n = 6, M = 76,927,621, SD = 57,326,843) compared to CON (n = 13, M = 14,483,527, SD = 19,883,947, p = 0.044; Figure 9). CHOP protein levels were also significantly higher in ALS (n = 6, M = 135,170,418, SD = 63,426,144) compared to CON (n = 12, M = 74,113,150, SD = 47,347,041, p = 0.035; Figure 9) psoas muscles. The CON-SMA group only contained one psoas muscle, so this group was not included in these analyses. Two of the 3 SMA

psoas samples were on the same membrane and potentially skewed, hence, this data was excluded from these analyses (see discussion).

Similar trends in protein levels for ALS and CON groups were observed when including all muscles vs. only the psoas muscle. The degree of differences in protein levels between ALS and CON did vary between all muscles vs. only the psoas muscles in some cases, but differences were always in the same direction regardless of muscle group. With the exception of PDI—which showed low signal and little variability across all samples— differences in protein levels between ALS and CON were in the hypothesized directions. Figure 9A shows differences in protein for each target protein between ALS and CON samples for all muscles. As hypothesized, levels of Akt and Grp78/BiP were significantly higher in ALS compared to CON. Although not statistically significant, differences in expression of SERCA1, SERCA2, and CHOP were in the predicted directions. SERCA1 expression was ~40% lower in ALS than in CON. Expression of CHOP was 22% higher in ALS compared to CON. As shown in figure 9B, similar trends were observed when only the psoas muscle was included in analyses.

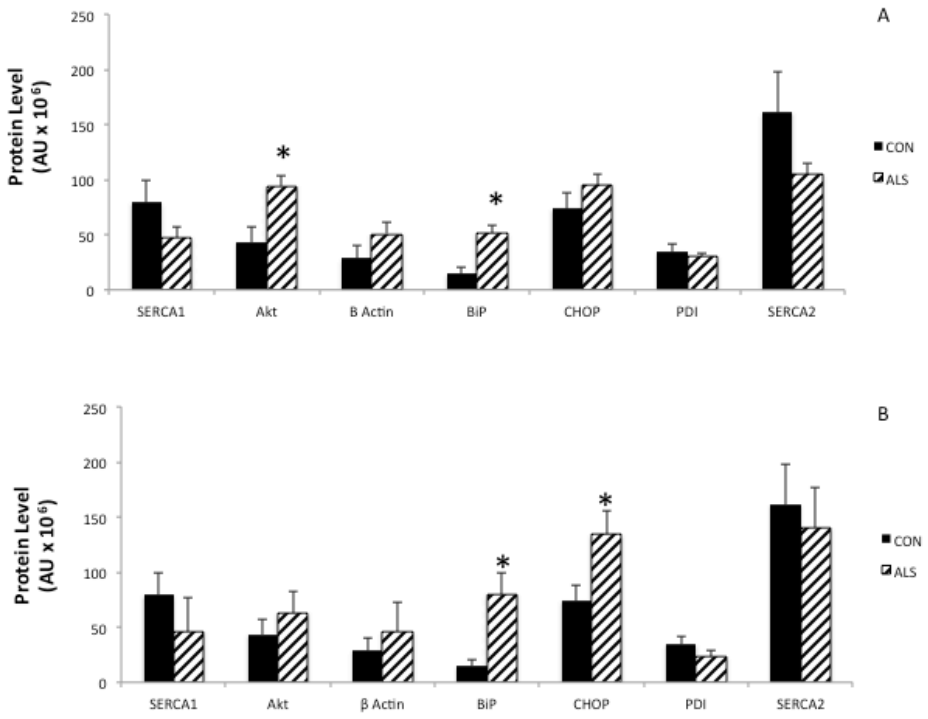

Figure 9. Bar graphs showing mean protein levels (AU x 10^6) + SE for each target protein in ALS and CON samples for all muscles combined (A) and only psoas muscles (B). *Indicates significant difference from CON, $p < 0.05$.

Differences in protein levels in C9orf72 cases. ANOVA in target protein levels between ALS cases that had a C9orf72 mutation (defined clinically as a GGGGCC repeat expansion of > 30; +C9orf72; n = 7), ALS cases that were confirmed to lack a C9orf72 mutation (30 or less repeat expansions; - C9orf72; n = 13), and CON samples (n = 13) revealed significant differences between these three groups (Figure 10; Appendix L) in levels of SERCA1 ($p = 0.022$), and Grp78/BiP ($p = 0.015$). Specifically, SERCA1 was significantly higher in CON (M = 79,243,663, SD = 73,560,886) than in + C9orf72 (M = 13,175,395, SD = 11,620,125; $p = 0.016$) and – C9orf72 (M = 27,433,424, SD = 46,187,598; $p = 0.023$; Figure 10A and 10C) and Grp78/BiP was significantly lower in CON (M = 14,483,527, SD = 19,883,948) compared to + C9orf72 (M =52,020,565, SD

=37,976,519; p = 0.031) and – C9orf72 (M =54,622,042, SD = 44,734,804; p = 0.007; Figure 10B and 10C). No significant differences were found in any other target proteins between + C9orf72, - C9orf72, and CON groups.

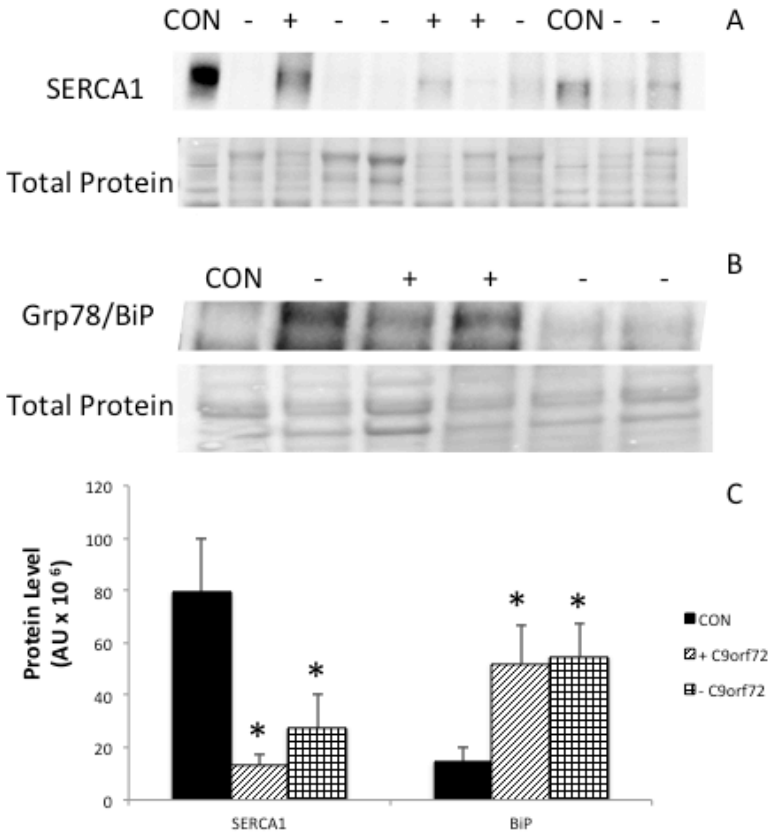

Figure 10. Western blot results for SERCA1 (A) and Grp78/BiP (B) in cases that had a C9orf72 mutation (+), cases that did not have a C9orf72 mutation (-), and CON. Chemiluminescent image for SERCA1 and Grp78/BiP is shown with image of total protein stain below demonstrating equal loading across lanes. Panel C shows mean protein signal (AU x 10^6 + SE) of SERCA1 and Grp78/BiP for the three groups. *Indicates significant difference from CON, $p < 0.05$.

Differences in protein levels between sALS and fALS. There was a significant effect of group on SERCA1 (p = 0.025) and Grp78/BiP (p = .004) protein levels between cases with a known familial linkage (fALS), those that were known to lack a familial

linkage (sALS) and CON (Figure 11; Appendix M). Post hoc analyses revealed that mean SERCA1 levels in the fALS (n = 3, M = 6,556,413, SD = 5,035,731), and sALS (n = 17, M = 26,360,052, SD = 40,920,870) groups were significantly higher than mean protein levels in the CON group (n = 13, M = 79,243,663, SD = 73,560,886; p = 0.049 vs. fALS; p = 0.014 vs. sALS). Grp78/BiP was significantly higher in the sALS group (n = 18, M = 63,994,984, SD = 46,540,662,) compared to the CON group (n = 13, M = 14,483,527, SD = 19,883,948, p < 0.001). No significant differences in any target proteins were found between the fALS and sALS groups.

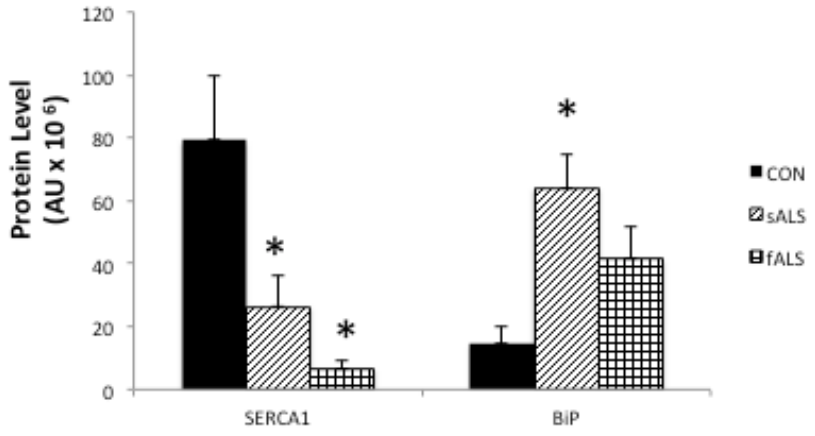

Figure 11. Bar graph showing mean SERCA1 and Grp78/BiP protein levels in sALS, fALS and CON. Data shown are mean protein levels (AU x 10^6) + SE. *Indicates significant difference from CON, p < 0.05.

Differences in target protein levels in limb vs. bulbar onset. There were significant differences between groups in Akt (p = 0.027), β-actin (p = 0.025), and Grp78/BiP (p = 0.010) protein levels between limb onset, bulbar onset, and CON (Figure 12; Appendix N). Specifically, Akt and Grp78/BiP protein levels were significantly higher in bulbar (Akt n = 12, M = 101,777,107, SD = 52,314,906, p = 0.017; Grp78/BiP

n = 13, M = 58,747,807, SD = 47,405,522, p = 0.004) and limb (n = 25; Akt M = 93,481,494, SD = 66,853,987, p = 0.017; Grp78/BiP M = 47,534,620, SD = 38,619,318, p = 0.013) onset compared to CON (n = 13; Akt M = 43,353,982, SD = 47,917,747; Grp78/BiP M = 14483527, SD = 19883948); β-actin expression was significantly higher in limb onset (n = 25, M = 62,736,136, SD = 66,491,068) compared to bulbar onset (n = 12, M = 14,112,736, SD = 17,299,646, p = 0.012). Although not statistically significant (p = 0.071), expression of SERCA1 was 59% higher in CON than in bulbar (Figure 12).

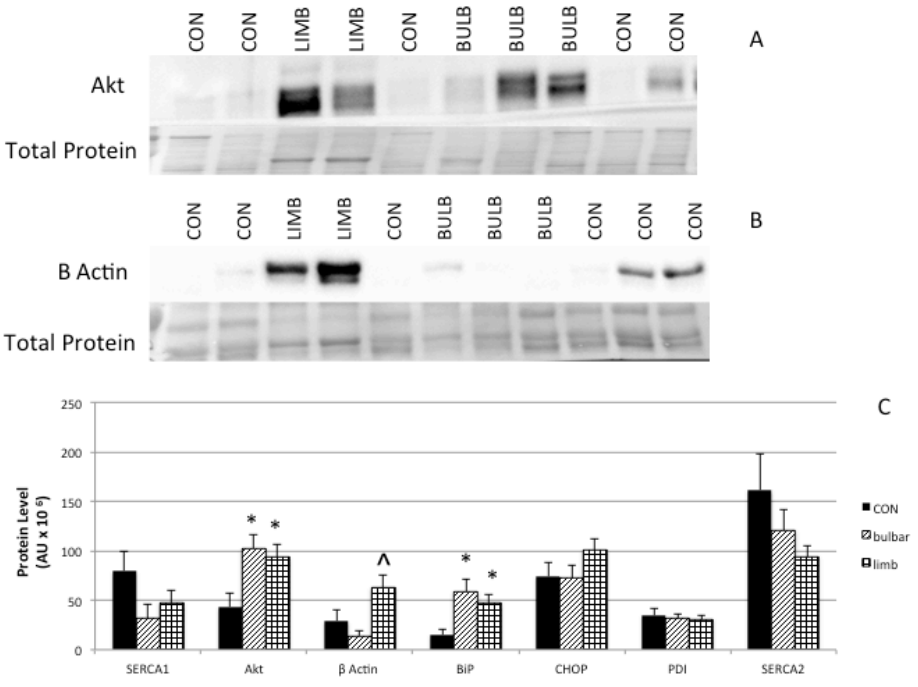

Figure 12. Western blot results showing differences in mean protein levels of all target proteins between limb onset, bulbar onset, and CON. Raw chemiluminescent western blot images for Akt (A) and β-actin (B) are shown with total protein image below showing equal loading across lanes. Data shown in C are mean protein levels (AU x 10^6) + SE. *Indicates significant difference from CON, $p < 0.05$. ^ Indicates significant difference from bulbar, $p < 0.05$.

Differences in protein levels in different muscles. ANOVA showed no significant effect of muscle source (psoas, quadriceps, diaphragm, biceps and intercostals were included; see Table 3 for n of each muscle) on level of any target proteins in the ALS group. However, independent samples t-tests revealed that SERCA1, Grp78/BiP, CHOP, and the SERCA1/SERCA2 ratio (previously discussed) levels were significantly different in ALS diaphragm muscles compared to quadriceps muscles (Appendix J). Specifically, SERCA1 was significantly lower in diaphragm muscles (M = 9185196, SD = 3360297) compared to quad muscles (M = 95500456.57, SD = 75348323.22, p = 0.023), and Grp78/BiP and CHOP were significantly higher in diaphragm muscles (Grp78/BiP M = 79,825,420, SD = 45,631,721; CHOP M = 103860866, SD = 31070804) compared to quadriceps muscles (Grp78/BiP M = 28779310, SD = 22110883, p = 0.038; CHOP M = 50441546, SD = 23977617, p = 0.017).

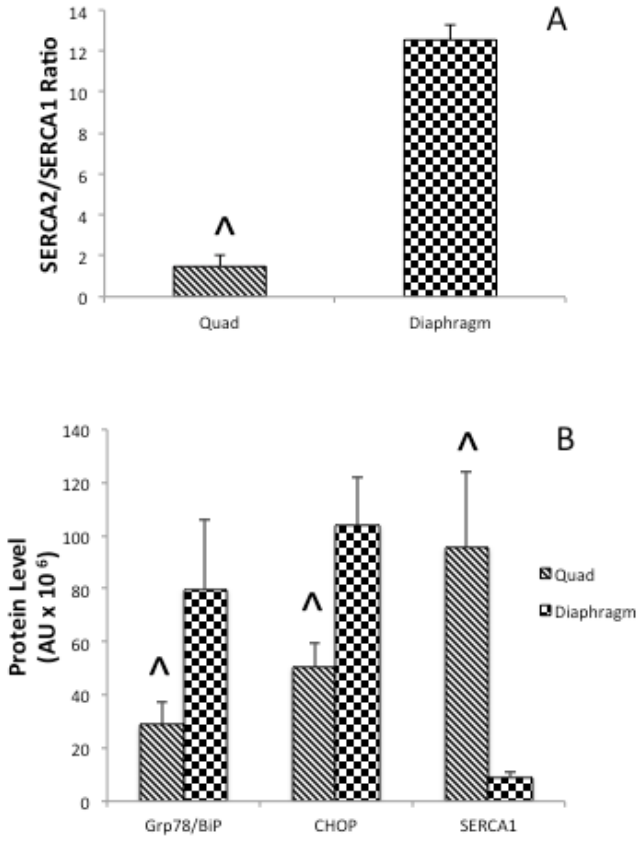

Figure 13. Mean SERCA2/SERCA1 ratio (A) and protein levels for Grp78/BiP, CHOP, and SERCA1 (B) in quadriceps and diaphragm muscles of ALS. Data shown are mean (A) ratio and (B) protein levels (AU x 10^6) + SE. * Indicates significant difference from diaphragm, $p < 0.05$.

ALS biceps (n = 7) and ALS quadriceps (n = 7) muscles were also analyzed separately since there was one of each muscle type from the same individual (for 7 individuals). No significant differences in protein levels of any target proteins were observed.

Table 2. *Location of muscle samples for each disease/ control group*

Muscle	CON	ALS	CON-SMA	SMA	Total
Deltoid		2			2
Psoas	13	6	1	3	23
Quad		7*			7
Diaphragm		3	2	4	9
Bicep		7*			7
Tongue		1			1
Intercostals		2			2
Unknown		11			11
Total	13	39	3	7	62

*Quad and bicep samples were obtained from the same individual.

Chapter 4: Discussion

Analysis of skeletal muscle autopsy samples from ALS patients and various controls demonstrated that the ER stress protein (Grp78/BiP), and Akt, a protein involved in protein synthesis, were higher in ALS compared to CON. The SERCA2/SERCA1 ratio was also higher as well as significantly more variable in ALS compared to CON. Furthermore, differences in protein levels of SERCA1, Grp78/BiP, and CHOP were observed in ALS diaphragm compared to quadriceps muscles. This study identifies the possibility that Ca^{2+} regulatory proteins, ER stress proteins, and proteins involved in protein syntheses are altered in ALS human skeletal muscle, and that different muscles may be differentially affected.

Western Blot Loading Controls with Diseased Muscle

When utilizing the immunoblotting technique, it is important to ensure equal amounts of total protein from each sample are loaded into the SDS-PAGE gel for electrophoresis. If protein loading is not equal, differences detected between samples in target proteins could be due to differences in the amount of total protein loaded rather than true differences between samples in the amount of the target protein. To ensure equal loading, researchers often probe membranes for "housekeeping" or "loading control" proteins—proteins that should be present in equal amounts in samples. The housekeeping proteins GAPDH, β- Actin, and α- Actin, commonly used in the literature, were tested in this study in order to determine which (if any) were the most reliable.

GAPDH showed much variability across samples in the present experiment (see Figure A6). This protein is a glycolytic enzyme that catalyzes the conversion of

glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate in the glycolytic pathway. Because the glycolytic capacity of type I muscle fibers is low and that of type II fibers is high, one would expect higher expression of GAPDH in type II fibers. ALS mice have shown selective denervation of large, type II motor units, while no denervation in the smaller, type I motor units was observed. These mice had significantly less type IIB muscle fibers compared to wild type mice (72). It is plausible that ALS muscle tissue would show decreased expression of GAPDH compared to healthy tissue, due to having less type II muscle fibers compared to healthy tissue. If GAPDH was used as a loading control in these cases, the quantity of total protein loaded for ALS samples would be underestimated compared to healthy tissue. Glycolytic demands may also differ between tissue that is in a normal state compared to a diseased state, contributing further to differences in GAPDH expression (10). Additionally, GAPDH is inactivated by particular oxidants (122). Given the known involvement of reactive oxygen species in ALS, it is possible that alterations in oxidant levels could be present in ALS muscle and effect GAPDH expression. GAPDH levels will also differ between the different skeletal muscle used and may also be different between individuals. Hence, there are numerous factors that could result in differences in GAPDH levels between ALS and control muscle, and its reliability as a housekeeping protein in this population is questionable. Although differences in GAPDH protein expression between ALS and control muscles were not specifically assessed, the potential for differences between groups based on the literature and the variability that was observed in the present experiment between samples lead to this protein being excluded as a loading control.

The cytoskeletal proteins tubulin and actin are abundant in cells, and therefor frequently used as a western blot loading control. Most often the α or β Tubulin families are used, which represent the major components of microtubules in the eukaryotic cytoskeleton. Given its abundance in cells, Tubulin may be a reliable loading control in many circumstances, however, recent research has discovered a high occurrence of mutation in the gene that encodes an α-tubulin (TUBA4A) in ALS (126). Similarly, alterations in the PFN1 gene, which plays a crucial role in actin dynamics, has also been linked to fALS. PFN1 mutants show reduced expression of F/G-actin (148). Hence, it is plausible neither tubulin nor actin are reliable loading controls for ALS samples.

α- Actin and β-actin were investigated in the present experiment as loading controls, and showed much variability between samples (Figure A6). Indeed, other researchers have also shown β-actin is not a reliable loading control in western blots (46, 50). After observing β-actin variability, the researchers decided to include β-actin in the target protein experiments and analyze data to determine if there were certain conditions (or muscles) that tended to show higher or lower levels of this protein. β-actin was ~4.5 times higher in limb onset compared to bulbar onset (Figure 12). Interestingly, this was the only significant difference in all of the analyses conducted in which two ALS subsets showed differences in protein levels in opposite directions relative to CON samples; β-actin level in limb onset was over 2 times that of CON, while levels in bulbar onset were approximately half that of CON.

Total protein has recently been established as a reliable loading control for western blots (2, 50, 117). Although others have been successful using the Bio-Rad stain free technique to visualize total protein (57), this method resulted in very high

background and the inability to accurately quantify total protein signal in the present experiment. Ultimately, total protein visualized with Memcode PVDF stain was selected as a loading control. This method is similar to a Ponceau or Coomassie blue stain, both of which have been reported as reliable loading controls for western blots (50, 117).

Chemiluminescent Signal Increases Linearly With Increasing Amounts of Protein

It was necessary to characterize the relationship between amount of protein and chemiluminescent signal, especially at very high or low ends of the signal range. For example, if chemiluminescent signals began to plateau at high values, a two-fold increase in amount of protein would result in a less-than-two fold increase in chemiluminescent signal. This would likely result in an underestimation of differences in protein levels when signal is very high, and have implications for accurate reporting of differences in signal. However, signal appeared to increase linearly with increasing amount of protein at both low and high ranges (Figure 1). For one of the two proteins tested (SERCA1), there was a decrease in signal (compared to what was expected with a linear increase) when 20µl (corresponding to 20µg of protein) was loaded into a well. Since this occurred in a sample that showed low signal (in addition to one that showed high signal), it is possible the drop off observed was due to some other confounding factor, and not due to the signal saturating at high values. The drop off observed in the sample showing low signal occurred around 20,000,000 AUs—a value that was well within the linear range with the sample showing high signal. Although the wells in the gels used were supposed to fit 20µl, it is likely that some of the sample was not contained within the well when 20µl was loaded. Attempting to fill the wells with 20µl of sample likely resulted in overflow, and less than 20µg of protein (20µl of sample) in the well. 14µg of protein

(corresponding to ~14µl of sample) was loaded per well for the western blots that investigated differences in target proteins. Hence, the possibility of overflow in these cases was highly unlikely.

Limitations Regarding Disease Controls

It is possible that results for the SMA condition in the psoas only analyses would have been skewed, hence this group was excluded from analyses. Two of the three SMA psoas samples were on the same membrane. In a group with only n = 3, having two of the three samples on the same membrane could lead to protein expression for this group being over-estimated or under-estimated, if that particular membrane showed very high or very low signal. Indeed, signal for SERCA1 on this particular membrane was well above the norm. Hence, comparisons to the SMA group in the psoas only analyses were not made.

In addition to possible limitations related to the lineup of SMA samples on the membrane, the disease control samples (SMA) used in this experiment were not age matched to ALS samples. Healthy control samples that were age matched to the SMA samples were obtained as well as healthy control samples age matched to ALS. In this study SMA was chosen as the disease CON since its etiology, also being a motor neuron-specific neurodegenerative disease, was closest to that of ALS. However, SMA and CON-SMA samples were from infants, since SMA types I and II have a pediatric lethality, while the average age of individuals from which the ALS samples came was 63 years. As such, it is most likely that differences observed between ALS or ALS age-matched healthy controls and SMA or SMA age matched controls (CON-SMA) ware due

to difference in age, rather than disease (or control) conditions. No significant differences in any target proteins were found between SMA and CON-SMA, further supporting the possibility that age affected protein expression. As such, this discussion will focus on differences between ALS and healthy, age-matched controls only.

