INVESTIGATING THE INTERPLAY OF INTRINSIC TO EXTRINSIC FACTORS
INFLUENCING AMYOTROPHIC LATERAL SCLEROSIS DISEASE PROGRESSION

A Dissertation in

Molecular Medicine

by

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a relentlessly progressive, neurodegenerative disorder primarily affecting lower motor neurons in the spinal cord and brainstem, and upper motor neurons in the motor cortex. The disorder has a worldwide incidence of 1.7-2.3 per 100,000 population per year, and a prevalence of 4-6 per 100,000 population. The average age of symptom onset is 62 years, the median survival time is 28 months from onset, and the 4-year survival rate is 40%. The disease causes paralysis of the voluntary muscles. ALS is uniformly fatal, and death results from respiratory failure. Riluzole is the only compound approved by the FDA to treat ALS. It extends lifespan by an average of 1-2 months. All of the more than 50 other compounds tested in clinical trials have failed to demonstrate any benefits for patients. The etiology of ALS, especially sporadic ALS (SALS), which comprises approximately 90% of total cases, remains largely unknown. Improved patient outcomes depend on understanding the pathways responsible for disease, and uncovering novel targets for future therapies.

The pathogenesis of ALS begins before a diagnosis can be made in the clinic. Significant motor neuron loss occurs prior to the discernable onset of symptoms. Ideally, research would be able to pinpoint the precise temporal and spatial progression of pathophysiological processes underlying ALS, close to their initiating events. However, this is difficult at present. Many experimental models of ALS exist, which should theoretically permit detailed investigation of ALS pathogenesis. However, other than riluzole, no compound with efficacy in pre-clinical models has had measurable clinical benefits, highlighting differences between animal and human biology as well as the importance of translational research. Given the difficulties in analyzing the events precipitating ALS, analysis of the ongoing processes influencing disease progression becomes an important strategy to elucidate disease mechanisms and pinpoint novel drug targets.
This dissertation investigated factors influencing ALS disease progression, using the framework that the interplay of a range of extrinsic and intrinsic factors determine phenotypes. Intrinsic factors, such as a patient’s genetic makeup, remain relatively stable throughout life. Extrinsic factors, such as drug therapies, vary with time and environment. Intermediate factors, such as proteins that can function as biomarkers, depend on and reflect the individual’s innate genetic and metabolic profile as well as the environment. The interplay of these factors help determine the quality of life and disease duration of a patient with ALS.

Our analysis of intrinsic, genetic factors focused on the H63D polymorphism in the HFE iron regulatory gene. This polymorphism was previously associated with neurodegenerative disorders, including ALS. It is also believed to increase ALS risk. Our results suggest that homozygosity for H63D HFE is correlated with approximately 2-year longer disease duration in patients with ALS, a large effect size compared to the median survival of 2-4 years. H63D HFE polymorphism was also associated with decreased levels of soluble, wild-type (WT) superoxide dismutase (SOD1) protein in muscle. We propose that H63D HFE induces misfolding and aggregation of proteins such as SOD1, leading to mild and chronic endoplasmic reticulum (ER) stress. This cellular stress increases the risk for ALS, but also paradoxically promotes adaptive mechanisms that prolong survival in those individuals with H63D HFE who develop ALS.

Studies analyzing intermediate factors influencing ALS disease progression focused on protein biomarkers. Biomarkers are objective measures that reflect disease status, progression or the effects of treatment. We analyzed 35 protein biomarkers in cerebrospinal fluid (CSF) and plasma of patients with ALS. Using statistical algorithms, we generated multivariable models incorporating panels of select biomarkers, which predicted ALS prognosis with reasonable accuracy. The biomarker panels included inflammatory cytokines, growth factors, and markers
of iron metabolism. We then focused on a specific biomarker, ferritin, which positively correlated with longer disease duration in our models. Interestingly, our results suggest ferritin is elevated in the blood of patients with ALS versus healthy controls and those with non-ALS neurological diseases. The intra-group variance in ferritin values made it difficult to use alone as a diagnostic biomarker. However, the results implicate perturbed iron homeostasis in ALS disease progression. Ferritin sequesters and stores free iron, as well as other trivalent metals to a lesser extent, in a non-toxic, soluble form. We propose that the elevated ferritin seen in ALS patients is an adaptive response to oxidative damage. Furthermore, our biomarker results support the neuroprotective role of immunomodulation by M2 macrophages and microglia.