Differences in Protein Levels Between ALS and Healthy Controls

Levels of Akt and Grp78/BiP were significantly different between ALS and healthy controls. In congruence with the results observed in ALS mice, Akt and Grp78/BiP levels were higher in ALS compared to CON. Akt is a protein involved in protein synthesis, and is activated upon phosphorylation. Interestingly, Leger et al (2006) found no differences in Akt mRNA between ALS and controls, but a decrease in phosphorylated Akt (the active form)/ Akt ratio in ALS skeletal muscle. Taken together with the results of the present experiment, it is possible there is a decreased ability to phosphorylate Akt in ALS. Thus, more of the protein remains in the inactive form.

The ER chaperone protein Grp78/BiP is up-regulated when an unfolded protein stress response is detected in the cell (77). The present experiment shows that Grp78/BiP is up-regulated in ALS skeletal muscle, providing support for the presence of the UPR in ALS skeletal muscle. Although other studies have investigated Grp78/BiP expression in spinal cord sections in ALS (147), the present study is the first to show alterations in Grp78/BiP in skeletal muscle.

Calcium regulation is impaired in motor neurons of individuals with ALS (17) and in skeletal muscle of ALS mice (28). Although not statistically significant, SERCA1 levels in the present study in all muscles combined were ~40% lower in ALS compared

to healthy controls (Figure 6). Impaired calcium homeostasis has a direct effect on mitochondrial function, can stimulate mitochondrial ROS production, and activate apoptotic pathways (15). Mitochondrial dysfunction is well established in ALS (17, 48, 86), and several authors have suggested mitochondrial pathogenesis is outside of the mitochondria itself (86, 141). Although more research is necessary, results from the present study present the possibility that mitochondrial dysfunction could be caused (at least in part) by misregulated calcium homeostasis resulting from reduced calcium clearance proteins in the SR. The present study extends previous research addressing alterations in calcium regulating proteins and ER stress proteins in motoneurons and spinal cord sections in individuals with ALS and in skeletal muscle of a mouse model of ALS to human skeletal muscle (Figure 14).

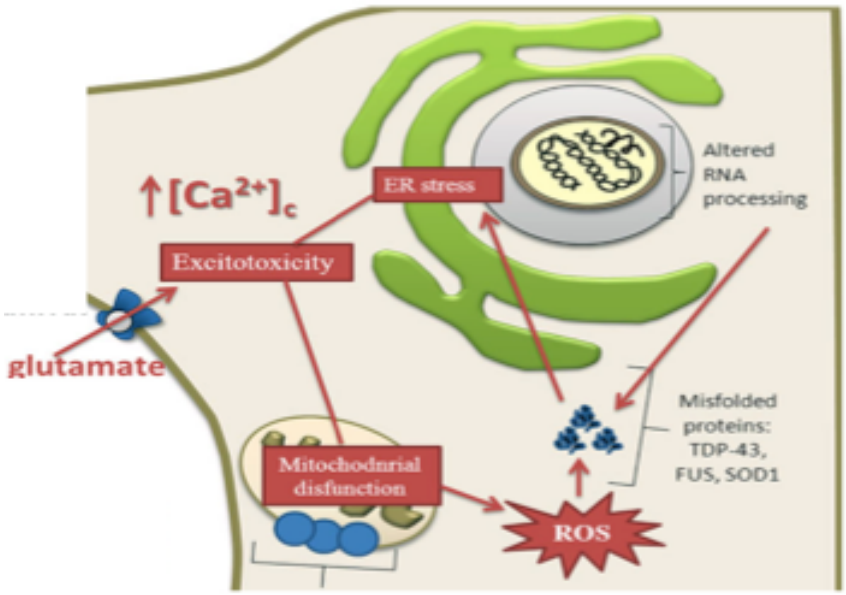

Figure 14. ER stress, mitochondrial dysfunction, calcium misregulation and reactive oxygen species (ROS) are implicated in ALS pathology (132).

The ratio of the SR/ER calcium pumps SERCA2 (found in type I muscle fibers), to SERCA1 (found in type II muscle fibers) was significantly lower in human ALS skeletal muscle compared to control muscle. Diaphragm muscles showed a particularly high SERCA2/SERCA1 ratio. Because none of the control samples in this study were from diaphragm muscles, it cannot be determined if the high SERCA2/SERCA1 ratio in diaphragm is specific to ALS or occurs in control diaphragms as well. If specific to ALS, a higher SERCA2/SERCA1 ratio could indicate a fiber type shift from type II to type I fibers. More likely, this increased SERCA2/SERCA1 ratio in diaphragm muscles could be due to decreased levels of SERCA1 protein independent of a fiber type shift, since SERCA2 levels were not different between diaphragm and quadriceps muscles. It is possible that diaphragm muscles are affected more than quadriceps muscles in ALS, which is logical considering death most often occurs due to respiratory failure. Grp78/BiP and CHOP levels were also significantly higher in diaphragm compared to quad muscles in ALS, perhaps indicative of increased ER stress. Future research that includes quadriceps and diaphragm control muscles is necessary to clarify if these differences are unique to ALS.

Differences in Protein Levels in Different Muscles

Having only psoas muscles for healthy controls and many different muscles for ALS cases presented both an opportunity to investigate differences in protein levels in different muscles in ALS and a limitation, because differences found in ALS could not be compared to control muscle. Results of analyses involving psoas muscles only were similar to those of all muscles, indicating that psoas muscles in ALS are fairly representative of the "average" muscle. However, vast differences were seen between

diaphragm and psoas muscles, indicating that these muscles could be particularly affected (or unaffected) in ALS. These findings bring to light the possibility that different muscles are affected differently in ALS. This information is useful in guiding future research—both in considerations for study designs and potential areas to investigate further.

Protein levels were not affected by C9orf72

Target protein levels were not significantly different between individuals that had a C9orf72 mutation and those that did not. For many individuals genetic testing was not performed (or if so the results were not obtained), in which case these individuals were not included in C9orf72 analyses. This resulted in fewer samples per group, reducing statistical power. Regardless, protein expression in ALS cases that had or lacked this mutation tended to be similar, so it is unlikely that differences would occur even if more samples were analyzed.

Significant differences were observed between the group with the mutation and controls, as well as the group lacking the mutation and controls. This, along with the lack of a difference in expression between the +/- C972orf groups, indicates that the mechanisms underlying skeletal muscle pathology investigated in the present experiment are not affected by a C9orf72 mutation. Not surprisingly, Grp78/BiP levels were higher in +/- C9orf72 ALS cases compared to controls, as was also the case when considering all ALS samples compared to controls. Interestingly, SERCA1 levels were significantly lower in +/- C9orf72 cases compared to controls, but not significantly different between all ALS and controls. This is potentially due to different muscles being included in +/-

C9orf72 cases compared to all ALS cases. There were no quadriceps or bicep muscles in the +/- C9orf72 cases, and these two muscles showed the highest levels of SERCA1 protein when all ALS cases were considered. The remainder of the target proteins showed similar trends to what was observed when all ALS samples were compared to controls.

Protein Levels Were Not Significantly Different Between fALS and sALS

Similar to the C9orf72 results, analyses of protein levels in fALS, sALS and controls showed similar trends to what was observed when all ALS samples were included. No significant differences were found between fALS and sALS, but differences were observed for SERCA1 and Grp78/BiP in either of these ALS groups when compared to controls. As was the case for the C9orf72 analyses, fALS and sALS cases did not contain any quadriceps or bicep muscles, resulting in lower SERCA1 levels compared to all ALS cases. Considering the overlap between genes involved in sALS and fALS, it is not surprising that no significant differences between these two groups were observed.

β-actin Was Significantly Different Between Limb and Bulbar Onset

β-actin was the only target protein for which expression was significantly different between an ALS sub-group—in this case, limb and bulbar onset. Levels of β-actin in limb onset were significantly higher than levels in bulbar onset and controls, while levels in bulbar onset were lower than that of controls. This provides further evidence that β-actin is not a reliable loading control in ALS muscle.

Not surprisingly, expression trends for bulbar/limb onset compared to controls tended to be similar to observed differences when considering all target proteins, with the

exception of CHOP. CHOP showed similar expression between bulbar onset and controls, whereas CHOP expression tended to be higher in ALS than in controls.

Study Limitations and Future Directions

Clinical information on autopsy samples was incomplete. For example, site of onset of symptoms and muscle from which the sample came is not known for all samples. As a result of this, the number of samples that were included in analyses comparing differences in proteins between such subgroups was limited, resulting in reduced power to detect differences. In addition, the amount of time from death to when samples were taken differs between samples. Although the manner in which this effects protein expression is unknown, it is possible this uncontrolled variable affected the results of the experiment.

The nature in which samples were assigned to different membranes was not ideal for the exploratory analyses of various subgroups. In this study, samples were randomized across 8 membranes. Ideally, separate western blots would be run for each of the different sub analyses, and all membranes would contain an equal number of samples from each sub group that is being analyzed (e.g., for the site of onset analysis, a separate western blot would be run with only the samples to be used in this specific analysis, alternating groups across lanes [i.e., limb, bulbar, CON, limb, bulbar, CON], with an equal amount of samples from each group on the membrane). Due to limited resources, conducting separate western blots for each of the different exploratory analyses was not feasible. Hence, the number of samples in each subgroup was not completely balanced between the different membranes. As was the case with the

analyses of psoas muscles in the SMA group, it is possible that other sub analyses could have been affected by distribution of samples across membranes. The exploratory analyses conducted were useful to identify potential differences in protein levels in a cost effective manner. The results of the present study can be used to guide follow-up experiments on alterations in skeletal muscle proteins in various subgroups of ALS.

As mentioned previously, the lack of age-matched disease controls was also a limitation in this study. Ideally, analyses would involve three age-matched groups: ALS, healthy control and diseased control.

Future studies may consider investigating the effect of exercise on protein expression in ALS muscle. To this author's knowledge, no previous studies have investigated changes in SERCA1, SERCA2, PDI, Grp78/BiP, CHOP, or Akt in ALS muscle in response to exercise training in individuals with ALS. Akt activation is known to increase with exercise (38). Perhaps an exercise intervention in ALS could "rescue" some of the decline in Akt activation that presumably occurs. The greatest effects on survival and motor function observed in ALS mice to date were seen with combined exercise training and IGF-I therapy (76). However, it is not known if increases in IGF-I associated with exercise training (rather than due to a drug intervention) would also elicit improvements in motor function and increased longevity.

Chapter 5: Review of Literature

ALS is a complicated disease that is not completely understood. A number of different pathophysiological mechanisms and ALS-linked genes have been identified and investigated. The ALS phenotype is very heterogeneous, and often difficult to diagnose clinically. Clinical features and diagnostic criteria, ALS genetics, pathophysiological mechanisms, and treatments will be reviewed.

Disease Statistics

ALS, commonly known as Lou Gehrig's disease after the renowned New York Yankees first baseman who contracted the disease in 1939, is the most common fatal neurodegenerative disorder in adults, with approximately 4 cases of ALS per 100,000 persons in the United States (103). Significant deviations from this incidence rate have been found in several ethnic populations; American Indians and Alaska Natives show lower incidence (0.63 cases per 100,000 persons (64), while incidence in two towns in Japan's Kii Peninsula was found to be significantly higher (33 and 20 persons per 100,000 (7)). Incidence of ALS in the Chamorro population on the Marianas island of Guam was reported at one time to be 50-100 times greater than anywhere else in the world—400 per 100,000 individuals (9).

Approximately 30,000 Americans have ALS at any given time and on average 15 new cases are diagnosed daily. The disease is 1.6 times more common in men vs. women and twice as common in Caucasians than in African Americans (103). The average age at diagnosis is 59 (83), and the highest prevalence rates are seen between 70-79 years of age (103). There are individuals with age at onset < 25 years old (termed Juvenile ALS).

In these cases, the disease tends to progress much more slowly than what is observed with adult onset (110). Average survival time from diagnosis is 19 months and from onset is 30 months, although much variability in these averages are seen (93). Indeed, 10% of ALS patients live longer than 10 years after diagnosis (110). Factors associated with shorter survival include older age at symptom onset, severe cognitive impairment, shorter interval between symptom onset and diagnosis, poorer motor function at first visit and bulbar onset of symptoms (62, 63, 65).

Diagnosing ALS

Individuals with ALS show vast variability in symptoms in the early stages of the disease. No biological diagnostic marker exists, and a progression of motor neuron degeneration must be observed to warrant an ALS diagnosis. As a result, ALS diagnostic timelines are often long, and frequent misdiagnoses occur. Median diagnostic time (defined as time from symptom onset to confirmed diagnosis) for individuals with ALS is approximately 11.5 months. On average patients see three physicians before an ALS diagnosis is given, and half receive an alternate diagnosis first. Diagnostic timelines tend to be longer for individuals with sALS, limb onset, and those over the age of 60 (134) (109).

El Escorial criteria for diagnosing ALS. ALS is marked by progressive degeneration of both UMNs (motor neurons in the cerebral cortex and brainstem) and LMNs (motor neurons in the spinal cord) and progressive muscle weakness. To warrant an ALS diagnosis, progressive UMN and LMN findings must be observed by examination and history (65). El Escorial criteria have been widely utilized for

diagnosing ALS. According to the El Escorial criteria, requirements for an ALS diagnosis include: evidence of LMN and UMN degeneration and progressive spread of symptoms, along with absence of evidence of other disease processes that could explain the degeneration (23).

ALS may be diagnosed clinically without pathological confirmation, based on the presence of UMN and LMN signs. In these cases, the terms Clinically Definite, Clinically Probable, or Clinically Possible ALS are used, each having specific criteria relating to the region affected and number of UMD and LMN signs that are present (23). See Table 3 for a summary of this criteria.

Table 3. *El Escorial Criteria for the Clinical Diagnosis of ALS (*from P. H. Gordon, 2013b).

Clinically possible ALS	UMN and LMN sings in one region, or UMN signs in at least two regions, or UMN and LMN signs in two regions with no UMN signs rostral to LMN signs
Laboratory-supported probable ALS	UMN signs in one or more regions and LMN signs defined by EMG in a least two regions
Clinically probable ALS	UMN and LMN signs in two regions with some UMN signs rostral to the LMN signs
Clinically definite ALS	UMN and LMN signs in three regions

EMG – electromyogram; LMN- lower motor neuron; UMN – upper motor neuron

Clinicians may also specify numerous ALS and ALS-like syndromes. When ALS is present in at least one other generation, it is recognized as Genetically- determined (familial/hereditary) ALS (fALS). In these familial cases, if a pathogenic mutation has

been determined, the diagnosis may be upgraded to Clinically Definite Familial ALS-Laboratory- supported. When no genetic link is established, the case is considered sporadic (sALS). ALS-Plus Syndromes may also be recognized, if other neurological diseases develop in parallel with ALS. Common ALS-Plus Syndromes include dementia and extra-pyramidal features. When symptoms are caused by non-ALS pathology, the terminology ALS-Mimic syndrome can be used. These syndromes include endocrineopathies, post-poliomyelitis, multifocal motor neuropathies, lead intoxication, and various infections (23).

Electrophysiological, neuroimaging, and neuropathalogical studies. When a clinical diagnosis of ALS is made, electrophysiological studies are typically performed to confirm LMN dysfunction in clinically affected areas or potentially detect LMN dysfunction in areas that were clinically uninvolved. Conventional electromyographic (EMG) needle examinations can provide electrophysiological evidence of active and chronic denervation (both of which are requirements of an ALS diagnosis), which often appear clinically as fibrillations and fasciculations. To support an ALS diagnosis, EMG signs must be observed in at least two of the following locations: bulbar/cranial motor neurons in the brainstem, anterior horn motor neurons in the cervical, thoracic, or lumbar spinal cord. The presence of fasciculation potentials in EMG recordings is also characteristic of ALS and can assist in diagnoses. Other techniques may be used to assess denervation, including single fiber or macro EMG, quantitative motor unit potential analyses, and motor unit number estimates. Nerve conduction and neuroimaging studies are also administered, primarily to exclude other disorders that may mimic ALS (23).

Detecting UMN involvement in ALS early in progression is difficult. Single-voxel magnetic resonance spectroscopy (MRS), diffusion tensor imaging (DTI) and transcranial magnetic stimulation (TMS) may be used as objective tests for clinical UMN signs, but their sensitivity early on is questionable (78, 143). Brain and spinal MRI are useful in excluding other diseases that affect UMNs, such as cervical spondylosis (23). Transcortical motor stimulation has recently been utilized in research settings, and does show cortical motor hyperexcitability in early stages of ALS. This technique identified UMN signs that were not detected by conventional exam, resulting in earlier diagnosis for many individuals. Although much more research and development is needed before this technique can be made available in clinical settings, researchers are hopeful it will allow for earlier diagnosis of ALS in the near future (56, 130).

Muscle and/or biopsy studies may support or exclude a diagnosis of ALS; however, the only method of definitively proving an ALS diagnosis is by autopsy examination (23).

Clinical Symptoms and Phenotypes

Phenotypically, ALS is markedly heterogeneous—even when considering family members with the same genetic mutation. The site of symptom onset may vary, UMNs and LMNs may be differentially affected, and vast differences in cognitive and behavioral effects may be present. Indeed, some suggest ALS is not a distinct disease, but a syndrome that exhibits a continuum of UMN/ LMN involvement and cognitive impairment, and other neurodegenerative diseases may fall under the ALS syndrome umbrella. Given the vast heterogeneity, ALS is often characterized by the site of

symptom onset, degree of involvement of UMNs and LMNs, and presence or absence of cognitive impairments.

Limb versus bulbar onset. ALS includes two main forms of onset: limb (sometimes referred to as spinal) and bulbar. Of the two, limb onset is more common and is associated with longer survival (62, 64). The first symptoms noticed are typically unilateral and focal weakness in a limb. Individuals often have difficulty walking, frequently tripping or stumbling, develop a foot drop, or have difficulty writing or holding utensils due to loss of dexterity in the hand.

In the case of bulbar onset, individuals typically begin to have difficulty speaking and swallowing. Dysarthria and dysphagia often progress to anarthria and malnutrition due to difficulties eating. Tongue atrophy and fasciculations are classic characteristics of bulbar onset ALS. Median survival time from onset of symptoms is ~27 months in individuals with bulbar onset, but highly variable (134). The prognosis for bulbar onset is worse than that for limb onset, in part because bulbar onset patients are prone to aspiration and malnutrition and potentially due to earlier respiratory dysfunction. Although ALS is more common in males than females, bulbar onset ALS has a female predominance, suggesting possible involvement of sex hormones in location of onset (24).

A small percentage of ALS patients (~4%) are characterized by respiratory onset. Spinal and/or bulbar involvement is often mild or absent in these cases, and prognosis is very poor (1.4 years on average) (29).

Involvement of UMNs and LMNs. Physicians consider muscle weakness, atrophy, hyporeflexia, cramps, and fasciculations signs of LMN involvement, while mood lability, hyperreflexia, muscle spasticity, and hypertonia are designated as UMN signs (65, 131). "Typical" or "classical" ALS is marked by simultaneous UMN and LMN weakness. If the involvement of either the UMNs or LMNs is drastically skewed, special designations are given: UMN-dominant (UMN-D) or LMN-dominant (LMN-D) ALS. Differentiating between these ALS subtypes and other neurodegenerative diseases with similar symptoms, such as Primary Lateral Sclerosis (PLS) and Progressive Muscle atrophy (PMA), is often challenging.

A diagnosis of PLS is designated for individuals exhibiting only UMN involvement. EMG is typically used to assess nerve dysfunction. Many individuals initially presenting with only UMN involvement eventually develop LMN sign, so frequent re-evaluations of patients showing only UMN signs is recommended (59). A "clinically pure" PLS diagnosis is associated with a lack of LMN involvement (indicated by negative EMG) four years after the onset of symptoms, since only ~23% of individuals who develop LMN involvement due so after 4 years from diagnosis (60). Individuals with PLS typically survive for decades. Even slight involvement of LMNs (EMG-positive findings) in individuals diagnosed with PLS is associated with a poorer prognosis, similar to that of individuals with classical ALS showing predominantly UMN signs (UMN-D ALS), suggesting possible misdiagnosis (59).

UMN-D ALS is less common than LMN-D ALS, and is not as well characterized. Mean age at onset in UMN-D ALS (52 years) tends to be lower than in classic ALS (61.4 years), and the disease tends to progress slower than what is observed in classic ALS

(disease durations of 56 and 33 months, respectively) (119).

On the other end of the spectrum, PMA is characterized by dysfunction of LMNs only. In essence, PMA is equivalent to ALS without UMN involvement. It is generally accepted that PMA evolves into ALS in some cases. In a review of 91 charts of individuals diagnosed with PMA, 20 of the patients later developed UMN symptoms, typically within 2 years of the PMA diagnosis, which qualifies them as having LMN-D ALS (79). Additionally, among individuals with a PMA diagnosis that had MRS studies conducted, over half showed UMN abnormalities. Interestingly, the presence of UMN signs in these cases was not significantly related to survival time although prognosis for individuals with PMA tends to be better than ALS prognoses. Risk factors for poor survival are congruent between the two diagnoses. Furthermore, mutations in the SOD1 gene have also been identified in PMA patients (27). Given the parallels between the two diseases, it has been suggested that PMA should be considered a form of ALS rather than a separate disease (79).

In some individuals with ALS, lower motor neuron involvement is limited to upper limbs or lower limbs for at least 12 months, known as flail arm syndrome and flail leg syndrome, respectively. In either case motor neuron degeneration spreads to all limbs, however, progression in these cases appears to be slightly slower than in classic ALS (81) (145).

The ALS spectrum. Multiple lines of evidence suggest that ALS, PLS, and PMA represent differing ends of a spectrum of the same disease (see Figure 15)(Su, 2014). However, differential diagnoses are helpful for researchers attempting to select homogenous participants for clinical trials (or at least as homogeneous as possible, given

the heterogeneity of ALS phenotypes) and for providing individuals with prognoses. Patients showing only LMN involvement (PMA) have a slightly better prognosis than individuals diagnosed with ALS (79), while those with only UMN involvement tend to have a much better prognosis (59). Consistent with the spectrum, prognosis for individuals with UMN-D ALS falls between that of classic ALS and PLS ((29, 119).

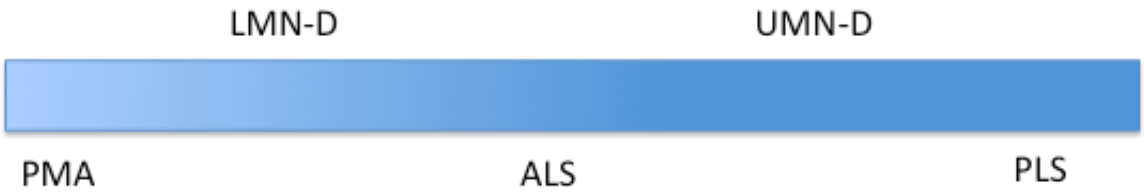

Figure 15. The PMA-ALS-PLS spectrum. The spectrum is based on involvement of UMNs and LMNs. In PMA (far left), only LMNs are affected, while in PLS (far right) only UMNs are affected. In ALS, both UMNs and LMNs are involved, however, the degree to which each is affected can vary (UMN-D ALS and LMN-D ALS). (Adapted from Swinnen, 2014)

Cognitive impairment in ALS. The degree and nature of cognitive impairments associated with ALS varies between individuals. Historically cognitive impairments with ALS were considered uncommon, but more recent research reveals this is not the case. An investigation of 131 French patients with ALS showed 40% had cognitive impairment and 10% had overt frontotemporal lobar degeneration (FTLD) (61). These results were corroborated in a recent study in which one-third of participants with ALS showed diminished performance on a simple frontal task involving word generation. In a subset of patients that consented to further neurological testing, nearly all of the individuals with diminished word generation met strict research criteria for FTLD and approximately 25% of the individuals that did not display diminished word generation met criteria for FTLD.

Many of these patients showed new onset personality changes, which is associated with frontotemporal dementia (FTD), a sub-classification of FTLD (94).

ALS-FTLD is associated with shorter survival time and is more likely to occur in patients with bulbar onset compared to individuals with classic ALS (61, 108). Individuals with ALS-FTLD were two times more likely to be noncompliant with noninvasive positive-pressure ventilation (NPPV) and percutaneous endoscopic gastrostomy (PEG). Noncompliance rates were 75% and 72%, respectively, compared to 38% and 31%, respectively, in individuals with classic ALS (108). It is unclear if shorter survival time for individuals with cognitive impairment is due to non-compliance or other underlying factors.

Interestingly, individuals with comorbid FTD were 3.5 times more likely to carry a genetic mutation than individuals that did not present with FTD (33). These findings highlight a genetic link between ALS and FTD. Similar to the PMA-ALS-PLS spectrum, researchers have also proposed an ALS-FTD spectrum, in which individuals are characterized by the degree of motor neuron and frontotemporal neuron involvement (see Figure 16) (131).

Figure 16. The ALS-FTD spectrum. The spectrum is based on the involvement of motor neurons and frontotemporal neurons. Individuals with classic ALS (far left) do not show signs of cognitive abnormality (no involvement of frontotemporal neurons; strictly motor neuron involvement), while those with FTD (far right) show no motor abnormalities. Moving from left

to right on the spectrum: individuals with ALS that show behavioral impairment (ALSbi) and cognitive impairment (ALSci) indicate some involvement of frontotemporal neurons. Those with ALS-FTD meet criteria for both diagnosis, and thus exhibit involvement of both frontotemporal neurons and motor neurons. Individuals with FTD-MND have some motor neuron involvement but do not meet criteria for an ALS diagnosis (Adapted from Swinnen, 2014).

Treatments for ALS

Therapeutic interventions. There is currently no cure for ALS. The only FDA-approved drug for treating ALS is Riluzole, which increases lifespan by approximately 3 months (104). Over 100 compounds have been as potential therapeutic interventions for ALS (see Wu et al 2014 for a complete list of compounds). Although many were effective in mice, translating the results to humans has not been successful (23). Examples of compounds that were successful in mice but failed in clinical trials include: creatine monohydrate (68), Vitamin E (67), COX2 inhibitors (37), and IGF-I (21, 87, 127). Questions have arisen on the ability of the common mouse model to accurately represent the pathophysiology underlying the majority of human ALS cases. It appears the SOD1 pathophysiology (seen in the most common mouse model) is quite different from that of other ALS linked genes, offering a possible explanation for why interventions that were successful in this model failed in ALS clinical trials (95).

Clinical trials involving the use of stem cell therapies are currently underway. Although stem cell research as a therapeutic intervention for ALS is in the early stages, it appears treatments that focus on increasing neural protection are promising, while those

involving motor neuron transplants are unlikely to be successful. The length of time it typically takes for ALS to be diagnosed presents a barrier for stem cell interventions; often, neural degeneration is well advanced by the time the disease is diagnosed, at which point cells may be past the point of repair. Hence, diagnosing ALS as early as possible will likely be imperative to the effectiveness of stem cell treatments (101).

Exercise as a therapeutic intervention for ALS. In healthy individuals, free radical production as a result of oxidative phosphorylation during exercise results in increased antioxidant enzymes and resistance to oxidative stress (112). This led researchers to explore exercise as a possible intervention to reduce oxidative stress in ALS patients. However, the relationship between exercise and ALS patient outcomes remains unclear. Some studies suggest that high intensity endurance training hastens a decrease in motor performance and death in SOD1 mice (98). As a result of these findings and studies associating competitive sport participation and ALS development, it is not uncommon for ALS patients to be advised to avoid physical activity, in hopes of preserving muscle strength and minimizing muscle damage (124). However, research has also shown positive effects of moderate exercise in mouse models of ALS (4, 6)(80) as well as positive effects of moderate exercise on disability in ALS patients (8, 19, 47). Furthermore, it is possible outcomes are dependent on specific exercise type (42), intensity (76), and sex (98, 140).

Although the precise relationship between exercise and ALS risk and disease progression remains unclear, several studies suggest that muscle exercise may be beneficial. In an ALS patient case study, strength gains were seen in 14 of 18 muscle groups following 75 days of resistance training (19). Short-term positive effects were

also seen in male ALS patients from a muscle exercise program. Patients who participated in two 15 minute sessions of daily muscle training exercises designed to improve endurance, involving modest loads and significant changes in length, showed significantly less deterioration in muscle spasticity and global functioning as assessed by the ALS Functional Rating Scale (ALSFRS) (1) than patients who did not perform this type of exercise. Although no significant differences were found between groups at 6 months, there was a trend toward less deterioration in the treated group (47). Significant differences have been observed at the 6 month timeframe between exercising and non-exercising ALS patient groups on the ALSFRS and in patient quality of life assessed by the SF-36 (8), with individuals who participated in a resistance training program reporting less decline in global functioning and quality of life than those who did not. These individuals also showed less decline in leg strength than individuals who did not participate in resistance training. However, no differences were seen in fatigue severity as assessed by the Fatigue Severity Scale (7) between groups (3).

Although few studies have been conducted on the efficacy of resistance training in ALS patients, there does appear to be some degree of short-term benefit for patient quality of life. Muscle exercise may help to attenuate the declines in muscle strength and spasticity seen in ALS patients and increase patient quality of life. To this author's knowledge, no studies to date have investigated the effects of any other types of exercise in ALS patients aside from resistance exercise. More research is needed to determine if other types of exercise may be beneficial for ALS patients.

Evidence for exercise training efficacy in mouse models of ALS. Research on the effects of exercise training in mouse models of ALS has yielded conflicting results.

Some studies report exercise training hastens onset of motor performance declines and decreases lifespan (98), others report beneficial effects of exercise on motor function (42, 80), and still others report exercise has no effect on motor function and survival (91). Different exercise intensities (4) and types of exercise (5) have been shown to produce differential effects on outcome measures. Differences in methods between studies may account for inconsistencies in results, as quantifications of exercise intensities, duration, and exercise type vary greatly. Furthermore, particular outcomes may also be dependent on gender (140).

Altogether, the data suggest a potential protective effect of moderate exercise (corresponding to speeds around 13 m/min) in the form of running on motor performance, motor neuron survival and lifespan in ALS mice. Moderate exercise appears to delay the onset of symptoms, at least in female mice (140), and decrease the rate of decline in motor performance after symptom onset (26). Combined IGF-I therapy and exercise show synergistic effects in ALS mice; the combined treatment resulted in the greatest increase in lifespan observed of any study to date involving exercise with ALS mice (76). It appears that intense running (corresponding to speeds around 22 m/min) may hasten the onset of motor performance deficits and potentially decrease lifespan in ALS mice (98). Given that ALS mice exhibit increased free radical production (5)(2) it is possible that high intensity exercise "tips the scale" so to speak, increasing oxidative stress to the point of cellular damage and death, while moderate exercise increases free radical production enough to increase antioxidant capacity, but not so much that cells are harmed. Measures of oxidative stress in future studies would be

helpful in determining the exact role of oxidative stress in the results seen at different exercise intensities.

Exercise in the form of swimming. Different types of exercise elicit different cellular responses and adaptations (120). In addition to exercise intensity, type of exercise appears to play a critical role in effecting motor function and life span in SOD1 mice. Specifically, when comparing male G93A*SOD1 mice trained in swimming, running, and sedentary mice, mice trained in swimming show delayed onset of symptoms (16 days in swimming vs. sedentary ALS mice; no significant difference in running vs. sedentary mice), less deterioration in motor function and strength, and increased life span (25 days in swimming vs. sedentary ALS mice) (42).

Current care for individuals with ALS. Participation in multidisciplinary care results in increased quality of life (89) and longer survival (133) in individuals with ALS. In these setting, individuals may interact with physicians, respiratory therapists, speech therapists, physical therapists and case coordinators or social workers, among other service providers. Delivering services in a multidisciplinary fashion helps to provide continuity and consistency of care for patients, and reduce the burden of traveling to multiple locations for various appointments with different service providers. (51)

The majority of ALS patients die from respiratory failure, resulting from an inability to contract the diaphragm. Respiratory support, such as noninvasive ventilators (NIV), improve sleep quality (70) and increase survival (22) when used at least 4 hours per day. All care for individuals with ALS is palliative. In the later stages of the disease,

the primary goal is to avoid suffering. Medications can be given for pain, anxiety, and to assist with sleep (65).

Healthcare and Caregivers

Healthcare costs for ALS patients are substantial and increase drastically as the disease progresses. Annual costs are estimated at approximately $5,000 per year in the early stages of the disease and $80,000 per year in later stages of the disease. Due to the severity and extremely fast progression of the disease, ALS patients are typically unable to work within a rather short timeframe of being diagnosed. Although insurance companies may cover some costs, coverage is not consistent. Sources of costs include medications to help manage symptoms and reduce pain, non-invasive ventilators and mechanical ventilators, and frequent emergency room and office visits. During the later stages, patients typically need a fulltime caregiver, often resulting in lost wages of family members taking on the responsibility (105). It is estimated that caregivers of individuals with ALS spend an average of 11 hours per day with the patient, even when paid assistance is also being received (84). Interestingly, although nearly half of primary caregivers report feeling physically and/or psychologically unwell (84), rates of caregiver depression were shown to decline from earlier on in disease progression to patient death or near death (111).

Potential Causes of ALS

About 5-10% of ALS cases are genetically inherited (fALS), most often as an autosomal dominant trait. The remaining 90-95% of cases are sporadic (sALS) and the cause is unknown, although sALS is thought to have both genetic and environmental

influences (64, 131). Genetic predispositions, exposure to toxins, and trauma may play a role in some cases of ALS. Although the cause is unknown, military Gulf War veterans are approximately twice as likely to develop ALS (3). Some researchers suggest a relationship exists between sports participation and development of ALS, but the literature on this topic is conflicting.

Genes linked to ALS. At least 22 genes have been identified that are linked to ALS (see Figure 18) (35). The most common genes associated with ALS, which account for just over half of fALS cases, are superoxide dismutase 1 (*SOD1*), transactive response DNA-binding protein of 43 kD (*TARDP*), fused in sarcolemma/ translated in liposarcoma (*FUS/TLS*) and *c9orf72* (see Figure 17). An additional 10-15% of fALS cases are accounted for by several other recently discovered genes, including *OPTN, VCP, SQSTM1, PFN1*, and *UBQLN2* (115). These ALS-linked genes serve numerous cellular functions, including RNA processing (TARDP and FUS/TLS), endosomal trafficking and cell signaling (C9ORF72 and OPTN), oxidative stress (SOD1), and ubiquitin/protein degradation (UBQLN2, VCP, and SQSTM1). Additionally, mutations in many of the same genes have been discovered in at least 9% of sALS cases (115); very recent research utilizing more advanced genome mapping techniques suggest this number may actually be as high as 27% (25).

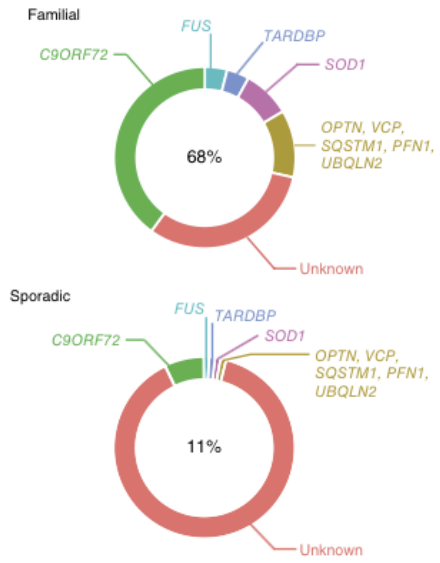

Figure 17. ALS linked genes in fALS (top) and sALS (bottom). At least 22 genes have been linked to ALS, the most common of which are shown here. Interestingly, similar mutations are found in both fALS and sALS. (115).

SOD1. In the early 1990s, researchers identified a close genetic linkage between fALS and the *SOD1* gene. SOD1 is an antioxidant enzyme that combats the free radical superoxide anion (118). Currently, 15% of fALS cases are due to mutations in *SOD1* and 1% of sALS patients also carry similar *SOD1* mutations (30). Mean age at onset is approximately three years earlier for individuals with a *SOD1* mutation (M= 46.9, SD= 12.5) compared to those lacking a mutation (M= 50.5, SD= 11.5) (36), and bulbar onset in SOD1 cases is extremely rare (131). Although over 160 mutations in the gene have been identified (65), a genetic link has only been observed in a portion of these (4). There is considerable phenotypic variability associated with different *SOD1* mutations. The most common mutation is an alanine for valine substitution at codon 4 (A4V), which is associated with a severe form of ALS that progresses rapidly and is correlated with

shorter survival. Conversely, G37R, G41D, and G93C mutations predict longer survival (36).

The discovery that ~15% of fALS patients carry a point mutation on the *SOD1* gene (118) led to the development of a transgenic mouse model of ALS in which the mutant human *SOD1* gene is overexpressed. The G93A*SOD1 mouse model is the most common ALS mouse model, in which there is a point mutation in the 93rd codon, where a glycine is replaced with an alanine (69). These mice show motor neuron degeneration similar to that seen in humans with ALS, expressing symptoms such as hind limb weakness and atrophy beginning around 3 months of age, progressing to complete paralysis typically around 5 months of age. The G93A*SOD1 mouse model most closely resembles the severe cases of human ALS with rapid progression and poor prognosis (40). Although the development of this mouse model has provided a platform for examination of the underlying disease mechanisms, the model's use for testing potential pharmacological treatment approaches for humans is questionable (95).

Formation of protein aggregates/inclusions in the cytoplasm of degenerating motor neurons is characteristic of neurodegenerative diseases. In ALS, ubiquinated inclusions containing TDP-43 (most often) and/or FUS (in some cases) proteins are a hallmark of the disease (6). Mutations in the *SOD1* gene are toxic, gain-of-function mutations that increase cellular oxidative stress and result in ubiquinated TDP-43 negative SOD1 protein aggregates in affected tissues. The aggregates are found in the highest abundance in the brain stem and spinal cord anterior horn, and have shown resistance to a protease digest that successfully degrades wild type SOD1 (129). The mechanism underlying SOD1 toxicity in motor neurons is not completely understood, but

there is evidence to suggest that non-neuronal cells may contribute to the damage of motor neurons. Indeed, lowering mutant SOD1 expression in microglia of ALS mice significantly slowed disease progression (20). Less neuronal death and prolonged survival was also seen in mice that expressed wild type microglia compared to those that expressed microglia with a SOD1 mutation (13), suggesting microglia play a causative role in neurodegeneration in SOD1 ALS cases.

Because of the distinct difference in presence/absence of TDP-43 in these protein aggregates, it has been suggested that *SOD1* ALS is caused by a different mechanism than all other forms, and may require selective treatment. This may also explain (at least in part) the lack of efficacy of therapeutic interventions in humans that were successful in the SOD1 mouse model (97, 115).

TARDP. The discovery that TDP-43 protein is a primary constituent of cytoplasmic neuronal inclusions in ALS (6) led to the linkage of *TARDP* (the gene that encodes the TDP-43 protein) gene mutations to fALS (128). As the name suggests, normal TAR DNA-binding Protein-43 is a DNA/RNA binding protein that regulates transcription, alternative splicing and microRNA biogenesis, and is typically localized to the nucleus. Conversely, pathological TDP-43 is hyperphosphorylated, ubiquinated, and cleaved at the N-terminus. Localization to the cytoplasm occurs, presumably due to cleavage of the nuclear localization signal at the N-terminus. A number of specific mutations in *TARDP* have been identified that are linked to fALS, accounting for approximately 4% of fALS cases (33). TDP-43 mutations have also been identified in a small percentage of sALS cases, however, the presence of TDP-43 pathology in ALS is much greater than the mutational frequency of the gene. Moreover, TDP-43 positive

ubiquinated inclusions are also hallmark of FTLD, which shows frequent co-occurrence with ALS (107).

FUS/TLS. Not long after the discovery of *TARDP* involvement in ALS, an additional protein with similar function to TDP-43 was liked to ALS. Mutations in FUS/TLS, a nuclear RNA and DNA binding protein that is involved in transcription and RNA splicing, were discovered in approximately 5% of fALS cases and are often linked to Juvenile ALS (115). Similar to TDP-43, FUS protein aggregates were a primary constituent of cytoplasmic inclusions in individuals with a mutated *FUS* gene. The functional homology between FUS and TARDP suggests that the two may function in the same pathway, sharing a common pathophysiological mechanism that leads to neurodegeneration (85, 139).

C9orf72. Of the common genes associated with fALS, *C9orf72* is the most recently discovered (44, 116) and the most common, accounting for ~40% of fALS cases (99). Furthermore, it accounts for 7% of sALS cases, making it the only gene whose alteration has been linked to a significant number of sALS cases (compared to all other genes which account for less than this amount combined). It was also found to account for ~24% of familial FTD, providing genetic evidence for the overlap between the two diseases (99). Contrary to individuals with SOD1 mutations, bulbar onset is very frequent in cases of C9orf72 (131). Shorter survival time is also seen in individuals with C9orf72 mutations compared to the average ALS survival time (33).

Specifically, massive hexanucleotide repeat expansions in the non-coding region of C9orf72 are observed in C9orf72 linked ALS and FTD, making C9orf72 unique from

other ALS-associated genes. Penetrance for individuals with the expansion is ~50% by age 58, and nearly 100% by 80 years of age (99). A number of modifying genes have been identified that are associated with the heterogeneity observed in individuals with C9orf72 repeats, including UBAP1, metallothionein, UNC13A, and ELP3. These genes code for proteins with varying functions, including protein sorting, antioxidant defense, neurotransmitter release, and RNA synthesis, and were shown to modify disease characteristics such as age at onset and survival after onset (136).

Genes involved in degradation and endosomal trafficking. Other genes have recently been identified that account for a small portion of fALS cases. Several are involved in ubiquitin and protein degradation, while others function in endosomal trafficking and cell signaling. Valosin-contining protein (VCP) mutations, known to cause inclusion body myopathy with FTD, are present in ~1-2% of fALS cases (74, 82). The presence of VCP gene mutations in both ALS and FTD provide further evidence for a genetic linkage between the two diseases. TANK-Band Kinase 1 (TBK1) was very recently implicated as an ALS gene; this protein plays an important role in autophagy and inflammation, and phosphorylates both Sequestome 1 (SQSTM1) and Optineurin (OPTN), both of which have previously been implicated in ALS (53, 102). SQSTM1 and OPTN serve as cargo receptors for ubiquinated proteins' engulfment into autophagosomes, and OPTN specifically functions in the autophagy of damaged mitochondria (146). Interestingly, OPTN is present in both SOD1 and TDP-43 positive inclusions, suggesting it may be involved in broader ALS pathogenesis (102). SigR1 mutations, also recently linked to ALS (1), appear to lead to impaired autophagic degradation as well. SigR1 loss of function also causes ER pathology, mitochondrial

abnormalities, and destabilization of lipid rafts (142). VCP, TBK1, SQSTM1, OPTN and SigR1 all appear to play critical roles in the removal of pathological protein aggregates via degradation and autophagy, highlighting the involvement of this malfunction in the pathophysiology of ALS (34).

Other ALS linked genes. In 2012, Wu et al discovered that mutations in the *Profilin 1* (*PFN1*) gene account for ~1-2% of fALS (148). The authors suggest mutant PFN1 protein alters dynamics of growing actin filaments, inhibiting axon outgrowth. They also provide evidence for the possibility of PFN1 inducing aggregation of TDP-43 (148). Additionally, mutations in Ubiquilin 2 (UBQLN2) have been identified as the cause of an X-linked juvenile and adult-onset form of fALS. UBQLN2 function is similar to that of other ALS-linked genes (VCP and SQSTM1), as it is involved in protein degradation, specifically of ubiquinated proteins (45). See Su et al 2014 for a complete list of ALS- associated genes.

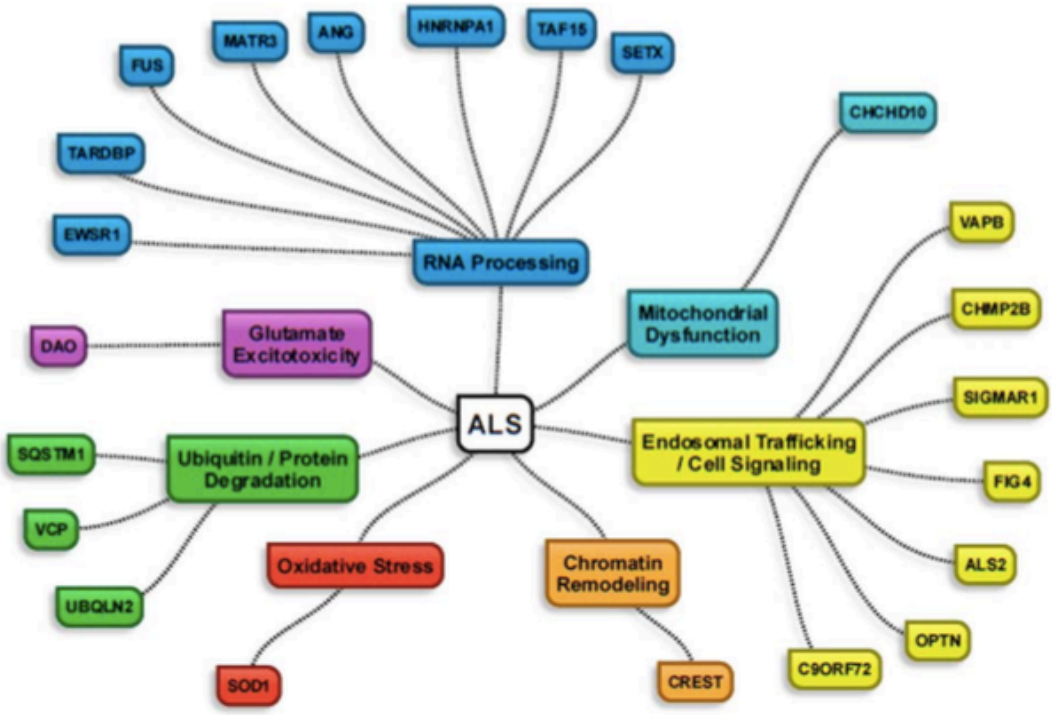

Figure 18. ALS- linked genes (35).

fALS versus sALS. The discovery that many genes responsible for fALS are also found in some sALS cases is important when considering the fALS verses sALS classification system. Some have proposed the possibility of an underlying genetic component in all of ALS (88) that is influenced by environmental factors, making certain individuals more or less susceptible to the disease (115). Recently, researchers have begun to investigate an oligogenic model of ALS, in which mutations in two or more genes leads to development of the disease (88). It has been suggested that a genetic variant leads to susceptibility to ALS and a second locus effects specifics of the disease such age of onset, progression, and/or phenotype (137). Indeed, inhibition of EPHA4 signaling increases survival in mouse and rat models of ALS, and expression of EPHA4

in humans is inversely correlated with disease onset and survival (138). Moreover, in SOD1 ALS mice, concomitant mutations in the homologue of human H63D HFE, a protein involved in oxidative stress and alterations in iron homeostasis, resulted in decreased survival and acceleration of disease progression (106). A comprehensive study that investigated 17 ALS-linked genes in individuals with ALS found that 3.8% of subjects had variants in >1 gene, and disease onset for these individuals was 10 years earlier, on average. Additionally, these researchers found that 27.8% of sALS individuals carried a known ALS-linked mutation—a much larger percentage that what has been previously found (25). Although significant progress has been made since the discovery of SOD1 ~25 years ago, much research is still needed to fully understand the involvement of genetic factors in ALS.

A complicated relationship between pathogenesis and phenotype. ALS is characterized by upper and lower motor neuron death and progressive muscle weakness, but vast phenotypic differences are seen in limb versus bulbar onset, the degree of associated cognitive deficits, and differential involvement of UMNs and LMNs. The distinctiveness of these phenotypes would seem to suggest different causal mechanisms, however, there are no neuropathic or genetic factors that can definitively distinguish between them (114). Conversely, different gene mutations associated with fALS result in highly similar, if not identical, phenotypes and sALS and fALS are phenotypically indistinguishable from one another. Although specific mutations may tend to show certain phenotypes (e.g. bulbar onset is very frequent with c9orf72 mutations and rare in cases with SOD1 mutations), vast variability is still observed. Any of the known genetic mutations may be associated with a number of observed clinical phenotypes (i.e. bulbar

onset, limb onset, age at onset, rate of progression, predominantly UMN/LMN, classical ALS, etc.). This suggests the involvement of other modifying factors, such as environmental factors and/or other genes that may serve a protective function or exacerbate the disease. Many different gene mutations yield the same phenotype, suggesting there must be numerous different underlying mechanisms with similar pathophysiological pathways that have the potential to cause ALS. Additionally, the ability of a specific gene mutation to result in a number of different clinical phenotypes highlights the complexity of the underlying disease mechanism and the possibility of extensive gene-gene or gene-environment interactions that can significantly affect phenotype.

Sports participation and development of ALS. Early epidemiological studies showed a relationship between previous participation in competitive sports and ALS incidence. 36% of ALS patients reported earning varsity letters in high school, while only 12% of diseased controls and 20% of healthy controls reported this achievement (54). These findings were supported by a more recent study, in which incidence of motor neuron disease was 1.7 times higher in patients who were varsity athletes vs. those who were not (121). Increased risk for development of ALS was reported among Italian soccer players, in particular midfielders and individuals with careers lasting longer than 5 years (31). It has been proposed that the increased risk observed in this population may be due to increased trauma or abuse of both legal and illegal drugs (32). However, subsequent studies failed to show an association between competitive sports or sports related traumas and development of ALS (135). Performance enhancing dietary supplements, specifically branched chain amino acids, have recently received further

attention as a possible contributing factor to increased ALS incidence in athletes (14, 100). At this time, the relationship between competitive sports and ALS development remains unclear.

ALS Pathophysiology is Complex

Although the pathophysiology of ALS is not completely understood, several theories have been proposed, including oxidative stress, impaired calcium signaling, mitochondrial dysfunction, protein misfolding and glutamate toxicity (20, 132). Two organelles receiving growing interest in ALS pathophysiology research are the sarco/endoplasmic reticulum (SR/ER) and mitochondria.

Sarco/endoplasmic reticulum stress is linked to ALS pathology. The ER, a multifunctional organelle, has two major functions in the cell: it is a site of Ca^{2+} storage and release, and is involved in protein synthesis and facilitation of proper protein folding (16). Alterations in one of the primary functions of the ER can greatly affect the other. Very sensitive to changes in homeostasis, during times of stress many ER stress-signaling pathways are activated resulting in alterations in protein expression. Ongoing protein synthesis is turned off, proteins that enhance cell survival may be up- regulated and/or apoptotic pathways may be initiated. Under conditions of ER stress, misfolded proteins can accumulate in the ER, inducing an unfolded protein response (UPR) (77). A primary cause of protein misfolding and activation of the UPR is deficient Ca^{2+} stores in the ER lumen. Conversely, the accumulation of misfolded proteins can cause misregulated Ca^{2+} release or uptake in the ER (16). Impaired Ca^{2+} regulation and misfolded protein accumulation have both been implicated in ALS pathology.

Calcium regulation is impaired in ALS. G93A*SOD1 transgenic mice have shown increased intracellular Ca^{2+} in single muscle fibers, likely due to decreased Ca^{2+} clearance proteins (28). After skeletal muscle contraction, the sarco/endoplasmic reticulum Calcium-ATPases (SERCA1 and SERCA2) function as pumps, moving Ca^{2+} from the cytosol back into the ER lumen for storage. Protein levels of SERCA1, found in type II muscle fibers, as well as SERCA2, located in type I fibers, were drastically reduced in G93A*SOD1 transgenic mice gastrocnemius muscle. In addition, the Ca^{2+} handling protein PV, involved in relaxation in type II fibers, was also reduced. Mutations in SigR1, known to cause a juvenile form of ALS, have been shown to affect Ca^{2+} homeostasis by destabilization of lipid rafts. Loss of SigR1 function has also been linked to deformities in the ER ultrastructure (142). Furthermore, mutant SOD1 expression in astrocytes was shown to result in elevated intra-mitochondrial Ca^{2+} levels in motor neurons and decreased mitochondrial membrane potential (17).

Reactive oxygen species are linked to motor neuron death and Ca^{2+} handling. An additional known cause of ER stress and the UPR, also linked to ALS, is the accumulation of ROS. Oxidative stress is caused by an imbalance between the production of ROS or free radicals and the cell's ability to detoxify these intermediates or repair damage caused by ROS. The SOD1 mutation found in the G93A*SOD1 mouse model is a gain of function mutation, increasing SOD1 activity, resulting in increased production of reactive oxygen species (ROS) (18). G93A*SOD1 mice show increased free radical production that results in protein damage (5). High levels of oxidative stress leading to cell damage have also been implicated in motor neuron death in individuals with ALS (11, 12). The hydroxyl radical, production of which is increased in G93A*SOD1 mice,

has been shown to inhibit SERCA protein function (149). Given the role of SERCA1 in Ca^{2+} regulation, it is not surprising that ROS production is also linked to altered Ca^{2+} regulation (16).

SR-mitochondrial coupling, ROS, and Ca^{2+} signaling. Mitochondria are plentiful in skeletal muscle, providing ATP for contraction and playing a key role in Ca^{2+} homeostasis (48). Often found in very close proximity to the SR, the two are "coupled," with various signaling mechanisms between them, and alterations in one organelle often resulting in changes in the other. Formation of mitochondrial networks within muscle fibers (i.e., mitochondrial fusion) has been observed, and is essential to proper bioenergetics and excitation-contraction coupling (52). Ca^{2+} released from the SR upon activation of the ryanodine receptor—such as during muscle contraction— accumulates in intermyofibrillar mitochondria, activating enzymes in the mitochondrial matrix that enhance ATP production (excitation-oxidative metabolism coupling) (75, 123). Movement of Ca^{2+} from the ER/SR lumen into the mitochondria can also stimulate ROS production in the mitochondria and activate apoptotic pathways. Conversely, mitochondrial ROS can leak into the ER and regulate ER Ca^{2+} release channels, creating bidirectional information transmission between the ER and the mitochondria that is Ca^{2+} dependent (16).

Evidence for mitochondrial alterations in ALS is plentiful (17, 48, 86, 141, 144), although it appears that mitochondrial dysfunction may be a result of pathophysiology upstream or outside of the mitochondria itself. Expression of multiple mitochondrial DNA (mtDNA) genes is reduced in ALS spinal cord tissue (e.g. COX3, ND2, ND4). This decrease is thought to be the result of alterations in upstream PGC-1α signaling,

rather than a primary pathogenic event (86). Reduced expression of mtDNA coding for COX and NADH:coQ oxidoreductase has also been observed in ALS skeletal muscle (141, 144). In this case, it was proposed that oxygen radicals, specifically SOD, were to blame for the mitochondrial alterations (141).

The superoxide anion radical, which is the SOD substrate, is the major reactive oxygen species produced in mitochondria (125). Mutant SOD1 has been shown to induce leakage of the outer mitochondrial membrane and expansion of the intermembrane space, resulting in mitochondrial degeneration and vacuolation (73). When mutant G93A SOD1 neuroblastoma, non-neuronal Madin-Darby Canine Kidney (MDCK), and NSC-34 motoneuronal-like cell lines were investigated, mitochondrial alterations were seen only in motoneuronal-like cell lines, suggesting selective vulnerability of motoneuronal mitochondria in particular to mutant G93A SOD1 (113). Muscle mitochondria uncoupling results in destruction of the neuromuscular junction and mild degeneration of motor neurons, suggesting that motor neuron death may be caused by pathology outside of the motor neuron (49).

Although the precise mechanisms underlying ALS pathophysiology are not clear, there is compelling evidence to suggest that dysfunction in skeletal muscle Ca^{2+} regulation and ROS production in the ER/SR and mitochondria play a role. Further research is needed to understand the precise mechanisms behind ALS pathophysiology.

Preferential motor unit loss in ALS. The cause of motor neuron death in ALS is not well understood. Research in G93A*SOD1 transgenic mice indicates that motor unit death occurs selectively in units of larger muscle fibers and prior to motor neuron

loss. Decreases in force production in muscles composed of primarily type II fibers, but not in muscles composed primarily of type I fibers, were seen at 80 days of age, prior to declines in the number of motor neurons and onset of overt symptoms. At 40 and 80 days of age, ALS mice had a 22% and 48% (respectively) reduction in motor units in muscles composed primarily of type II fibers, but no significant reduction in motor units in muscles composed primarily of type I fibers, in comparison to controls (71). Furthermore, at 60 days of age, muscle from the tibialis anterior (primarily type II fibers) of G93A*SOD1 mice contained 40% less innervated fibers, 60% less motor units, and a 44% higher innervations ratio when compared to wild type mice, however, force production per motor unit was lower in ALS mice. The same mice showed 65% less IIB fibers, 28% less IID/X fibers and about twice as many IIA fibers than the wild type mice. This indicates selective denervation of fast-twitch fatigable and fatigue intermediate fibers and reinnervation of these muscle cells by slower fatigue resistant type IIA motor neurons (72). Decreased competence for synaptic sprouting-- in type IIB fibers, specifically-- is likely to play a role in the selective denervation seen in ALS. There is a strong inverse relationship between susceptibility to synapse loss and the ability to produce synaptic sprouting in ALS mice (55).

Alterations in Human Skeletal Muscle Protein Expression in ALS

Previous studies have shown mRNAs and proteins involved in muscle hypertrophy and atrophy are altered in ALS skeletal muscle. mRNA and protein expression of Atrogin-1, a protein involved in muscle atrophy, are significantly elevated in ALS mice and human skeletal muscle (90). Akt, which has anabolic effects and is thought to be an upstream regulator of Atrogin-1 (58), is also altered in ALS. Skeletal

muscle biopsies from individuals with ALS showed a 68% decrease in phosphorylated Akt (the active form) to Akt ratio when compared to healthy control muscle. However, downstream Akt target proteins $p70^{s6k}$ and GSK-3β were not different between ALS and control muscle (90). The IGF-I family proteins, also upstream regulators of Akt, are altered in ALS as well. IGF-I family proteins include IGF-I, a trophic factor that is implicated in muscle and nerve anabolism, and IGF- Binding Proteins which may potentiate or inhibit IGF-I activity. IGF-I and IGF-Binding Proteins that have the ability to potentiate IGF-I showed decreased expression in ALS human skeletal muscle, and the IGF-I receptor β subunit showed increased expression.

Recent work discovered a correlation between Akt, as well as Factor XIIIB, and overall survival in individuals with ALS. In a screening of 143 cell-function related proteins and phosphoproteins, significant differences between human ALS and healthy control skeletal muscle were found in seventeen proteins. Individuals that showed high expression of Akt or Factor XIIIB had a better prognosis than individuals with low expression. In addition, expression of four proteins (p-GSK-3α/$\beta^{Ser21/9}$, p-RB^{Ser780}, p-$PKC\alpha^{Ser657}$ and SYK) was significantly correlated with ALS-Functional Rating Scale (Revised) scores <40, with lower scores indicating lower level of function. Increased expression of the protein SK3 was related to greater muscle atrophy (150).

The SR/ER transmembrane protein stromal interaction molecule 1 (STIM1) is also altered in ALS skeletal muscle (66). STIM1 functions as a Ca^{2+} sensor on the SR/ ER membrane, activating store-operated Ca^{2+} entry (SOCE) channels when Ca^{2+} stores in the ER lumen are depleted. Mutations that decrease STIM1 affinity for Ca^{2+} induce a permanent SOCE resulting in Ca^{2+} misregulation (92). STIM1 is an important signaling

molecule in the initiation of myoblast differentiation that subsequently forms contractile myotubes (41). Accumulation of STIM1 protein was found in partially and completely atrophied ALS muscle fibers, but was not present in healthy fibers. Given that autophagy is regulated by Ca^{2+} homeostasis, it is possible that STIM1 plays a part in autophagy regulation in skeletal muscle (66).

Summary

Although there is still much to understand about ALS pathophysiology, significant gains in understanding the underlying disease mechanisms and ALS genetics have been made in the past 20 years. Although many different genes with varying functions and various molecular pathways may be involved, the relationship between these pathways is beginning to come to light. With this deeper understanding comes the potential for development of new treatments for this devastating disease for which there is no cure.

Appendices

Appendix A

SERCA1 expression was significantly lower in G93A SOD1 mice compared to controls (A) and also tended to be lower in individuals with the SOD1 mutation compared to other ALS subtypes and diseased controls (B). Grp78/BiP was significantly higher in ALS mice compared to controls, and also tended to be higher in individuals with sALS and a C9orf72 mutation compared to SOD1 mutation and diseased controls.

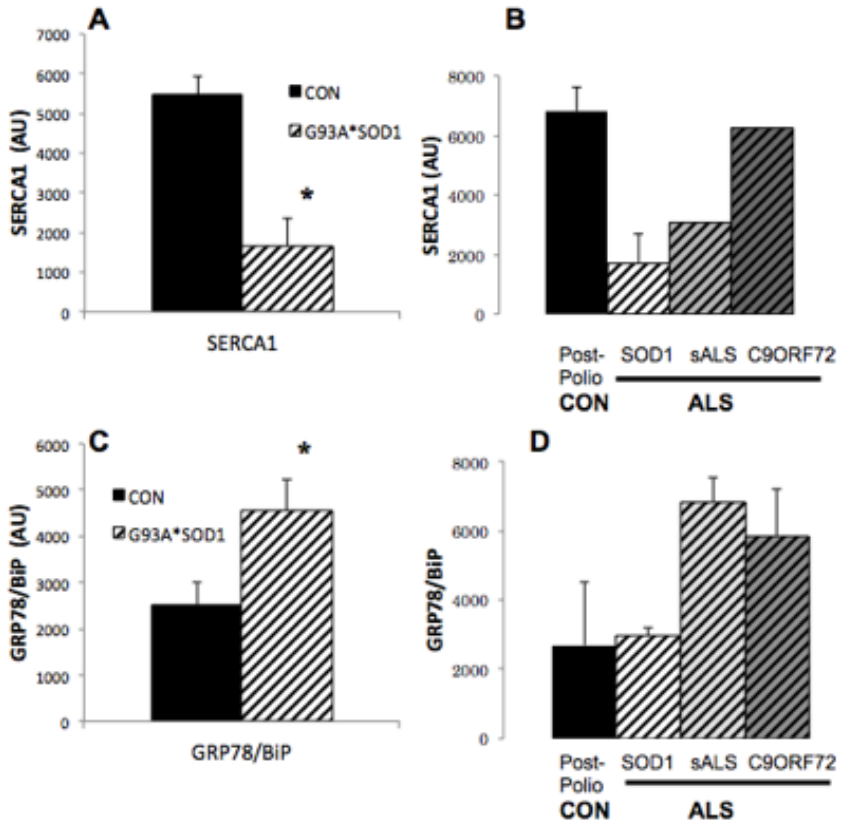

Figure A1. Alterations in skeletal muscle proteins in ALS mice and human autopsy samples.

Appendix B

Data in Figure A2 shows differences in Akt protein level between diseased control and different sub-groups of ALS. This figure also demonstrates the approach in blinded analysis of protein levels (panels A and B) followed by un-blinded data analysis to examine trends across groups. Protein level of Akt tends to be higher in SOD1 and sALS subgroups compared to C9orf72 subgroups.

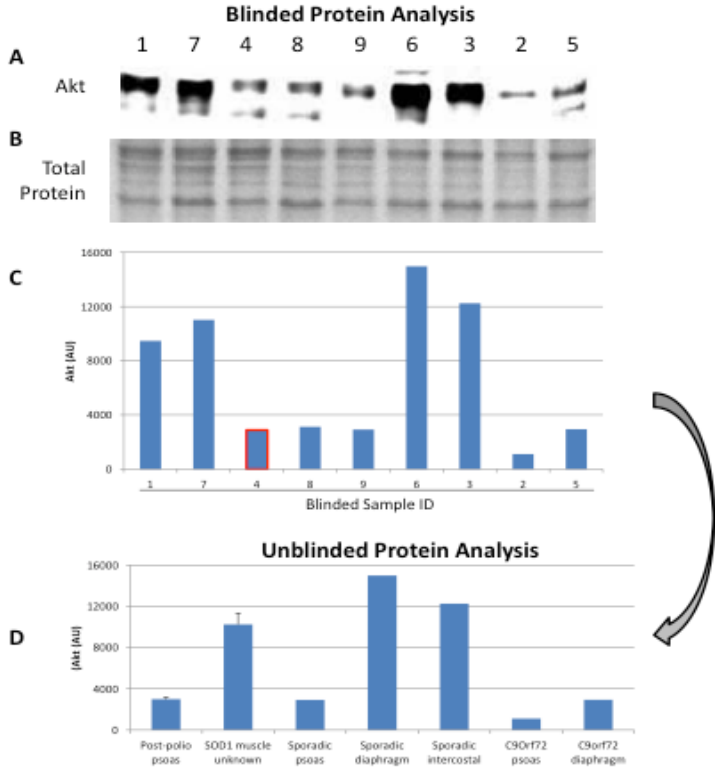

Figure A2. Akt western blot results. Akt western blot results (A) and membrane total protein stain (B) showing equal loading across lanes. Akt signal for each lane is displayed (C), then researcher is un-blinded to sample identity for analyses (D).

Appendix C

Chemiluminescent western blot results showing Akt antibody binding (A) and Alexa image (B) showing protein marker of known molecular weights were overlaid (C), allowing for identification of target protein bands by molecular weight. Panel B also shows map of protein marker (provided by Bio-Rad) used to identify molecular weights of bands on the marker.

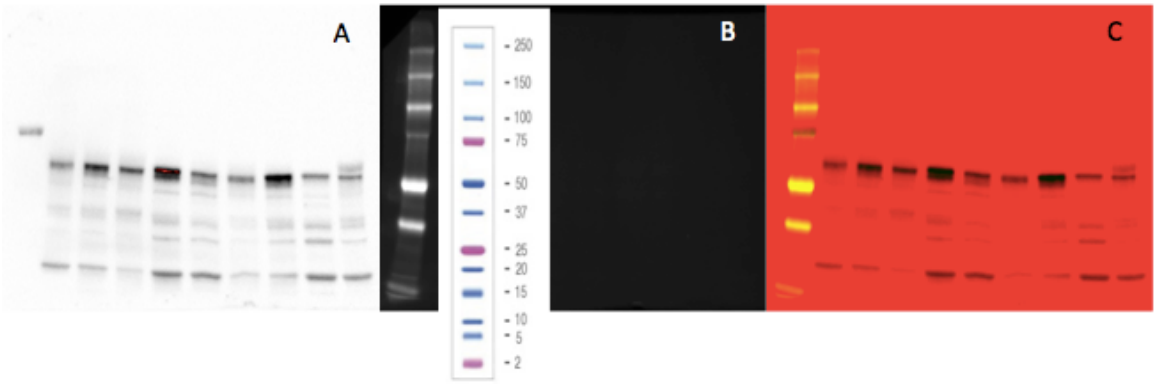

Figure A3. Method for identifying target proteins.

Appendix D

An image was taken of all proteins on the SDS-PAGE gel immediately after electrophoresis (visualized via Bio-Rad Stain Free gels; panel A). Stain free gels were imaged after proteins were transferred to PVDF membrane under the turbo transfer setting (proteins visible are those that remained on the gel after transfer and were not successfully transferred to PVDF membrane; panel B). Total protein on the PVDF membrane was visualized with Bio-Rad stain free technique after probing for target protein (C). Stain free images showed weak total protein signal. A second PVDF membrane was inserted behind the target PVDF membrane to determine if proteins over-transferred (through the first membrane; panel D).

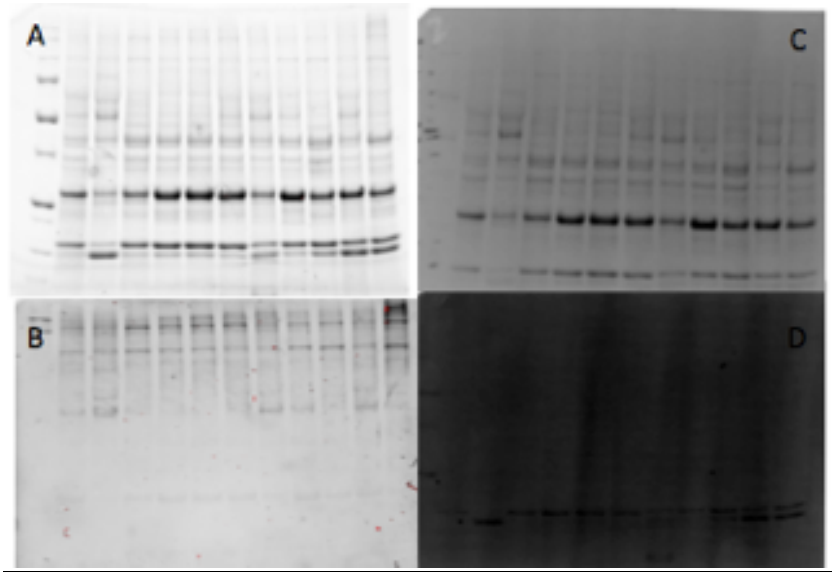

Figure A4. Results of condition optimization experiments for optimal western blotting transfer times.

Appendix E

Western blot chemiluminescent images of target bands with 7.5 % gels (A) tended to be diffuse and difficult to accurately quantify, hence, 10% (B) and 12% (C) gels were used.

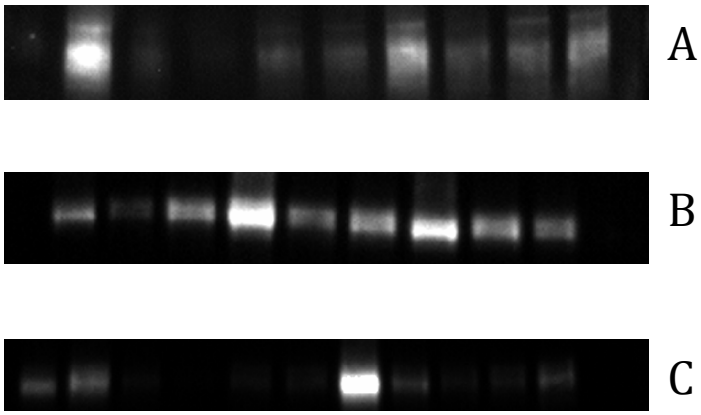

Figure A5. Western blot target protein chemiluminescent images with 7.5% (A), 10% (B), and 12% (C) gels.

Appendix F

Stain-free images were taken of total protein on the gel (A) and PVDF membrane (B). Total protein images on PVDF membranes obtained via stain-free technology were too faint and had too much background to accurately quantify. Total protein images obtained with Memcode PVDF Stain (E) showed clear, prominent bands. Chemiluminescent images of GAPDH (C) and β-actin (D) on the same membrane showed much variability across lanes. Chemiluminescent image of ACTA1 (F) also showed much variability. Total protein stain obtained via Memcode PVDF Stain produced the most reliable results, and was selected as the loading control.

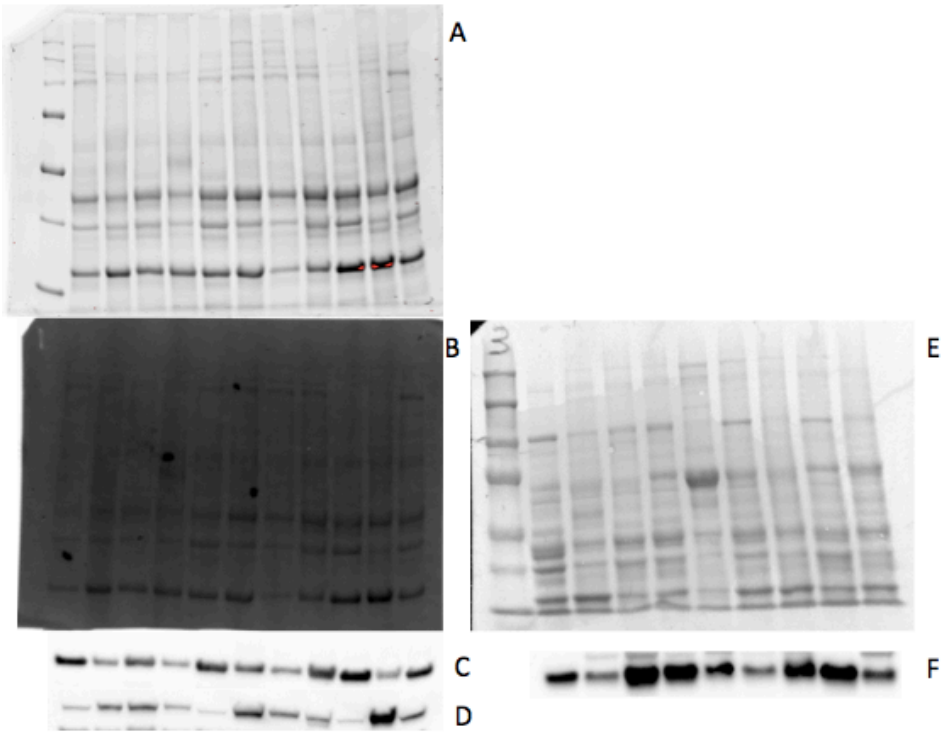

Figure A6. Western blot results of loading control experiments. Note: A-D are from the same gel/ membrane and E-F are from the same membrane.

Appendix G

IRE1α chemiluminescent images showed high background and non-specific binding. Target band was unidentifyable, therefore, this protein was excluded from the study.

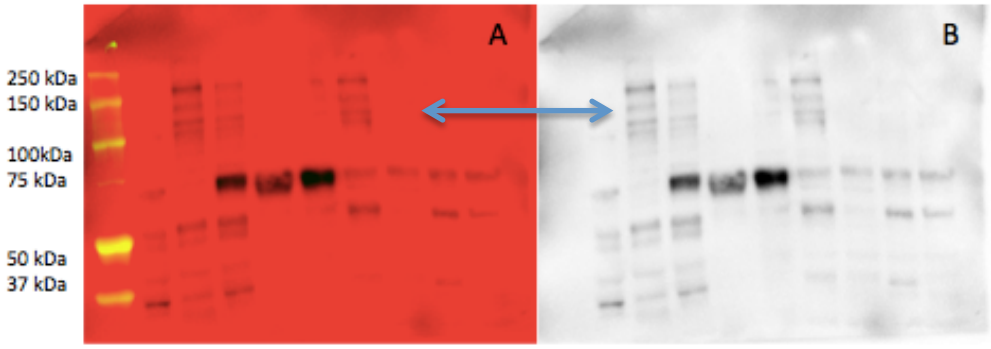

Figure A7. Western blot results showing inability to identify target band corresponding to IRE1α (130 kDa). Panel A is an overlay of Alexa image (showing protein ladder) and chemiluminescent image. Panel B is chemiluminescent image only. Double-headed arrow shows where target band was expected.

Appendix H

SPSS data from comparisons of protein levels between disease groups (ALS, CON, SMA, CON-SMA) for each target protein are shown. One-way between subjects ANOVA with LSD post hoc analyses were used.

Table A1. *Descriptive statistics for disease groups.*

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
SERCA1	ALS	38	47,470,872	64,191,641	10,413,259	26,371,605	68,570,139	1.20E+06	2.24E+08
	healthy control	13	79,243,663	73,560,886	20,402,119	34,791,264	123,696,061	1.29E+07	2.26E+08
	SMA	7	132,463,641	213,618,593	80,740,239	-65,100,606	330,027,889	450840.00	5.89E+08
	SMA control	3	105,349,353	151,680,232	87,572,623	-271,445,231	482,143,937	1.33E+06	2.79E+08
	Total	61	66,841,874	98,999,750	12,675,619	41,486,861	92,196,887	450840.00	5.89E+08
Akt	ALS	38	94,261,937	62,180,996	10,087,090	73,823,552	114,700,322	1.00E+07	2.35E+08
	healthy control	13	43,353,982	47,917,747	13,289,992	14,397,577	72,310,386	2.26E+06	1.71E+08
	SMA	7	117,595,063	36,943,467	13,963,318	83,428,054	151,762,071	7.84E+07	1.82E+08
	SMA control	3	97,537,068	25,463,201	14,701,186	34,282,971	160,791,166	8.02E+07	1.27E+08
	Total	61	86,251,345	59,682,291	7,641,534	70,966,000	101,536,689	2.26E+06	2.35E+08
BActin	ALS	38	50,856,808	63,639,054	10,323,618	29,939,171	71,774,444	1.35E+06	2.18E+08
	healthy control	13	28,556,806	42,686,415	11,839,081	2,761,664	54,351,949	1.53E+06	1.63E+08
	SMA	7	111,434,993	64,084,267	24,221,576	52,166,931	170,703,056	1.28E+07	2.05E+08
	SMA control	3	125,095,703	75,905,545	43,824,087	-63,464,124	313,655,529	5.61E+07	2.06E+08
	Total	61	56,707,037	65,125,046	8,338,408	40,027,738	73,386,335	1.35E+06	2.18E+08
Bip	ALS	39	51,634,409	41,020,106	6,568,474	38,337,228	64,931,589	5.13E+06	1.74E+08
	healthy control	13	14,483,527	19,883,948	5,514,815	2,467,778	26,499,277	1.26E+06	6.37E+07
	SMA	5	51,997,328	56,827,614	25,414,082	-18,563,475	122,558,130	5.36E+06	1.49E+08
	SMA control	3	57,134,183	50,535,448	29,176,655	-68,402,830	182,671,196	170232.00	9.66E+07

	Total	60	43,890,283	41,404,547	5,345,304	33,194,354	54,586,212	170232.00	1.74E+08
CHOP	ALS	38	95,146,570	62,225,605	10,094,326	74,693,523	115,599,618	1.79E+07	2.67E+08
	healthy control	12	74,113,151	47,347,042	13,667,914	44,030,275	104,196,026	2.20E+07	1.72E+08
	SMA	6	41,525,999	41,799,636	17,064,630	-2,340,029	85,392,027	893404.00	9.91E+07
	SMA control	3	13,152,370	17,349,400	10,016,681	-29,945,930	56,250,670	645021.00	3.30E+07
	Total	59	81,246,450	59,970,048	7,807,435	65,618,171	96,874,730	645021.00	2.67E+08
PDI	ALS	39	30,764,036	18,891,170	3,025,008	24,640,227	36,887,845	2.73E+06	1.01E+08
	healthy control	13	34,479,876	25,647,774	7,113,413	18,981,081	49,978,671	2.43E+06	8.67E+07
	SMA	7	29,638,620	31,777,751	12,010,861	249,102	59,028,138	3.06E+06	9.10E+07
	SMA control	3	19,733,108	21,232,211	12,258,423	-33,010,628	72,476,843	403410.00	4.25E+07
	Total	62	30,882,346	21,791,901	2,767,574	25,348,239	36,416,453	403410.00	1.01E+08
Serca2	ALS	38	104,466,528	63,880,313	10,362,755	83,469,591	125,463,464	1.26E+07	3.03E+08
	healthy control	13	161,107,268	133,779,109	37,103,649	80,265,361	241,949,175	3.90E+07	5.43E+08
	SMA	7	93,078,427	51,220,138	19,359,392	45,707,701	140,449,154	6.48E+06	1.75E+08
	SMA control	3	128,035,716	54,791,964	31,634,155	-8,075,068	264,146,501	6.62E+07	1.71E+08
	Total	61	116,389,814	83,974,379	10,751,817	94,882,978	137,896,651	6.48E+06	5.43E+08

Table A2. *ANOVA results for disease groups.*

		Sum of Squares	df	Mean Square	F	Sig.
SERCA1	Between Groups	5.E+16	3	2.E+16	1.798	.158
	Within Groups	5.E+17	57	9.E+15		
	Total	6.E+17	60			
Akt	Between Groups	3.E+16	3	1.E+16	3.547	.020
	Within Groups	2.E+17	57	3.E+15		
	Total	2.E+17	60			
BActin	Between Groups	5.E+16	3	2.E+16	4.259	.009
	Within Groups	2.E+17	57	4.E+15		
	Total	3.E+17	60			
Bip	Between Groups	1.E+16	3	5.E+15	3.108	.034
	Within Groups	9.E+16	56	2.E+15		
	Total	1.E+17	59			
CHOP	Between Groups	3.E+16	3	1.E+16	3.240	.029
	Within Groups	2.E+17	55	3.E+15		
	Total	2.E+17	58			
PDI	Between Groups	6.E+14	3	2.E+14	.376	.771
	Within Groups	3.E+16	58	5.E+14		
	Total	3.E+16	61			
Serca2	Between Groups	4.E+16	3	1.E+16	1.746	.168
	Within Groups	4.E+17	57	7.E+15		
	Total	4.E+17	60			

Table A3. *LSD post hoc analyses of disease groups.*

Dependent Variable			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
SERCA1	ALS	healthy control	-31772791	31192836	.313	-94235368	30689786
		SMA	-84992769*	39929943	.038	-164951101	-5034438
		SMA control	-57878480	58220074	.324	-174462168	58705206
	healthy control	ALS	31772790	31192836	.313	-30689786	94235368
		SMA	-53219978	45512171	.247	-144356529	37916572
		SMA control	-26105690	62181463	.676	-150621923	98410542
	SMA	ALS	84992769*	39929943	.038	5034438	164951101
		healthy control	53219978	45512171	.247	-37916572	144356529
		SMA control	27114288	66992102	.687	-107035082	161263658
	SMA control	ALS	57878480	58220074	.324	-58705206	174462168
		healthy control	26105690	62181463	.676	-98410542	150621923
		SMA	-27114288	66992102	.687	-161263658	107035082
Akt	ALS	healthy control	50907955*	18060899	.007	14741628	87074283
		SMA	-23333125	23119753	.317	-69629633	22963382
		SMA control	-3275131	33709884	.923	-70778010	64227748
	healthy control	ALS	-50907955*	18060899	.007	-87074283	-14741628
		SMA	-74241080*	26351908	.007	-127009866	-21472296
		SMA control	-54183086	36003560	.138	-126278972	17912799
	SMA	ALS	23333125	23119753	.317	-22963382	69629633
		healthy control	74241080*	26351908	.007	21472296	127009866
		SMA control	20057994	38788958	.607	-57615554	97731543
	SMA control	ALS	3275131	33709884	.923	-64227748	70778010
		healthy control	54183086	36003560	.138	-17912799	126278972

		SMA	-20057994	38788958	.607	-97731543	57615554
BActin	ALS	healthy control	22300001	19403828	.255	-16555493	61155496
		SMA	-60578185*	24838836	.018	-110317096	-10839275
		SMA control	-74238894*	36216402	.045	-146760988	-1716801
	healthy control	ALS	-22300001	19403828	.255	-61155496	16555493
		SMA	-82878187*	28311319	.005	-139570625	-26185749
		SMA control	-96538896*	38680625	.015	-173995512	-19082281
	SMA	ALS	60578185*	24838836	.018	10839275	110317096
		healthy control	82878187*	28311319	.005	26185749	139570625
		SMA control	-13660709	41673133	.744	-97109718	69788300
	SMA control	ALS	74238894*	36216402	.045	1716801	146760988
		healthy control	96538896*	38680625	.015	19082281	173995512
		SMA	13660709	41673133	.744	-69788300	97109718
Bip	ALS	healthy control	37150881*	12601987	.005	11906068	62395694
		SMA	-362918	18691767	.985	-37807027	37081190
		SMA control	-5499774	23576158	.816	-52728495	41728945
	healthy control	ALS	-37150881*	12601987	.005	-62395694	-11906068
		SMA	-37513800	20707177	.075	-78995261	3967660
		SMA control	-42650655	25203973	.096	-93140282	7838970
	SMA	ALS	362918	18691767	.985	-37081190	37807027
		healthy control	37513800	20707177	.075	-3967660	78995261
		SMA control	-5136855	28736951	.859	-62703886	52430175
	SMA control	ALS	5499774	23576158	.816	-41728945	52728495
		healthy control	42650656	25203973	.096	-7838970	93140282
		SMA	5136856	28736951	.859	-52430175	62703886
CHOP	ALS	healthy control	21033419	18798763	.268	-16640144	58706983
		SMA	53620570*	24939378	.036	3640941	103600200

		SMA control	81994199*	34046008	.019	13764475	150223925
	healthy control	ALS	-21033419	18798763	.268	-58706983	16640144
		SMA	32587151	28385510	.256	-24298682	89472984
		SMA control	60960780	36645536	.102	-12478514	134400075
	SMA	ALS	-53620570*	24939378	.036	-103600200	-3640941
		healthy control	-32587151	28385510	.256	-89472984	24298682
		SMA control	28373629	40143173	.483	-52075087	108822345
	SMA control	ALS	-81994199*	34046008	.019	-150223925	-13764475
		healthy control	-60960780	36645536	.102	-134400075	12478514
		SMA	-28373629	40143173	.483	-108822345	52075087
Serca2	ALS	healthy control	-56640739*	26492091	.037	-109690238	-3591242
		SMA	11388101	33912521	.738	-56520550	79296752
		SMA control	-23569188	49446338	.635	-122583772	75445396
	healthy control	ALS	56640739*	26492091	.037	3591242	109690238
		SMA	68028841	38653510	.084	-9373477	145431159
		SMA control	33071552	52810748	.534	-72680147	138823250
	SMA	ALS	-11388101	33912521	.738	-79296752	56520550
		healthy control	-68028841	38653510	.084	-145431159	9373477
		SMA control	-34957289	56896426	.541	-148890416	78975838
	SMA control	ALS	23569188	49446338	.635	-75445396	122583772
		healthy control	-33071552	52810748	.534	-138823250	72680147
		SMA	34957289	56896426	.541	-78975838	148890416

*. The mean difference is significant at the 0.05 level.

Appendix I

The ratio of SERCA2/SERCA1 protein was significantly higher in ALS compared to CON. SPSS analyses showed Levene's test for equality of variances was significant (Table A5), so t-test results for equal variances not assumed were used.

Table A4. *Descriptive statistics for ratio of SERCA2/SERCA1 protein levels for ALS and CON.*

ALSCON		N	Mean	Std. Deviation	Std. Error Mean
SERCA21ratio	ALS	38	13.8702	21.46194	3.48159
	CON	13	3.8850	2.78616	.77274

Table A5. *T-test results for differences in ratio of SERCA2/SERCA1 protein levels between ALS and CON.*

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
SERCA21ratio	Equal variances assumed	6.76	.012	1.662	49	.103	9.98525	6.00865	-2.08959	22.06008
	Equal variances not assumed			2.800	40.433	.008	9.98525	3.56631	2.77986	17.19063

Appendix J

SPSS t-tests revealed significant differences in Grp78/BiP, CHOP, SERCA2/SERCA1 ratio, and SERCA1 between quadriceps and diaphragm muscles in ALS.

Table A6. *Descriptive statistics for target protein levels in quad and diaphragm muscles in ALS.*

muscleanalysis		N	Mean	Std. Deviation	Std. Error Mean
Bip	quad	7	28779310.71	22110883.62	8357128.48
	diaphragm	3	79825420.33	45631721.94	26345486.95
CHOP	quad	7	50441546.00	23977617.02	9062687.38
	diaphragm	3	103860866.33	31070804.76	17938737.49
SERCA21ratio	quad	7	1.48	1.43	0.54
	diaphragm	3	12.55	1.27	0.73
SERCA1	quad	7	95500456.57	75348323.22	28478989.28
	diaphragm	3	9185196.00	3360297.73	1940068.80
Akt	quad	7	134928642.71	61396441.33	23205673.59
	diaphragm	3	98735343.00	62603872.21	36144362.47
BActin	quad	7	61938114.43	56308980.96	21282794.31
	diaphragm	3	23280364.00	20264916.87	11699955.21
PDI	quad	7	30869133.14	14988937.97	5665286.04
	diaphragm	3	54513776.83	40197769.51	23208193.04
Serca2	quad	7	96000399.00	63151731.69	23869110.99
	diaphragm	3	116361138.67	50215266.43	28991797.59

Table A7. *T-test results for differences in target protein levels between quad and diaphragm muscles in ALS.*

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Bip	Equal variances assumed	3.521	.097	-2.483	8	.038	-51046110	20554595	-98445091	-3647128
	Equal variances not assumed			-1.847	2.415	.184	-51046110	27639216	-152392389	50300170
CHOP	Equal variances assumed	.449	.522	-2.985	8	.017	-53419320	17895774	-94687049	-12151592
	Equal variances not assumed			-2.658	3.084	.074	-53419320	20098025	-116403295	9564654
SERCA21ratio	Equal variances assumed	.001	.978	-11.510	8	.000	-11.07	0.96	-13.28	-8.85
	Equal variances not assumed			-12.141	4.342	.000	-11.07	0.91	-13.52	-8.61
SERCA1	Equal variances assumed	11.606	.009	1.916	8	.092	86315261	45044160	-17556758	190187279
	Equal variances not assumed			3.024	6.055	.023	86315261	28544994	16622858	156007664
Akt	Equal variances assumed	.111	.748	.850	8	.420	36193300	42577400	-61990360	134376960

BActin	Equal variances not assumed			.843	3.775	.450	36193300	42952511	-85920641	158307240
	Equal variances assumed	1.488	.257	1.125	8	.293	38657750	34369787	-40599120	117914621
	Equal variances not assumed			1.592	7.986	.150	38657750	24286751	-17364307	94679808
PDI	Equal variances assumed	10.792	.011	-1.432	8	.190	-23644644	16510694	-61718373	14429086
	Equal variances not assumed			-.990	2.243	.417	-23644644	23889657	-116472957	69183670
Serca2	Equal variances assumed	.859	.381	-.490	8	.637	-20360740	41527381	-116123053	75401574
	Equal variances not assumed			-.542	4.882	.612	-20360740	37553412	-117597938	76876459

Appendix K

SPSS t-test results for differences in target protein levels between ALS and CON in psoas muscles are shown. Grp78/BiP and CHOP protein levels were significantly higher in psoas muscles of ALS compared to CON. Levene's test for equality of variances was significant for Grp78/BiP (Table A7), so t-test results for equal variances not assumed were used.

Table A8. *Descriptive statistics for mean target protein levels in psoas muscles of ALS and CON.*

psoas		N	Mean	Std. Deviation	Std. Error Mean
SERCA21ratio	ALS	6	19	29	12
	CON	13	4	3	1
SERCA1	ALS	6	53039110	85337051	34838705
	CON	13	79243663	73560886	20402119
Akt	ALS	6	47472699	34089327	13916910
	CON	13	43353982	47917747	13289992
BActin	ALS	6	53525322	74877065	30568434
	CON	13	28556806	42686415	11839081
Bip	ALS	6	76927621	57326843	23403586
	CON	13	14483527	19883948	5514815
CHOP	ALS	6	135170418	63426144	25893615
	CON	12	74113151	47347042	13667914
PDI	ALS	6	23850033	17153310	7002809
	CON	13	34479876	25647774	7113413
Serca2	ALS	6	112754960	70222851	28668359
	CON	13	161107268	133779109	37103649

Table A9. *T-test results comparing means of target protein levels in psoas muscles of ALS and CON.*

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
SERCA21ratio	Equal variances assumed	16.259	.001	1.893	17	.076	15	8	-2	31
	not assumed			1.252	5.043	.265	15	12	-15	45
SERCA1	Equal variances assumed	.026	.875	-.688	17	.501	-26204553	38107410	-106604160	54195055
	not assumed			-.649	8.596	.533	-26204553	40373033	-118192761	65783655
Akt	Equal variances assumed	.447	.513	.188	17	.853	4118717	21864642	-42011646	50249080
	not assumed			.214	13.574	.834	4118717	19243291	-37275901	45513335
BActin	Equal variances assumed	3.038	.099	.934	17	.363	24968516	26739206	-31446276	81383308
	not assumed			.762	6.551	.473	24968516	32780985	-53636108	103573140
Bip	Equal variances assumed	14.263	.002	3.585	17	.002	62444094	17419272	25692643	99195544
	not assumed			2.597	5.564	.044	62444094	24044563	2471981	122416207
CHOP	Equal variances assumed	.646	.433	2.308	16	.035	61057268	26449700	4986408	117128127
	not assumed			2.085	7.896	.071	61057268	29279535	-6616899	128731434
PDI	Equal variances assumed	1.169	.295	-.918	17	.372	-10629843	11583936	-35069812	13810125
	not assumed			-1.065	14.299	.305	-10629843	9981983	-31997192	10737505
Serca2	Equal variances assumed	.534	.475	-.826	17	.421	-48352308	58571162	-171926659	75222043
	not assumed			-1.031	16.495	.317	-48352308	46888758	-147510051	50805435

Appendix L

SPSS results for ANOVA in target protein levels between +C9orf72 cases, -C9orf72 cases and CON.

Table A10. *Descriptive statistics for mean target protein levels in +/- C9orf72 cases and CON.*

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
SERCA1	yes	7	13175395	11620125	4391994	2428572	23922218	5.77E+06	3.89E+07
	no	13	27433424	46187598	12810135	-477463	55344310	1.96E+06	1.68E+08
	con	13	79243663	73560886	20402119	34791264	123696061	1.29E+07	2.26E+08
	Total	33	44819087	60644577	10556866	23315456	66322719	1.96E+06	2.26E+08
Akt	yes	7	76668092	56324671	21288725	24576459	128759724	1.81E+07	1.92E+08
	no	13	67681704	45380562	12586303	40258505	95104903	1.00E+07	1.67E+08
	con	13	43353982	47917747	13289992	14397577	72310386	2.26E+06	1.71E+08
	Total	33	60004259	49249029	8573155	42541313	77467205	2.26E+06	1.92E+08
BActin	yes	7	16438855	16750467	6331081	947256	31930453	4.56E+06	5.35E+07
	no	13	41510771	52600260	14588687	9724753	73296790	1.94E+06	1.94E+08
	con	13	28556806	42686415	11839081	2761664	54351949	1.53E+06	1.63E+08
	Total	33	31089409	43210312	7521950	15767698	46411119	1.53E+06	1.94E+08
Bip	yes	7	52020565	37976519	14353775	16898143	87142988	1.22E+07	1.32E+08
	no	13	54622042	44734804	12407202	27589071	81655014	1.60E+07	1.74E+08
	con	13	14483527	19883948	5514815	2467778	26499277	1.26E+06	6.37E+07
	Total	33	38258072	39356995	6851173	24302688	52213455	1.26E+06	1.74E+08
CHOP	yes	7	92878565	40354943	15252735	55556468	130200663	3.13E+07	1.38E+08
	no	13	99309800	57676742	15996650	64456094	134163507	2.12E+07	2.15E+08
	con	12	74113151	47347042	13667914	44030275	104196026	2.20E+07	1.72E+08
	Total	32	88454224	50317239	8894915	70312925	106595523	2.12E+07	2.15E+08
PDI	yes	7	44499675	27587386	10427052	18985598	70013752	2.48E+07	1.01E+08
	no	13	26641546	15309307	4246038	17390225	35892868	2.73E+06	4.90E+07
	con	13	34479876	25647774	7113413	18981081	49978671	2.43E+06	8.67E+07
	Total	33	33517461	22874319	3981908	25406580	41628342	2.43E+06	1.01E+08
Serca2	yes	7	106400652	37321589	14106235	71883940	140917365	4.55E+07	1.71E+08
	no	13	76713981	50421017	13984274	46244865	107183096	1.37E+07	1.78E+08
	con	13	161107268	133779109	37103649	80265361	241949175	3.90E+07	5.43E+08
	Total	33	116256994	96951107	16877021	81879626	150634362	1.37E+07	5.43E+08

Table A11. *ANOVA results for differences in mean target protein levels between +C9orf72 cases, - C9orf72 cases, and CON.*

		Sum of Squares	df	Mean Square	F	Sig.
SERCA1	Between Groups	2.63E+16	2	1.32E+16	4.326	.022
	Within Groups	9.13E+16	30	3.04E+15		
	Total	1.18E+17	32			
Akt	Between Groups	6.31E+15	2	3.16E+15	1.328	.280
	Within Groups	7.13E+16	30	2.38E+15		
	Total	7.76E+16	32			
BActin	Between Groups	3.00E+15	2	1.50E+15	.792	.462
	Within Groups	5.68E+16	30	1.89E+15		
	Total	5.97E+16	32			
Bip	Between Groups	1.22E+16	2	6.08E+15	4.873	.015
	Within Groups	3.74E+16	30	1.25E+15		
	Total	4.96E+16	32			
CHOP	Between Groups	4.14E+15	2	2.07E+15	.807	.456
	Within Groups	7.43E+16	29	2.56E+15		
	Total	7.85E+16	31			
PDI	Between Groups	1.47E+15	2	7.35E+14	1.445	.252
	Within Groups	1.53E+16	30	5.09E+14		
	Total	1.67E+16	32			
Serca2	Between Groups	4.72E+16	2	2.36E+16	2.789	.077
	Within Groups	2.54E+17	30	8.45E+15		
	Total	3.01E+17	32			

Table A12. *LSD post hoc analyses for differences in mean target protein levels between +C9orf72 cases, - C9orf72 cases, and CON.*

Dependent Variable			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
SERCA1	yes	no	-14258029	25868666	.586	-67088892	38572834
		control	-66068267*	25868666	.016	-118899131	-13237405
	no	yes	14258029	25868666	.586	-38572834	67088892
		control	-51810239*	21643278	.023	-96011710	-7608768
	control	yes	66068267*	25868666	.016	13237405	118899131
		no	51810239*	21643278	.023	7608768	96011710
Akt	yes	no	8986387	22854995	.697	-37689738	55662514
		control	33314110	22854995	.155	-13362016	79990236
	no	yes	-8986388	22854995	.697	-55662514	37689738
		control	24327722	19121860	.213	-14724327	63379771
	control	yes	-33314110	22854995	.155	-79990236	13362016
		no	-24327722	19121860	.213	-63379771	14724327
BActin	yes	no	-25071917	20390057	.228	-66713968	16570134
		control	-12117952	20390057	.557	-53760003	29524099
	no	yes	25071917	20390057	.228	-16570134	66713968
		control	12953965	17059545	.454	-21886274	47794205
	control	yes	12117952	20390057	.557	-29524099	53760003
		no	-12953965	17059545	.454	-47794205	21886274
Bip	yes	no	-2601477	16555420	.876	-36412156	31209202
		control	37537037*	16555420	.031	3726359	71347716
	no	yes	2601477	16555420	.876	-31209202	36412156
		control	40138514*	13851258	.007	11850472	68426558
	control	yes	-37537037*	16555420	.031	-71347716	-3726359
		no	-40138514*	13851258	.007	-68426558	-11850472
CHOP	yes	no	-6431235	23737476	.788	-54979825	42117355
		control	18765415	24081160	.442	-30486087	68016917
	no	yes	6431235	23737476	.788	-42117355	54979825

		control	25196650	20269729	.224	-16259602	66652901
	control	yes	-18765415	24081160	.442	-68016917	30486087
		no	-25196650	20269729	.224	-66652901	16259602
PDI	yes	no	17858129	10577667	.102	-3744350	39460608
		control	10019799	10577667	.351	-11582679	31622278
	no	yes	-17858129	10577667	.102	-39460608	3744350
		control	-7838330	8849911	.383	-25912260	10235601
	control	yes	-10019799	10577667	.351	-31622278	11582679
		no	7838329	8849911	.383	-10235601	25912260
Serca2	yes	no	29686672	43105370	.496	-58346238	117719582
		control	-54706615	43105370	.214	-142739525	33326295
	no	yes	-29686672	43105370	.496	-117719582	58346238
		control	-84393287*	36064540	.026	-158046904	-10739670
	control	yes	54706615	43105370	.214	-33326295	142739525
		no	84393287*	36064540	.026	10739670	158046904

*. The mean difference is significant at the 0.05 level.

Appendix M

SPSS results for ANOVA in target protein levels between sALS cases, fALS cases and CON.

Table A13. *Descriptive statistics for mean target protein levels in sALS, fALS, and CON.*

		N	Mean	Std. Deviation	Std. Error
SERCA1	sALS	17	26360052	40920870	9924769
	fALS	3	6556413	5035731	2907381
	control	13	79243663	73560886	20402119
	Total	33	45392659	60559079	10541983
Akt	sALS	17	65043431	43881703	10642876
	fALS	3	94845698	84536338	48807078
	control	13	43353982	47917747	13289992
	Total	33	59208400	50097513	8720858
BActin	sALS	17	34318033	47573193	11538194
	fALS	3	27372913	22640666	13071595
	control	13	28556806	42686415	11839081
	Total	33	31417084	43084432	7500037
Bip	sALS	18	63994984	46540662	10969739
	fALS	3	41340191	18219872	10519248
	control	13	14483527	19883948	5514815
	Total	34	43065180	42903958	7357968
CHOP	sALS	17	109095750	55088439	13360909
	fALS	3	68893182	37304727	21537894
	control	12	74113151	47347042	13667914
	Total	32	92208284	52794913	9332910
PDI	sALS	18	31406260	24550817	5786683
	fALS	3	33385712	7960616	4596064
	control	13	34479876	25647774	7113413
	Total	34	32756124	23574307	4042960
Serca2	sALS	17	103500456	70587718	17120036
	fALS	3	92011072	27432181	15837977
	control	13	161107268	133779109	37103649
	Total	33	125149559	100632345	17517843

Table A14. *ANOVA results for differences in mean target protein levels between sALS, fALS, and CON.*

		Sum of Squares	df	Mean Square	F	Sig.
SERCA1	Between Groups	25579420271194600	2	12789710135597300	4.181	.025
	Within Groups	91777446485642500	30	3059248216188080		
	Total	117356866756837000	32			
Akt	Between Groups	7656573567603200	2	3828286783801600	1.581	.222
	Within Groups	72655771874718500	30	2421859062490620		
	Total	80312345442321700	32			
BActin	Between Groups	298484982459270	2	149242491229635	.076	.927
	Within Groups	59102098679829800	30	1970069955994330		
	Total	59400583662289100	32			
Bip	Between Groups	18513788160520200	2	9256894080260090	6.795	.004
	Within Groups	42230948945970600	31	1362288675676470		
	Total	60744737106490800	33			
CHOP	Between Groups	10408158700322200	2	5204079350161100	1.986	.156
	Within Groups	75998229189721600	29	2620628592749020		
	Total	86406387890043800	31			
PDI	Between Groups	72614711938039	2	36307355969020	.062	.940
	Within Groups	18267066923224800	31	589260223329833		
	Total	18339681635162900	33			
Serca2	Between Groups	28070538708476800	2	14035269354238400	1.423	.257
	Within Groups	295989266315132000	30	9866308877171070		
	Total	324059805023609000	32			

Table A15. *LSD post hoc analyses for differences in mean target protein levels between sALS, fALS, and CON.*

Dependent Variable			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
SERCA1	sALS	fALS	19803639.01961	34636761	.572	-50934063.0690	90541341.1083
		control	-52883610.91629*	20378483	.014	-94502025.1428	-11265196.6897
	fALS	sALS	-19803639.01961	34636761	.572	-90541341.1083	50934063.0690
		control	-72687249.93590*	35427055	.049	-145038947.5696	-335552.3022
	control	sALS	52883610.91629*	20378483	.014	11265196.6897	94502025.1428
		fALS	72687249.93590*	35427055	.049	335552.3022	145038947.5696
Akt	sALS	fALS	-29802267.39216	30817992	.341	-92741004.3096	33136469.5253
		control	21689449.17195	18131717	.241	-15340456.3322	58719354.6761
	fALS	sALS	29802267.39216	30817992	.341	-33136469.5253	92741004.3096
		control	51491716.56410	31521155	.113	-12883069.8432	115866502.9714
	control	sALS	-21689449.17195	18131717	.241	-58719354.6761	15340456.3322
		fALS	-51491716.56410	31521155	.113	-115866502.9714	12883069.8432
BActin	sALS	fALS	6945119.82353	27795260	.804	-49820373.1667	63710612.8138
		control	5761226.59276	16353297	.727	-27636660.6906	39159113.8762
	fALS	sALS	-6945119.82353	27795260	.804	-63710612.8138	49820373.1667
		control	-1183893.23077	28429454	.967	-59244583.1259	56876796.6643
	control	sALS	-5761226.59276	16353297	.727	-39159113.8762	27636660.6906
		fALS	1183893.23077	28429454	.967	-56876796.6643	59244583.1259
Bip	sALS	fALS	22654792.16667	23016927	.333	-24288539.9847	69598124.3180
		control	49511456.11538*	13434066	.001	22112496.9955	76910415.2353
	fALS	sALS	-22654792.16667	23016927	.333	-69598124.3180	24288539.9847
		control	26856663.94872	23640805	.265	-21359075.7655	75072403.6630
	control	sALS	-49511456.11538*	13434066	.001	-76910415.2353	-22112496.9955
		fALS	-26856663.94872	23640805	.265	-75072403.6630	21359075.7655
CHOP	sALS	fALS	40202567.79412	32057721	.220	-25362833.8256	105767969.4138
		control	34982599.21078	19301304	.080	-4493000.0242	74458198.4458
	fALS	sALS	-40202567.79412	32057721	.220	-105767969.4138	25362833.8256

		control	-5219968.58333	33044343	.876	-72803237.6493	62363300.4827
	control	sALS	-34982599.21078	19301304	.080	-74458198.4458	4493000.0242
		fALS	5219968.58333	33044343	.876	-62363300.4827	72803237.6493
PDI	sALS	fALS	-1979451.58333	15137924	.897	-32853451.8383	28894548.6717
		control	-3073616.16026	8835405	.730	-21093544.2754	14946311.9549
	fALS	sALS	1979451.58333	15137924	.897	-28894548.6717	32853451.8383
		control	-1094164.57692	15548241	.944	-32805010.2287	30616681.0749
	control	sALS	3073616.16026	8835405	.730	-14946311.9549	21093544.2754
		fALS	1094164.57692	15548241	.944	-30616681.0749	32805010.2287
Serca2	sALS	fALS	11489384.29412	62202417	.855	-115544899.5715	138523668.1597
		control	-57606811.55204	36596693	.126	-132347229.8974	17133606.7933
	fALS	sALS	-11489384.29412	62202417	.855	-138523668.1597	115544899.5715
		control	-69096195.84615	63621667	.286	-199028973.1909	60836581.4985
	control	sALS	57606811.55204	36596693	.126	-17133606.7933	132347229.8974
		fALS	69096195.84615	63621667	.286	-60836581.4985	199028973.1909

Appendix N

SPSS results for ANOVA in target protein levels between bulbar onset, limb onset, and CON.

Table A16. *Descriptive statistics for mean target protein levels in bulbar onset, limb onset, and CON.*

		N	Mean	Std. Deviation	Std. Error
SERCA1	bulbar	12	32174944	46207930	13339080
	limb	25	47760815	62942364	12588473
	control	13	79243663	73560886	20402119
	Total	50	52205747	63618041	8996950
Akt	bulbar	12	101777107	52314906	15102012
	limb	25	93481494	66853987	13370797
	control	13	43353982	47917747	13289992
	Total	50	82439288	62649148	8859927
BActin	bulbar	12	14112736	17299646	4993978
	limb	25	62736136	66491068	13298214
	control	13	28556806	42686415	11839081
	Total	50	42179894	56005269	7920341
Bip	bulbar	13	58747807	47405522	13147926
	limb	25	47534620	38619318	7723864
	control	13	14483527	19883948	5514815
	Total	51	41968095	40438060	5662461
CHOP	bulbar	12	72024888	46700688	13481327
	limb	25	100250676	61152316	12230463
	control	12	74113151	47347042	13667914
	Total	49	86937211	55428899	7918414
PDI	bulbar	13	32045029	14152833	3925290
	limb	25	30344643	21499895	4299979
	control	13	34479876	25647774	7113413
	Total	51	31832154	20754887	2906265
Serca2	bulbar	12	120610186	74609170	21537812
	limb	25	93862280	57271206	11454241
	control	13	161107268	133779109	37103649
	Total	50	117765474	89615462	12673540

Table A17. *ANOVA results for differences in mean target protein levels between bulbar onset, limb onset, and CON.*

		Sum of Squares	df	Mean Square	F	Sig.
SERCA1	Between Groups	14812367812374600	2	7406183906187300	1.897	.161
	Within Groups	183503136339966000	47	3904322049786500		
	Total	198315504152340000	49			
Akt	Between Groups	27395268049953200	2	13697634024976600	3.904	.027
	Within Groups	164925602053484000	47	3509055362840090		
	Total	192320870103438000	49			
BActin	Between Groups	22429812438888700	2	11214906219444300	4.016	.025
	Within Groups	131263107833596000	47	2792832081565870		
	Total	153692920272485000	49			
Bip	Between Groups	14255137485863600	2	7127568742931820	5.068	.010
	Within Groups	67506699219585400	48	1406389567074700		
	Total	81761836705449100	50			
CHOP	Between Groups	9073215189496310	2	4536607594748160	1.508	.232
	Within Groups	138400200365677000	46	3008700007949490		
	Total	147473415555173000	48			
PDI	Between Groups	147041975090847	2	73520987545424	.165	.848
	Within Groups	21391223941285500	48	445650498776782		
	Total	21538265916376400	50			
Serca2	Between Groups	38801820250424800	2	19400910125212400	2.571	.087
	Within Groups	354713797616209000	47	7547102076940620		
	Total	393515617866634000	49			

Table A18. *LSD post hoc analyses for differences in mean target protein levels between bulbar onset, limb onset, and CON.*

Dependent Variable			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
SERCA1	bulbar	limb	-15585871.11667	21943861	.481	-59731226.1073	28559483.8740
		control	-47068718.68590	25013849	.066	-97390091.8979	3252654.5261
	limb	bulbar	15585871.11667	21943861	.481	-28559483.8740	59731226.1073
		control	-31482847.56923	21365986	.147	-74465666.7319	11499971.5935
	control	bulbar	47068718.68590	25013849	.066	-3252654.5261	97390091.8979
		limb	31482847.56923	21365986	.147	-11499971.5935	74465666.7319
Akt	bulbar	limb	8295612.84333	20803449	.692	-33555528.5507	50146754.2373
		control	58423125.31410*	23713891	.017	10716930.4975	106129320.1307
	limb	bulbar	-8295612.84333	20803449	.692	-50146754.2373	33555528.5507
		control	50127512.47077*	20255605	.017	9378490.4577	90876534.4839
	control	bulbar	-58423125.31410*	23713891	.017	-106129320.1307	-10716930.4975
		limb	-50127512.47077*	20255605	.017	-90876534.4839	-9378490.4577
BActin	bulbar	limb	-48623400.24000*	18559345	.012	-85959986.6599	-11286813.8201
		control	-14444070.23077	21155832	.498	-57004115.3384	28115974.8769
	limb	bulbar	48623400.24000*	18559345	.012	11286813.8201	85959986.6599
		control	34179330.00923	18070598	.065	-2174024.5527	70532684.5712
	control	bulbar	14444070.23077	21155832	.498	-28115974.8769	57004115.3384
		limb	-34179330.00923	18070598	.065	-70532684.5712	2174024.5527
Bip	bulbar	limb	11213187.37538	12823393	.386	-14569971.3213	36996346.0721
		control	44264279.73077*	14709440	.004	14688968.3612	73839591.1003
	limb	bulbar	-11213187.37538	12823393	.386	-36996346.0721	14569971.3213
		control	33051092.35538*	12823393	.013	7267933.6587	58834251.0521
	control	bulbar	-44264279.73077*	14709440	.004	-73839591.1003	-14688968.3612
		limb	-33051092.35538*	12823393	.013	-58834251.0521	-7267933.6587
CHOP	bulbar	limb	-28225787.20333	19263255	.150	-67000708.8205	10549134.4138
		control	-2088262.16667	22393079	.926	-47163192.9713	42986668.6380
	limb	bulbar	28225787.20333	19263255	.150	-10549134.4138	67000708.8205

		control	26137525.03667	19263255	.181	-12637396.5805	64912446.6538
	control	bulbar	2088262.16667	22393079	.926	-42986668.6380	47163192.9713
		limb	-26137525.03667	19263255	.181	-64912446.6538	12637396.5805
PDI	bulbar	limb	1700386.68615	7218506	.815	-12813392.1913	16214165.5637
		control	-2434846.73077	8280194	.770	-19083292.8929	14213599.4313
	limb	bulbar	-1700386.68615	7218506	.815	-16214165.5637	12813392.1913
		control	-4135233.41692	7218506	.569	-18649012.2944	10378545.4606
	control	bulbar	2434846.73077	8280194	.770	-14213599.4313	19083292.8929
		limb	4135233.41692	7218506	.569	-10378545.4606	18649012.2944
Serca2	bulbar	limb	26747906.57333	30509167	.385	-34628620.2302	88124433.3769
		control	-40497081.51282	34777457	.250	-110460299.8942	29466136.8685
	limb	bulbar	-26747906.57333	30509167	.385	-88124433.3769	34628620.2302
		control	-67244988.08615*	29705730	.028	-127005208.6799	-7484767.4924
	control	bulbar	40497081.51282	34777457	.250	-29466136.8685	110460299.8942
		limb	67244988.08615*	29705730	.028	7484767.4924	127005208.6799

*. The mean difference is significant at the 0.05 level.

Appendix O

Letter for IRB exemption.

UNIVERSITY OF MARYLAND
INSTITUTIONAL REVIEW BOARD

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College Park, MD 20742-5125
TEL 301.405.4212
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DATE:	January 25, 2013
TO:	Eva Chin, PhD
FROM:	University of Maryland College Park (UMCP) IRB
PROJECT TITLE:	[404343-1] Analysis of human skeletal muscle biopsy samples for ALS biomarkers
SUBMISSION TYPE:	New Project
ACTION:	DETERMINATION OF EXEMPT STATUS
DECISION DATE:	January 25, 2013
REVIEW CATEGORY:	Exemption category # 4

Thank you for your submission of New Project materials for this project. The University of Maryland College Park (UMCP) IRB has determined this project is EXEMPT FROM IRB REVIEW according to federal regulations.

We will retain a copy of this correspondence within our records.

If you have any questions, please contact the IRB Office at 301-405-4212 or irb@umd.edu. Please include your project title and reference number in all correspondence with this committee.

This letter has been electronically signed in accordance with all applicable regulations, and a copy is retained within University of Maryland College Park (UMCP) IRB's records.

References

1. **Al-Saif A**, **Al-Mohanna F**, **Bohlega S**. A mutation in sigma-1 receptor causes juvenile amyotrophic lateral sclerosis. *Ann Neurol.* 70: 913–919, 2011.

2. **Aldridge GM**, **Podrebarac DM**, **Greenough WT**, **Weiler IJ**. The use of total protein stains as loading controls: an alternative to high-abundance single-protein controls in semi-quantitative immunoblotting. *J. Neurosci. Methods* 172: 250–254, 2008.

3. **ALSAssociation**. Research ALS Today Fall 2013.

4. **Andersen PM**. Amyotrophic lateral sclerosis associated with mutations in the CuZn superoxide dismutase gene. *Curr Neurol Neurosci Rep* 6: 37–46, 2006.

5. **Andrus PK**, **Fleck TJ**, **Gurney ME**, **Hall ED**. Protein oxidative damage in a transgenic mouse model of familial amyotrophic lateral sclerosis. *J. Neurochem.* 71: 2041–2048, 1998.

6. **Arai T**, **Hasegawa M**, **Akiyama H**, **Ikeda K**, **Nonaka T**, **Mori H**, **Mann D**, **Tsuchiya K**, **Yoshida M**, **Hashizume Y**, **Oda T**. TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Biochemical and Biophysical Research Communications* 351: 602–611, 2006.

7. **Araki S**, **Iwahashi Y**, **Kuroiwa Y**. Epidemiological study of amyotrophic lateral sclerosis and allied disorders in the Kii Peninsula (Japan). *J. Neurol. Sci.* 4: 279–287, 1967.

8. **Armon C**. A randomized controlled trial of resistance exercise in individuals with ALS. *Neurology* 71: 864–5– author reply 865–6, 2008.

9. **ARNOLD A**, **EDGREN DC**, **PALLADINO VS**. AMYOTROPHIC LATERAL SCLEROSIS. *The Journal of Nervous and Mental Disease* 117: 135–139, 1953.

10. **Barber RD**, **Harmer DW**, **Coleman RA**, **Clark BJ**. GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. *Physiol. Genomics* 21: 389–395, 2005.

11. **Barber SC**, **Mead RJ**, **Shaw PJ**. Oxidative stress in ALS: A mechanism of neurodegeneration and a therapeutic target. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* 1762: 1051–1067, 2006.

12. **Barber SC**, **Shaw PJ**. Free Radical Biology & Medicine. *Free Radical Biology and Medicine* 48: 629–641, 2010.

13. **Beers DR**, **Henkel JS**, **Xiao Q**, **Zhao W**, **Wang J**, **Yen AA**, **Siklos L**, **McKercher SR**, **Appel SH**. Wild-type microglia extend survival in PU.1 knockout mice with familial amyotrophic lateral sclerosis. *Proc. Natl. Acad. Sci. U.S.A.* 103: 16021–16026, 2006.

14. **Belli S**, **Vanacore N**. Proportionate mortality of Italian soccer players: Is amyotrophic lateral sclerosis an occupational disease? *Eur J Epidemiol* 20: 237–242, 2005.

15. **Berridge MJ**. The endoplasmic reticulum: a multifunctional signaling organelle. *Cell Calcium* 32: 235–249, 2002.

16. **Berridge MJ**. The endoplasmic reticulum: a multifunctional signaling organelle. *Cell Calcium* 32: 235–249, 2002.

17. **Bilsland LG**, **Nirmalananthan N**, **Yip J**, **Greensmith L**, **Duchen MR**. Expression of mutant SOD1 in astrocytes induces functional deficits in motoneuron mitochondria. *J. Neurochem.* 107: 1271–1283, 2008.

18. **Bogdanov MB**, **Ramos LE**, **Xu Z**, **Beal MF**. Elevated "hydroxyl radical" generation in vivo in an animal model of amyotrophic lateral sclerosis. *J. Neurochem.* 71: 1321–1324, 1998.

19. **Bohannon RW**. Results of resistance exercise on a patient with amyotrophic lateral sclerosis. A case report. *Phys Ther* 63: 965–968, 1983.

20. **Boillee S**, **Vandevelde C**, **Cleveland D**. ALS: A Disease of Motor Neurons and Their Nonneuronal Neighbors. *Neuron* 52: 39–59, 2006.

21. **Borasio GD**, **Robberecht W**, **Leigh PN**, **Emile J**. A placebo-controlled trial of insulin-like growth factor-I in amyotrophic lateral sclerosis. *Neurology*.

22. **Bourke SC**, **Tomlinson M**, **Williams TL**, **Bullock RE**, **Shaw PJ**, **Gibson GJ**. Effects of non-invasive ventilation on survival and quality of life in patients with amyotrophic lateral sclerosis: a randomised controlled trial. *Lancet Neurol* 5: 140–147, 2006.

23. **Brooks BR**, **Miller RG**, **Swash M**, **Munsat TL**, **World Federation of Neurology Research Group on Motor Neuron Diseases**. El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. Amyotrophic lateral sclerosis and other motor neuron disorders : official publication of the World Federation of Neurology, Research Group on Motor Neuron Diseases. 2000, p. 293–299.

24. **Brooks BR**. Managing amyotrophic lateral sclerosis: slowing disease progression and improving patient quality of life. *Ann Neurol.* 65 Suppl 1: S17–23, 2009.

25. **Cady J**, **Allred P**, **Bali T**, **Pestronk A**, **Goate A**, **Miller TM**, **Mitra RD**, **Ravits J**, **Harms MB**, **Baloh RH**. Amyotrophic lateral sclerosis onset is influenced by the burden of rare variants in known amyotrophic lateral sclerosis genes. *Ann Neurol.* 77: 100–113, 2015.

26. **Carreras I**, **Yuruker S**, **Aytan N**, **Hossain L**, **Choi J-K**, **Jenkins BG**, **Kowall NW**, **Dedeoglu A**. Moderate exercise delays the motor performance decline in a transgenic model of ALS. *Brain Research* 1313: 192–201, 2010.

27. **Cervenakova L**, **Protas II**, **Hirano A**, **Votiakov VI**. Progressive muscular atrophy variant of familial amyotrophic lateral sclerosis (PMA/ALS). *Journal of the ...* 177: 124–130, 2000.

28. **Chin ER**, **Chen D**, **Bobyk K**, **Mazala DAG**. Perturbations in Intracellular Ca2+ Handling in Skeletal Muscle in the G93A*SOD1 Mouse Model of Amyotrophic Lateral Sclerosis. *AJP: Cell Physiology* (September 24, 2014). doi: 10.1152/ajpcell.00237.2013.

29. **Chio A**, **Calvo A**, **Moglia C**, **Mazzini L**, **Mora G**, **PARALS study group**. Phenotypic heterogeneity of amyotrophic lateral sclerosis: a population based study. *J. Neurol. Neurosurg. Psychiatr.* 82: 740–746, 2011.

30. **Chio A**, **Traynor BJ**, **Lombardo F**, **Fimognari M**, **Calvo A**, **Ghiglione P**, **Mutani R**, **Restagno G**. Prevalence of SOD1 mutations in the Italian ALS population. *Neurology* 70: 533–537, 2008.

31. **Chiò A**, **Benzi G**, **Dossena M**, **Mutani R**, **Mora G**. Severely increased risk of amyotrophic lateral sclerosis among Italian professional football players. *Brain* 128: 472–476, 2005.

32. **Chiò A**, **Calvo A**, **Dossena M**, **Ghiglione P**, **Mutani R**, **Mora G**. ALS in Italian professional soccer players: the risk is still present and could be soccer-specific. *Amyotroph Lateral Scler* 10: 205–209, 2009.

33. **Chiò A**, **Calvo A**, **Mazzini L**, **Cantello R**, **Mora G**, **Moglia C**, **Corrado L**, **D'Alfonso S**, **Majounie E**, **Renton A**, **Pisano F**, **Ossola I**, **Brunetti M**, **Traynor BJ**, **Restagno G**, **PARALS**. Extensive genetics of ALS: a population-based study in Italy. *Neurology* 79: 1983–1989, 2012.

34. **Cirulli ET**, **Lasseigne BN**, **Petrovski S**, **Sapp PC**, **Dion PA**, **Leblond CS**, **Couthouis J**, **Lu Y-F**, **Wang Q**, **Krueger BJ**, **Ren Z**, **Keebler J**, **Han Y**, **Levy SE**, **Boone BE**, **Wimbish JR**, **Waite LL**, **Jones AL**, **Carulli JP**, **Day-Williams AG**, **Staropoli JF**, **Xin WW**, **Chesi A**, **Raphael AR**, **McKenna-Yasek D**, **Cady J**, **Vianney de Jong JMB**, **Kenna KP**, **Smith BN**, **Topp S**, **Miller J**, **Gkazi A**, **FALS Sequencing Consortium**, **Al-Chalabi A**, **van den Berg LH**, **Veldink J**, **Silani V**, **Ticozzi N**, **Shaw CE**, **Baloh RH**, **Appel S**, **Simpson E**, **Lagier-Tourenne C**, **Pulst SM**, **Gibson S**, **Trojanowski JQ**, **Elman L**, **McCluskey L**, **Grossman M**, **Shneider NA**, **Chung WK**, **Ravits JM**, **Glass JD**, **Sims KB**,

Van Deerlin VM, **Maniatis T**, **Hayes SD**, **Ordureau A**, **Swarup S**, **Landers J**, **Baas F**, **Allen AS**, **Bedlack RS**, **Harper JW**, **Gitler AD**, **Rouleau GA**, **Brown R**, **Harms MB**, **Cooper GM**, **Harris T**, **Myers RM**, **Goldstein DB**. Exome sequencing in amyotrophic lateral sclerosis identifies risk genes and pathways. *Science* (February 19, 2015). doi: 10.1126/science.aaa3650.

35. **Coatti GC**, **Beccari MS**, **Olávio TR**, **Mitne-Neto M**, **Okamoto OK**, **Zatz M**. Stem cells for amyotrophic lateral sclerosis modeling and therapy: Myth or fact? *Cytometry A* 87: 197–211, 2015.

36. **Cudkowicz ME**, **McKenna-Yasek D**, **Sapp PE**, **Chin W**, **Geller B**, **Hayden DL**, **Schoenfeld DA**, **Hosler BA**, **Horvitz HR**, **Brown RH**. Epidemiology of mutations in superoxide dismutase in amyotrophic lateral sclerosis. *Ann Neurol.* 41: 210–221, 1997.

37. **Cudkowicz ME**, **Shefner JM**, **Schoenfeld DA**, **Zhang H**, **Andreasson KI**, **Rothstein JD**, **Drachman DB**. Trial of celecoxib in amyotrophic lateral sclerosis. *Ann Neurol.* 60: 22–31, 2006.

38. **Cuthbertson DJ**, **Babraj J**, **Smith K**, **Wilkes E**, **Fedele MJ**, **Esser K**, **Rennie M**. Anabolic signaling and protein synthesis in human skeletal muscle after dynamic shortening or lengthening exercise. *Am. J. Physiol. Endocrinol. Metab.* 290: E731–8, 2006.

39. **Dal Bello-Haas FJKL V**. Therapeutic exercise for people with amyotrophic lateral sclerosis or motor neuron disease (Review).

40. **Dal Canto MC**, **Gurney ME**. Neuropathological changes in two lines of mice carrying a transgene for mutant human Cu,Zn SOD, and in mice overexpressing wild type human SOD: a model of familial amyotrophic lateral sclerosis (FALS). *Brain Research* 676: 25–40, 1995.

41. **Darbellay B**, **Arnaudeau S**, **König S**, **Jousset H**, **Bader C**, **Demaurex N**, **Bernheim L**. STIM1- and Orai1-dependent store-operated calcium entry regulates human myoblast differentiation. *J. Biol. Chem.* 284: 5370–5380, 2009.

42. **Deforges S**, **Branchu J**, **Biondi O**, **Grondard C**, **Pariset C**, **Lecolle S**, **Lopes P**, **Vidal PP**, **Chanoine C**, **Charbonnier F**. Motoneuron survival is promoted by specific exercise in a mouse model of amyotrophic lateral sclerosis. *The Journal of Physiology* 587: 3561–3572, 2009.

43. **DeJesus-Hernandez M**, **Mackenzie IR**, **Boeve BF**, **Boxer AL**, **Baker M**, **Rutherford NJ**, **Nicholson AM**, **Finch NA**, **Flynn H**, **Adamson J**, **Kouri N**, **Wojtas A**, **Sengdy P**, **Hsiung G-YR**, **Karydas A**, **Seeley WW**, **Josephs KA**, **Coppola G**, **Geschwind DH**, **Wszolek ZK**, **Feldman H**, **Knopman DS**, **Petersen RC**, **Miller BL**, **Dickson DW**, **Boylan KB**, **Graff-Radford NR**, **Rademakers R**. Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. *Neuron* 72: 245–256,

2011.

44. **DeJesus-Hernandez M**, **Mackenzie IR**, **Boeve BF**, **Boxer AL**, **Baker M**, **Rutherford NJ**, **Nicholson AM**, **Finch NA**, **Flynn H**, **Adamson J**, **Kouri N**, **Wojtas A**, **Sengdy P**, **Hsiung G-YR**, **Karydas A**, **Seeley WW**, **Josephs KA**, **Coppola G**, **Geschwind DH**, **Wszolek ZK**, **Feldman H**, **Knopman DS**, **Petersen RC**, **Miller BL**, **Dickson DW**, **Boylan KB**, **Graff-Radford NR**, **Rademakers R**. Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. *Neuron* 72: 245–256, 2011.

45. **Deng H-X**, **Chen W**, **Hong S-T**, **Boycott KM**, **Gorrie GH**, **Siddique N**, **Yang Y**, **Fecto F**, **Shi Y**, **Zhai H**, **Jiang H**, **Hirano M**, **Rampersaud E**, **Jansen GH**, **Donkervoort S**, **Bigio EH**, **Brooks BR**, **Ajroud K**, **Sufit RL**, **Haines JL**, **Mugnaini E**, **Pericak Vance MA**, **Siddique T**. Mutations in UBQLN2 cause dominant X-linked juvenile and adult-onset ALS and ALS/dementia. *Nature* 477: 211–215, 2011.

46. **Dittmer A**, **Dittmer J**. β-Actin is not a reliable loading control in Western blot analysis. *Electrophoresis* 27: 2844–2845, 2006.

47. **Drory VE**, **Goltsman E**, **Reznik JG**, **Mosek A**, **Korczyn AD**. The value of muscle exercise in patients with amyotrophic lateral sclerosis. *J. Neurol. Sci.* 191: 133–137, 2001.

48. **Duffy LM**, **Chapman AL**, **Shaw PJ**, **Grierson AJ**. Review: The role of mitochondria in the pathogenesis of amyotrophic lateral sclerosis. *Neuropathol. Appl. Neurobiol.* 37: 336–352, 2011.

49. **Dupuis L**, **Loeffler J-P**. Neuromuscular junction destruction during amyotrophic lateral sclerosis: insights from transgenic models. *Curr Opin Pharmacol* 9: 341–346, 2009.

50. **Eaton SL**, **Roche SL**, **Llavero Hurtado M**, **Oldknow KJ**, **Farquharson C**, **Gillingwater TH**, **Wishart TM**. Total protein analysis as a reliable loading control for quantitative fluorescent Western blotting. *PLoS ONE* 8: e72457, 2013.

51. **EFNS Task Force on Diagnosis and Management of Amyotrophic Lateral Sclerosis:**, **Andersen PM**, **Abrahams S**, **Borasio GD**, **de Carvalho M**, **Chiò A**, **Van Damme P**, **Hardiman O**, **Kollewe K**, **Morrison KE**, **Petri S**, **Pradat P-F**, **Silani V**, **Tomik B**, **Wasner M**, **Weber M**. EFNS guidelines on the clinical management of amyotrophic lateral sclerosis (MALS)--revised report of an EFNS task force. *Eur. J. Neurol.* 19: 360–375, 2012.

52. **Eisner V**, **Lenaers G**, **Hajnóczky G**. Mitochondrial fusion is frequent in skeletal muscle and supports excitation-contraction coupling. *The Journal of Cell Biology* 205: 179–195, 2014.

53. **Fecto F**. SQSTM1 Mutations in Familial and Sporadic Amyotrophic Lateral Sclerosis. *Arch Neurol* 68: 1440, 2011.

54. **Felmus MT**, **Patten BM**, **Swanke L**. Antecedent Events in Amyotrophic Lateral Sclerosis. *Neurology* 26: 167–172, 1976.

55. **Frey D**, **Schneider C**, **Xu L**, **Borg J**, **Spooren W**, **Caroni P**. Early and selective loss of neuromuscular synapse subtypes with low sprouting competence in motoneuron diseases. *Journal of Neuroscience* 20: 2534–2542, 2000.

56. **Geevasinga N**, **Menon P**, **Yiannikas C**, **Kiernan MC**, **Vucic S**. Diagnostic utility of cortical excitability studies in amyotrophic lateral sclerosis. *Eur. J. Neurol.* 21: 1451–1457, 2014.

57. **Gilda JE**, **Gomes AV**. Stain-Free total protein staining is a superior loading control to β-actin for Western blots. *Anal. Biochem.* 440: 186–188, 2013.

58. **Gomes MD**, **Lecker SH**, **Jagoe RT**, **Navon A**, **Goldberg AL**. Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. *Proc. Natl. Acad. Sci. U.S.A.* 98: 14440–14445, 2001.

59. **Gordon PH**, **Cheng B**, **Katz IB**, **Mitsumoto H**, **Rowland, L.P.** Clinical features that distinguish PLS, upper motor neuron-dominant ALS, and typical ALS. *Neurology* 72: 1948–1952, 2009.

60. **Gordon PH**, **Cheng B**, **Katz IB**, **Pinto M**, **Hays AP**, **Mitsumoto H**, **Rowland, L.P.** The natural history of primary lateral sclerosis. *Neurology* 66: 647–653, 2006.

61. **Gordon PH**, **Delgadillo D**, **Piquard A**, **Bruneteau G**, **Pradat P-F**, **Salachas F**, **Payan C**, **Meininger V**, **Lacomblez L**. The range and clinical impact of cognitive impairment in French patients with ALS: a cross-sectional study of neuropsychological test performance. *Amyotroph Lateral Scler* 12: 372–378, 2011.

62. **Gordon PH**, **Salachas F**, **Lacomblez L**, **Le Forestier N**, **Pradat P-F**, **Bruneteau G**, **Elbaz A**, **Meininger V**. Predicting survival of patients with amyotrophic lateral sclerosis at presentation: a 15-year experience. *Neurodegener Dis* 12: 81–90, 2013.

63. **Gordon PH**. Amyotrophic lateral sclerosis: pathophysiology, diagnosis and management. *CNS Drugs* 25: 1–15, 2011.

64. **Gordon PH**. Amyotrophic Lateral Sclerosis: An update for 2013 Clinical Features, Pathophysiology, Management and Therapeutic Trials. *Aging Dis* 4: 295–310, 2013.

65. **Gordon PH**. Amyotrophic Lateral Sclerosis: An update for 2013 Clinical

Features, Pathophysiology, Management and Therapeutic Trials. *Aging Dis* 4: 295–310, 2013.

66. **Goswami A**, **Jesse CM**, **Chandrasekar A**, **Bushuven E**, **Vollrath JT**, **Dreser A**, **Katona I**, **Beyer C**, **Johann S**, **Feller AC**, **Grond M**, **Wagner S**, **Nikolin S**, **Troost D**, **Weis J**. Accumulation of STIM1 is associated with the degenerative muscle fibre phenotype in ALS and other neurogenic atrophies. *Neuropathol. Appl. Neurobiol.* 41: 304–318, 2015.

67. **Graf M**, **Ecker D**, **Horowski R**, **Kramer B**, **Riederer P**, **Gerlach M**, **Hager C**, **Ludolph AC**, **Becker G**, **Osterhage J**, **Jost WH**, **Schrank B**, **Stein C**, **Kostopulos P**, **Lubik S**, **Wekwerth K**, **Dengler R**, **Troeger M**, **Wuerz A**, **Hoge A**, **Schrader C**, **Schimke N**, **Krampfl K**, **Petri S**, **Zierz S**, **Eger K**, **Neudecker S**, **Traufeller K**, **Sievert M**, **Neundörfer B**, **Hecht M**, **German vitamin E/ALS Study Group**. High dose vitamin E therapy in amyotrophic lateral sclerosis as add-on therapy to riluzole: results of a placebo-controlled double-blind study. *J. Neural Transm. Gen. Sect.* 112: 649–660, 2005.

68. **Groeneveld GJ**, **Veldink JH**, **van der Tweel I**, **Kalmijn S**, **Beijer C**, **de Visser M**, **Wokke JHJ**, **Franssen H**, **van den Berg LH**. A randomized sequential trial of creatine in amyotrophic lateral sclerosis. *Ann Neurol.* 53: 437–445, 2003.

69. **Gurney ME**, **Pu H**, **Chiu AY**, **Dal Canto MC**, **Polchow CY**, **Alexander DD**, **Caliendo J**, **Hentati A**, **Kwon YW**, **Deng HX**. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* 264: 1772–1775, 1994.

70. **Heckmatt JZ**, **Loh L**, **Dubowitz V**. Night-time nasal ventilation in neuromuscular disease. *Lancet* 335: 579–582, 1990.

71. **Hegedus J**, **Putman CT**, **Gordon T**. Time course of preferential motor unit loss in the SOD1 G93A mouse model of amyotrophic lateral sclerosis. *Neurobiology of Disease* 28: 154–164, 2007.

72. **Hegedus J**, **Putman CT**, **Tyreman N**, **Gordon T**. Preferential motor unit loss in the SOD1G93A transgenic mouse model of amyotrophic lateral sclerosis. *The Journal of Physiology* 586: 3337–3351, 2008.

73. **Higgins CMJ**, **Jung C**, **Xu Z**. ALS-associated mutant SOD1G93A causes mitochondrial vacuolation by expansion of the intermembrane space and by involvement of SOD1 aggregation and peroxisomes. *BMC Neurosci* 4: 16, 2003.

74. **Johnson JO**, **Mandrioli J**, **Benatar M**, **Abramzon Y**, **Van Deerlin VM**, **Trojanowski JQ**, **Gibbs JR**, **Brunetti M**, **Gronka S**, **Wuu J**, **Ding J**, **McCluskey L**, **Martinez-Lage M**, **Falcone D**, **Hernandez DG**, **Arepalli S**, **Chong S**, **Schymick JC**, **Rothstein J**, **Landi F**, **Wang Y-D**, **Calvo A**, **Mora G**, **Sabatelli M**, **Monsurrò MR**, **Battistini S**, **Salvi F**, **Spataro R**, **Sola P**, **Borghero G**, **ITALSGEN Consortium**, **Galassi G**, **Scholz SW**, **Taylor JP**,

Restagno G, **Chiò A**, **Traynor BJ**. Exome sequencing reveals VCP mutations as a cause of familial ALS. *Neuron* 68: 857–864, 2010.

75. **Jouaville LS**, **Ichas F**, **Holmuhamedov EL**, **Camacho P**, **Lechleiter JD**. Synchronization of calcium waves by mitochondrial substrates in Xenopus laevis oocytes. *Nature* 377: 438–441, 1995.

76. **Kaspar BK**, **Frost LM**, **Christian L**, **Umapathi P**, **Gage FH**. Synergy of insulin-like growth factor-1 and exercise in amyotrophic lateral sclerosis. *Ann Neurol.* 57: 649–655, 2005.

77. **Kaufman RJ**. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes & Development* 13: 1211–1233, 1999.

78. **Kaufmann P**, **Pullman SL**, **Shungu DC**, **Chan S**, **Hays AP**, **Del Bene ML**, **Dover MA**, **Vukic M**, **Rowland, L.P.**, **Mitsumoto H**. Objective tests for upper motor neuron involvement in amyotrophic lateral sclerosis (ALS). *Neurology* 62: 1753–1757, 2004.

79. **Kim W-K**, **Liu X**, **Sandner J**, **Pasmantier M**, **Andrews J**, **Rowland, L.P.**, **Mitsumoto H**. Study of 962 patients indicates progressive muscular atrophy is a form of ALS. *Neurology* 73: 1686–1692, 2009.

80. **Kirkinezos IG**, **Hernandez D**, **Bradley WG**, **Moraes CT**. Regular exercise is beneficial to a mouse model of amyotrophic lateral sclerosis. *Ann Neurol.* 53: 804–807, 2003.

81. **Kobayashi Z**, **Tsuchiya K**, **Arai T**, **Yokota O**, **Watabiki S**, **Ishizu H**, **Akiyama H**, **Mizusawa H**. Pseudopolyneuritic form of ALS revisited: clinical and pathological heterogeneity. *Neuropathology* 30: 372–380, 2010.

82. **Koppers M**, **van Blitterswijk MM**, **Vlam L**, **Rowicka PA**, **van Vught PWJ**, **Groen EJN**, **Spliet WGM**, **Engelen-Lee J**, **Schelhaas HJ**, **de Visser M**, **van der Kooi AJ**, **van der Pol W-L**, **Pasterkamp RJ**, **Veldink JH**, **van den Berg LH**. VCP mutations in familial and sporadic amyotrophic lateral sclerosis. *Neurobiol. Aging* 33: 837.e7–13, 2012.

83. **Körner S**, **Kollewe K**, **Ilsemann J**, **Müller-Heine A**, **Dengler R**, **Krampfl K**, **Petri S**. Prevalence and prognostic impact of comorbidities in amyotrophic lateral sclerosis. *Eur. J. Neurol.* 20: 647–654, 2013.

84. **Krivickas LS**, **Shockley L**, **Mitsumoto H**. Home care of patients with amyotrophic lateral sclerosis (ALS). *J. Neurol. Sci.* 152 Suppl 1: S82–9, 1997.

85. **Kwiatkowski TJ**, **Bosco DA**, **LeClerc AL**, **Tamrazian E**, **Vanderburg CR**, **Russ C**, **Davis A**, **Gilchrist J**, **Kasarskis EJ**, **Munsat T**, **Valdmanis P**, **Rouleau GA**, **Hosler BA**, **Cortelli P**, **de Jong PJ**, **Yoshinaga Y**, **Haines JL**,

Pericak-Vance MA, **Yan J**, **Ticozzi N**, **Siddique T**, **McKenna-Yasek D**, **Sapp PC**, **Horvitz HR**, **Landers JE**, **Brown RH**. Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. *Science* 323: 1205–1208, 2009.

86. **Ladd AC**, **Keeney PM**, **Govind MM**, **Bennett JP**. Mitochondrial oxidative phosphorylation transcriptome alterations in human amyotrophic lateral sclerosis spinal cord and blood. *Neuromolecular Med.* 16: 714–726, 2014.

87. **Lai EC**, **Felice KJ**, **Festoff BW**, **Gawel MJ**, **Gelinas DF**, **Kratz R**, **Murphy MF**, **Natter HM**, **Norris FH**, **Rudnicki SA**, **Group TNAAI-IS**. Effect of recombinant human insulin-like growth factor-I on progression of ALS A placebo-controlled study. *Neurology* 49: 1621–1630, 1997.

88. **Leblond CS**, **Kaneb HM**, **Dion PA**, **Rouleau GA**. Dissection of genetic factors associated with amyotrophic lateral sclerosis. *Exp. Neurol.* 262 Pt B: 91–101, 2014.

89. **Lee JR**, **Annegers JF**, **Appel SH**. Prognosis of amyotrophic lateral sclerosis and the effect of referral selection. *J. Neurol. Sci.* 132: 207–215, 1995.

90. **Léger B**, **Vergani L**, **Sorarù G**, **Hespel P**, **Derave W**, **Gobelet C**, **D'Ascenzio C**, **Angelini C**, **Russell AP**. Human skeletal muscle atrophy in amyotrophic lateral sclerosis reveals a reduction in Akt and an increase in atrogin-1. *FASEB J.* 20: 583–585, 2006.

91. **Liebetanz D**, **Hagemann K**, **Lewinski Von F**, **Kahler E**, **Paulus W**. Extensive exercise is not harmful in amyotrophic lateral sclerosis. *Eur. J. Neurosci.* 20: 3115–3120, 2004.

92. **Liou J**, **Kim ML**, **Heo WD**, **Jones JT**, **Myers JW**, **Ferrell JE**, **Meyer T**. STIM is a Ca2+ sensor essential for Ca2+-store-depletion-triggered Ca2+ influx. *Curr. Biol.* 15: 1235–1241, 2005.

93. **Logroscino G**, **Traynor BJ**, **Hardiman O**, **Chio A**, **Couratier P**, **Mitchell JD**, **Swingler RJ**, **Beghi E**, **EURALS**. Descriptive epidemiology of amyotrophic lateral sclerosis: new evidence and unsolved issues. *J. Neurol. Neurosurg. Psychiatr.* 79: 6–11, 2008.

94. **Lomen-Hoerth C**, **Murphy J**, **Langmore S**, **Kramer JH**, **Olney RK**, **Miller B**. Are amyotrophic lateral sclerosis patients cognitively normal? *Neurology* 60: 1094–1097, 2003.

95. **Ludolph AC**, **Bendotti C**, **Blaugrund E**, **Hengerer B**, **Löffler JP**, **Martin J**, **Meininger V**, **Meyer T**, **Moussaoui S**, **Robberecht W**, **Scott S**, **Silani V**, **van den Berg LH**, **ENMC Group for the Establishment of Guidelines for the Conduct of Preclinical and Proof of Concept Studies in ALS/MND Models**. Guidelines for the preclinical in vivo evaluation of pharmacological active drugs

for ALS/MND: report on the 142nd ENMC international workshop. *Amyotroph Lateral Scler* 8: 217–223, 2007.

96. **Lunetta C**, **Serafini M**, **Prelle A**, **Magni P**, **Dozio E**, **Ruscica M**, **Sassone J**, **Colciago C**, **Moggio M**, **Corbo M**, **Silani V**. Impaired expression of insulin-like growth factor-1 system in skeletal muscle of amyotrophic lateral sclerosis patients. *Muscle Nerve* 45: 200–208, 2012.

97. **Mackenzie IRA**, **Bigio EH**, **Ince PG**, **Geser F**, **Neumann M**, **Cairns NJ**, **Kwong LK**, **Forman MS**, **Ravits J**, **Stewart H**, **Eisen A**, **McClusky L**, **Kretzschmar HA**, **Monoranu CM**, **Highley JR**, **Kirby J**, **Siddique T**, **Shaw PJ**, **Lee VM-Y**, **Trojanowski JQ**. Pathological TDP-43 distinguishes sporadic amyotrophic lateral sclerosis from amyotrophic lateral sclerosis with SOD1 mutations. *Ann Neurol.* 61: 427–434, 2007.

98. **Mahoney DJ**, **Rodriguez C**, **Devries M**, **Yasuda N**, **Tarnopolsky MA**. Effects of high-intensity endurance exercise training in the G93A mouse model of amyotrophic lateral sclerosis. *Muscle Nerve* 29: 656–662, 2004.

99. **Majounie E**, **Renton AE**, **Mok K**, **Dopper EGP**, **Waite A**, **Rollinson S**, **Chiò A**, **Restagno G**, **Nicolaou N**, **Simón-Sánchez J**, **van Swieten JC**, **Abramzon Y**, **Johnson JO**, **Sendtner M**, **Pamphlett R**, **Orrell RW**, **Mead S**, **Sidle KC**, **Houlden H**, **Rohrer JD**, **Morrison KE**, **Pall H**, **Talbot K**, **Ansorge O**, **Chromosome 9-ALS/FTD Consortium**, **French research network on FTLD/FTLD/ALS**, **ITALSGEN Consortium**, **Hernandez DG**, **Arepalli S**, **Sabatelli M**, **Mora G**, **Corbo M**, **Giannini F**, **Calvo A**, **Englund E**, **Borghero G**, **Floris GL**, **Remes AM**, **Laaksovirta H**, **McCluskey L**, **Trojanowski JQ**, **Van Deerlin VM**, **Schellenberg GD**, **Nalls MA**, **Drory VE**, **Lu C-S**, **Yeh T-H**, **Ishiura H**, **Takahashi Y**, **Tsuji S**, **Le Ber I**, **Brice A**, **Drepper C**, **Williams N**, **Kirby J**, **Shaw P**, **Hardy J**, **Tienari PJ**, **Heutink P**, **Morris HR**, **Pickering-Brown S**, **Traynor BJ**. Frequency of the C9orf72 hexanucleotide repeat expansion in patients with amyotrophic lateral sclerosis and frontotemporal dementia: a cross-sectional study. *Lancet Neurol* 11: 323–330, 2012.

100. **Manuel M**, **Heckman CJ**. Stronger is not always better: could a bodybuilding dietary supplement lead to ALS? *Exp. Neurol.* 228: 5–8, 2011.

101. **Maragakis NJ**. Stem cells and the ALS neurologist. *Amyotroph Lateral Scler* 11: 417–423, 2010.

102. **Maruyama H**, **Morino H**, **Ito H**, **Izumi Y**, **Kato H**, **Watanabe Y**, **Kinoshita Y**, **Kamada M**, **Nodera H**, **Suzuki H**, **Komure O**, **Matsuura S**, **Kobatake K**, **Morimoto N**, **Abe K**, **Suzuki N**, **Aoki M**, **Kawata A**, **Hirai T**, **Kato T**, **Ogasawara K**, **Hirano A**, **Takumi T**, **Kusaka H**, **Hagiwara K**, **Kaji R**, **Kawakami H**. Mutations of optineurin in amyotrophic lateral sclerosis. *Nature* 465: 223–226, 2010.

103. **Mehta P**, **Antao V**, **Kaye W**, **Sanchez M**, **Williamson D**, **Bryan L**, **Muravov**

O, **Horton K**, **Division of Toxicology and Human Health Sciences, Agency for Toxic Substances and Disease Registry, Atlanta, Georgia**, **Centers for Disease Control and Prevention (CDC)**. Prevalence of amyotrophic lateral sclerosis - United States, 2010-2011. *MMWR Surveill Summ* 63 Suppl 7: 1–14, 2014.

104. **Miller RG**, **Mitchell JD**, **Lyon M**, **Moore DH**. Riluzole for amyotrophic lateral sclerosis (ALS)/motor neuron disease (MND). *Cochrane Database Syst Rev* : CD001447, 2002.

105. **Mitsumoto H**, **Bromberg M**, **Johnston W**, **Tandan R**, **Byock I**, **Lyon M**, **Miller RG**, **Appel SH**, **Benditt J**, **Bernat JL**, **Borasio GD**, **Carver AC**, **Clawson L**, **Del Bene ML**, **Kasarskis EJ**, **LeGrand SB**, **Mandler R**, **McCarthy J**, **Munsat T**, **Newman D**, **Sufit RL**, **Versenyi A**. Promoting excellence in end-of-life care in ALS. *Amyotroph. Lateral Scler. Other Motor Neuron Disord.* 6: 145–154, 2005.

106. **Nandar W**, **Neely EB**, **Simmons Z**, **Connor JR**. H63D HFE genotype accelerates disease progression in animal models of amyotrophic lateral sclerosis. *Biochim. Biophys. Acta* 1842: 2413–2426, 2014.

107. **Neumann M**, **Sampathu DM**, **Kwong LK**, **Truax AC**, **Micsenyi MC**, **Chou TT**, **Bruce J**, **Schuck T**, **Grossman M**, **Clark CM**, **McCluskey LF**, **Miller BL**, **Masliah E**, **Mackenzie IR**, **Feldman H**, **Feiden W**, **Kretzschmar HA**, **Trojanowski JQ**, **Lee VM-Y**. Ubiquitinated TDP-43 in Frontotemporal Lobar Degeneration and Amyotrophic Lateral Sclerosis. *Science* 314: 130–133, 2006.

108. **Olney RK**, **Murphy J**, **Forshew D**, **Garwood E**, **Miller BL**, **Langmore S**, **Kohn MA**, **Lomen-Hoerth C**. The effects of executive and behavioral dysfunction on the course of ALS. *Neurology* 65: 1774–1777, 2005.

109. **Paganoni S**, **Macklin EA**, **Lee A**, **Murphy A**, **Chang J**, **Zipf A**, **Cudkowicz M**, **Atassi N**. Diagnostic timelines and delays in diagnosing amyotrophic lateral sclerosis (ALS). *Amyotroph Lateral Scler Frontotemporal Degener* 15: 453–456, 2014.

110. **Pupillo E**, **Messina P**, **Logroscino G**, **Beghi E**, **SLALOM Group**. Long-term survival in amyotrophic lateral sclerosis: a population-based study. *Ann Neurol.* 75: 287–297, 2014.

111. **Rabkin JG**, **Albert SM**, **Rowland, L.P.** How common is depression among ALS caregivers? A longitudinal study. *Amyotrophic Lateral ...* (2009). doi: 10.3109/17482960802459889.

112. **Radák Z**, **Naito H**, **Kaneko T**, **Tahara S**, **Nakamoto H**, **Takahashi R**, **Cardozo-Pelaez F**, **Goto S**. Exercise training decreases DNA damage and increases DNA repair and resistance against oxidative stress of proteins in aged rat skeletal muscle. *Pfl□gers Archiv European Journal of Physiology* 445: 273–

278, 2002.

113. **Raimondi A**, **Mangolini A**, **Rizzardini M**, **Tartari S**, **Massari S**, **Bendotti C**, **Francolini M**, **Borgese N**, **Cantoni L**, **Pietrini G**. Cell culture models to investigate the selective vulnerability of motoneuronal mitochondria to familial ALS-linked G93ASOD1. *Eur. J. Neurosci.* 24: 387–399, 2006.

114. **Ravits J**, **Appel S**, **Baloh RH**, **Barohn R**, **Brooks BR**, **Elman L**, **Floeter MK**, **Henderson C**, **Lomen-Hoerth C**, **Macklis JD**, **McCluskey L**, **Mitsumoto H**, **Przedborski S**, **Rothstein J**, **Trojanowski JQ**, **van den Berg LH**, **Ringel S**. Deciphering amyotrophic lateral sclerosis: what phenotype, neuropathology and genetics are telling us about pathogenesis. *Amyotroph Lateral Scler Frontotemporal Degener* 14 Suppl 1: 5–18, 2013.

115. **Renton AE**, **Chiò A**, **Traynor BJ**. State of play in amyotrophic lateral sclerosis genetics. *Nature Publishing Group* 17: 17–23, 2014.

116. **Renton AE**, **Majounie E**, **Waite A**, **Simón-Sánchez J**, **Rollinson S**, **Gibbs JR**, **Schymick JC**, **Laaksovirta H**, **van Swieten JC**, **Myllykangas L**, **Kalimo H**, **Paetau A**, **Abramzon Y**, **Remes AM**, **Kaganovich A**, **Scholz SW**, **Duckworth J**, **Ding J**, **Harmer DW**, **Hernandez DG**, **Johnson JO**, **Mok K**, **Ryten M**, **Trabzuni D**, **Guerreiro RJ**, **Orrell RW**, **Neal J**, **Murray A**, **Pearson J**, **Jansen IE**, **Sondervan D**, **Seelaar H**, **Blake D**, **Young K**, **Halliwell N**, **Callister JB**, **Toulson G**, **Richardson A**, **Gerhard A**, **Snowden J**, **Mann D**, **Neary D**, **Nalls MA**, **Peuralinna T**, **Jansson L**, **Isoviita V-M**, **Kaivorinne A-L**, **Hölttä-Vuori M**, **Ikonen E**, **Sulkava R**, **Benatar M**, **Wuu J**, **Chiò A**, **Restagno G**, **Borghero G**, **Sabatelli M**, **Heckerman D**, **Rogaeva E**, **Zinman L**, **Rothstein JD**, **Sendtner M**, **Drepper C**, **Eichler EE**, **Alkan C**, **Abdullaev Z**, **Pack SD**, **Dutra A**, **Pak E**, **Hardy J**, **Singleton A**, **Williams NM**, **Heutink P**, **Pickering-Brown S**, **Morris HR**, **Tienari PJ**, **Traynor BJ**, **Consortium28 TI**. A Hexanucleotide Repeat Expansion in C9ORF72Is the Cause of Chromosome 9p21-Linked ALS-FTD. *Neuron* 72: 257–268, 2011.

117. **Romero-Calvo I**, **Ocón B**, **Martínez-Moya P**, **Suárez MD**, **Zarzuelo A**, **Martínez-Augustin O**, **de Medina FS**. Reversible Ponceau staining as a loading control alternative to actin in Western blots. *Anal. Biochem.* 401: 318–320, 2010.

118. **Rosen DR**, **Siddique T**, **Patterson D**, **Figlewicz DA**, **Sapp P**, **Hentati A**, **Donaldson D**, **Goto J**, **O'Regan JP**, **Deng HX**. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* 362: 59–62, 1993.

119. **Sabatelli M**, **Zollino M**, **Luigetti M**, **Grande AD**, **Lattante S**, **Marangi G**, **Monaco ML**, **Madia F**, **Meleo E**, **Bisogni G**, **Conte A**. Uncovering amyotrophic lateral sclerosis phenotypes: clinical features and long-term follow-up of upper motor neuron-dominant ALS. *Amyotroph Lateral Scler* 12: 278–282, 2011.

120. **Sanjak M**, **Reddan W**, **Brooks BR**. Role of muscular exercise in amyotrophic lateral sclerosis. *Neurol Clin* 5: 251–68– vi, 1987.

121. **Scarmeas N**, **Shih T**, **Stern Y**, **Ottman R**, **Rowland, L.P.** Premorbid weight, body mass, and varsity athletics in ALS. *Neurology* 59: 773–775, 2002.

122. **Shenton D**, **Perrone G**, **Quinn KA**, **Dawes IW**, **Grant CM**. Regulation of protein S-thiolation by glutaredoxin 5 in the yeast Saccharomyces cerevisiae. *J. Biol. Chem.* 277: 16853–16859, 2002.

123. **Shkryl VM**, **Shirokova N**. Transfer and tunneling of Ca2+ from sarcoplasmic reticulum to mitochondria in skeletal muscle. *J. Biol. Chem.* 281: 1547–1554, 2006.

124. **Sinaki M**. Physical therapy and rehabilitation techniques for patients with amyotrophic lateral sclerosis. *Amyotrophic Lateral Sclerosis* (1987). doi: 10.1007/978-1-4684-5302-7_36.

125. **Skulachev VP**. Role of uncoupled and non-coupled oxidations in maintenance of safely low levels of oxygen and its one-electron reductants. *Q. Rev. Biophys.* 29: 169–202, 1996.

126. **Smith BN**, **Ticozzi N**, **Fallini C**, **Gkazi AS**, **Topp S**, **Kenna KP**, **Scotter EL**, **Kost J**, **Keagle P**, **Miller JW**, **Calini D**, **Vance C**, **Danielson EW**, **Troakes C**, **Tiloca C**, **Al-Sarraj S**, **Lewis EA**, **King A**, **Colombrita C**, **Pensato V**, **Castellotti B**, **de Belleroche J**, **Baas F**, **Asbroek ten ALMA**, **Sapp PC**, **McKenna-Yasek D**, **McLaughlin RL**, **Polak M**, **Asress S**, **Esteban-Pérez J**, **Muñoz-Blanco JL**, **Simpson M**, **SLAGEN Consortium**, **van Rheenen W**, **Diekstra FP**, **Lauria G**, **Duga S**, **Corti S**, **Cereda C**, **Corrado L**, **Sorarù G**, **Morrison KE**, **Williams KL**, **Nicholson GA**, **Blair IP**, **Dion PA**, **Leblond CS**, **Rouleau GA**, **Hardiman O**, **Veldink JH**, **van den Berg LH**, **Al-Chalabi A**, **Pall H**, **Shaw PJ**, **Turner MR**, **Talbot K**, **Taroni F**, **García-Redondo A**, **Wu Z**, **Glass JD**, **Gellera C**, **Ratti A**, **Brown RH**, **Silani V**, **Shaw CE**, **Landers JE**. Exome-wide rare variant analysis identifies TUBA4A mutations associated with familial ALS. *Neuron* 84: 324–331, 2014.

127. **Sorenson EJ**, **Windbank AJ**, **Mandrekar JN**, **Bamlet WR**, **Appel SH**, **Armon C**, **Barkhaus PE**, **Bosch P**, **Boylan K**, **David WS**, **Feldman E**, **Glass J**, **Gutmann L**, **Katz J**, **King W**, **Luciano CA**, **McCluskey LF**, **Nash S**, **Newman DS**, **Pascuzzi RM**, **Pioro E**, **Sams LJ**, **Scelsa S**, **Simpson EP**, **Subramony SH**, **Tiryaki E**, **Thornton CA**. Subcutaneous IGF-1 is not beneficial in 2-year ALS trial. *Neurology* 71: 1770–1775, 2008.

128. **Sreedharan J**, **Blair IP**, **Tripathi VB**, **Hu X**, **Vance C**. TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. *Science* (2008). doi: 10.1126/science.1152337.

129. **Steinacker P**, **Berner C**, **Thal DR**, **Attems J**, **Ludolph AC**, **Otto M**. Protease-

resistant SOD1 aggregates in amyotrophic lateral sclerosis demonstrated by paraffin-embedded tissue (PET) blot. *Acta Neuropathol Commun* 2: 130, 2014.

130. **Swash M**. Early diagnosis of amyotrophic lateral sclerosis - a way forward? *Eur. J. Neurol.* 21: 1435–1435, 2014.

131. **Swinnen B**, **Robberecht W**. The phenotypic variability of amyotrophic lateral sclerosis. *Nature Publishing Group* 10: 661–670, 2014.

132. **Tadic V**, **Prell T**, **Lautenschlaeger J**, **Grosskreutz J**. The ER mitochondria calcium cycle and ER stress response as therapeutic targets in amyotrophic lateral sclerosis. *Front Cell Neurosci* 8: 147, 2014.

133. **Traynor BJ**, **Alexander M**, **Corr B**, **Frost E**, **Hardiman O**. Effect of a multidisciplinary amyotrophic lateral sclerosis (ALS) clinic on ALS survival: a population based study, 1996-2000. *J. Neurol. Neurosurg. Psychiatr.* 74: 1258–1261, 2003.

134. **Turner MR**, **Scaber J**, **Goodfellow JA**, **Lord ME**, **Marsden R**, **Talbot K**. The diagnostic pathway and prognosis in bulbar-onset amyotrophic lateral sclerosis. *J. Neurol. Sci.* 294: 81–85, 2010.

135. **Valenti M**, **Pontieri FE**, **Conti F**, **Altobelli E**, **Manzoni T**, **Frati L**. Amyotrophic lateral sclerosis and sports: a case-control study. *Eur. J. Neurol.* 12: 223–225, 2005.

136. **van Blitterswijk M**, **Mullen B**, **Wojtas A**, **Heckman MG**, **Diehl NN**, **Baker MC**, **DeJesus-Hernandez M**, **Brown PH**, **Murray ME**, **Hsiung G-YR**, **Stewart H**, **Karydas AM**, **Finger E**, **Kertesz A**, **Bigio EH**, **Weintraub S**, **Mesulam M**, **Hatanpaa KJ**, **White CL**, **Neumann M**, **Strong MJ**, **Beach TG**, **Wszolek ZK**, **Lippa C**, **Caselli R**, **Petrucelli L**, **Josephs KA**, **Parisi JE**, **Knopman DS**, **Petersen RC**, **Mackenzie IR**, **Seeley WW**, **Grinberg LT**, **Miller BL**, **Boylan KB**, **Graff-Radford NR**, **Boeve BF**, **Dickson DW**, **Rademakers R**. Genetic modifiers in carriers of repeat expansions in the C9ORF72 gene. *Mol Neurodegener* 9: 38, 2014.

137. **van Blitterswijk M**, **van Es MA**, **Hennekam EAM**, **Dooijes D**, **van Rheenen W**, **Medic J**, **Bourque PR**, **Schelhaas HJ**, **van der Kooi AJ**, **de Visser M**, **de Bakker PIW**, **Veldink JH**, **van den Berg LH**. Evidence for an oligogenic basis of amyotrophic lateral sclerosis. *Human Molecular Genetics* 21: 3776–3784, 2012.

138. **Van Hoecke A**, **Schoonaert L**, **Lemmens R**, **Timmers M**, **Staats KA**, **Laird AS**, **Peeters E**, **Philips T**, **Goris A**, **Dubois B**, **Andersen PM**, **Al-Chalabi A**, **Thijs V**, **Turnley AM**, **van Vught PW**, **Veldink JH**, **Hardiman O**, **Van Den Bosch L**, **Gonzalez-Perez P**, **Van Damme P**, **Brown RH**, **van den Berg LH**, **Robberecht W**. EPHA4 is a disease modifier of amyotrophic lateral sclerosis in animal models and in humans. *Nat. Med.* 18: 1418–1422, 2012.

139. **Vance C**, **Rogelj B**, **Hortobágyi T**, **De Vos KJ**, **Nishimura AL**, **Sreedharan J**, **Hu X**, **Smith B**, **Ruddy D**, **Wright P**, **Ganesalingam J**, **Williams KL**, **Tripathi V**, **Al-Saraj S**, **Al-Chalabi A**, **Leigh PN**, **Blair IP**, **Nicholson G**, **de Belleroche J**, **Gallo J-M**, **Miller CC**, **Shaw CE**. Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. *Science* 323: 1208–1211, 2009.

140. **Veldink JH**, **Bär PR**, **Joosten EAJ**, **Otten M**, **Wokke JHJ**, **van den Berg LH**. Sexual differences in onset of disease and response to exercise in a transgenic model of ALS. *Neuromuscular Disorders* 13: 737–743, 2003.

141. **Vielhaber S**, **Kunz D**, **Winkler K**, **Wiedemann FR**, **Kirches E**, **Feistner H**, **Heinze HJ**, **Elger CE**, **Schubert W**, **Kunz WS**. Mitochondrial DNA abnormalities in skeletal muscle of patients with sporadic amyotrophic lateral sclerosis. *Brain* 123 (Pt 7): 1339–1348, 2000.

142. **Vollrath JT**, **Sechi A**, **Dreser A**, **Katona I**, **Wiemuth D**, **Vervoorts J**, **Dohmen M**, **Chandrasekar A**, **Prause J**, **Brauers E**, **Jesse CM**, **Weis J**, **Goswami A**. Loss of function of the ALS protein SigR1 leads to ER pathology associated with defective autophagy and lipid raft disturbances. *Cell Death Dis* 5: e1290, 2014.

143. **Wang S**, **Melhem ER**. Amyotrophic lateral sclerosis and primary lateral sclerosis: The role of diffusion tensor imaging and other advanced MR-based techniques as objective upper motor neuron markers. *Ann. N. Y. Acad. Sci.* 1064: 61–77, 2005.

144. **Wiedemann FR**, **Winkler K**, **Kuznetsov AV**, **Bartels C**, **Vielhaber S**, **Feistner H**, **Kunz WS**. Impairment of mitochondrial function in skeletal muscle of patients with amyotrophic lateral sclerosis. *J. Neurol. Sci.* 156: 65–72, 1998.

145. **Wijesekera LC**, **Mathers S**, **Talman P**, **Galtrey C**, **Parkinson MH**, **Ganesalingam J**, **Willey E**, **Ampong MA**, **Ellis CM**, **Shaw CE**, **Al-Chalabi A**, **Leigh PN**. Natural history and clinical features of the flail arm and flail leg ALS variants. *Neurology* 72: 1087–1094, 2009.

146. **Wong YC**, **Holzbaur ELF**. Optineurin is an autophagy receptor for damaged mitochondria in parkin-mediated mitophagy that is disrupted by an ALS-linked mutation. *Proc. Natl. Acad. Sci. U.S.A.* 111: E4439–48, 2014.

147. **Wootz H**, **Hansson I**, **Korhonen L**, **Näpänkangas U**, **Lindholm D**. Caspase-12 cleavage and increased oxidative stress during motoneuron degeneration in transgenic mouse model of ALS. *Biochemical and Biophysical Research Communications* 322: 281–286, 2004.

148. **Wu C-H**, **Fallini C**, **Ticozzi N**, **Keagle PJ**, **Sapp PC**, **Piotrowska K**, **Lowe P**, **Koppers M**, **McKenna-Yasek D**, **Baron DM**, **Kost JE**, **Gonzalez-Perez P**, **Fox AD**, **Adams J**, **Taroni F**, **Tiloca C**, **Leclerc AL**, **Chafe SC**, **Mangroo D**, **Moore MJ**, **Zitzewitz JA**, **Xu Z-S**, **van den Berg LH**, **Glass JD**, **Siciliano G**,

Cirulli ET, **Goldstein DB**, **Salachas F**, **Meininger V**, **Rossoll W**, **Ratti A**, **Gellera C**, **Bosco DA**, **Bassell GJ**, **Silani V**, **Drory VE**, **Brown RH**, **Landers JE**. Mutations in the profilin 1 gene cause familial amyotrophic lateral sclerosis. *Nature* 488: 499–503, 2012.

149. **Xu KY**, **Zweier JL**, **Becker LC**. Hydroxyl radical inhibits sarcoplasmic reticulum Ca(2+)-ATPase function by direct attack on the ATP binding site. *Circulation Research* 80: 76–81, 1997.

150. **Yin F**, **Ye F**, **Tan L**, **Liu K**, **Xuan Z**, **Zhang J**, **Wang W**, **Zhang Y**, **Jiang X**, **Zhang DY**. Alterations of signaling pathways in muscle tissues of patients with amyotrophic lateral sclerosis. *Muscle Nerve* 46: 861–870, 2012.

151. **Yin F**, **Ye F**, **Tan L**, **Liu K**, **Xuan Z**, **Zhang J**, **Wang W**, **Zhang Y**, **Jiang X**, **Zhang DY**. Alterations of signaling pathways in muscle tissues of patients with amyotrophic lateral sclerosis. *Muscle Nerve* 46: 861–870, 2012.