We used the SOD1 G93A transgenic mouse model of ALS to study extrinsic factors modifying disease progression. Our results suggest HMG-CoA reductase inhibitors (statins), which are commonly prescribed to manage cholesterol, adversely impact ALS phenotype. SOD1 mice administered statins had accelerated disease progression and decreased survival, with double transgenic animals harboring both SOD1 G93A and H67D HFE, homologous to human H63D HFE, having the worst phenotype. This is consistent with clinical reports suggesting statins may accelerate disease progression in patients with ALS, and underscores the need for surveillance of disease progression in patients with ALS receiving statin therapy. Statin effects also depend on HFE genotype. However, there was little evidence of mitochondrial dysfunction, which has been implicated in the adverse effects of statins. We hypothesize statin-induced disruption of anti-inflammatory immunomodulation contributes, in part, to their adverse effects.

Our results suggest strategies to stratify patients in clinical trials, enabling more precise evaluation of outcomes that may uncover subgroup dependent effects; as well as therapeutic approaches that may improve the clinical situation for patients with ALS.
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<th>Definition</th>
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<tbody>
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<td>AAA</td>
<td>ATPases associated with diverse cellular activities</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>ALS2</td>
<td>alsin</td>
</tr>
<tr>
<td>ALSFRS-R</td>
<td>ALS Functional Rating Scale-Revised</td>
</tr>
<tr>
<td>ALSoD</td>
<td>ALS Online Database</td>
</tr>
<tr>
<td>ANG</td>
<td>angiogenin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATXN2</td>
<td>ataxin-2</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>β2M</td>
<td>beta-2 microglobulin</td>
</tr>
<tr>
<td>BCAA</td>
<td>branched-chain amino acids</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C9ORF72</td>
<td>chromosome 9 open reading frame 72</td>
</tr>
<tr>
<td>CHGB</td>
<td>chromogranin B</td>
</tr>
<tr>
<td>CHMP2B</td>
<td>charged multivesicular body protein 2B</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CNTF</td>
<td>ciliary neurotrophic factor</td>
</tr>
<tr>
<td>CpG</td>
<td>cytosine residues adjacent to guanines</td>
</tr>
<tr>
<td>CREST</td>
<td>nBAF component SS18L1</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>DAO</td>
<td>D-amino acid oxidase</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunoassay</td>
</tr>
<tr>
<td>ELP3</td>
<td>elongator protein 3</td>
</tr>
<tr>
<td>Eph</td>
<td>ephrin</td>
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<tr>
<td>EPH4A</td>
<td>ephrin 4A</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>ETC</td>
<td>electron transport chain</td>
</tr>
<tr>
<td>FALS</td>
<td>familial ALS</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FIG4</td>
<td>polyphosphoinositide phosphatase</td>
</tr>
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<td>FTD</td>
<td>frontotemporal dementia</td>
</tr>
<tr>
<td>FUS</td>
<td>fused in sarcoma / translated in liposarcoma</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>GRN</td>
<td>progranulin</td>
</tr>
<tr>
<td>HFE</td>
<td>human hemochromatosis protein</td>
</tr>
<tr>
<td>hnRNPA1</td>
<td>heterogeneous nuclear ribonucleoprotein A1</td>
</tr>
<tr>
<td>hnRNPA2B1</td>
<td>heterogeneous nuclear ribonucleoprotein A2B1</td>
</tr>
<tr>
<td>HO-1</td>
<td>hemeoxygenase-1</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HSP</td>
<td>hereditary spastic paraplegia</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IBMPFD</td>
<td>inclusion body myopathy, Paget disease and FTD</td>
</tr>
<tr>
<td>ICV</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor-1</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IL-1RA</td>
<td>interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IP-10</td>
<td>C-X-C motif chemokine 10</td>
</tr>
<tr>
<td>IQR</td>
<td>interquartile range</td>
</tr>
<tr>
<td>IRP</td>
<td>iron-responsive element-binding protein</td>
</tr>
<tr>
<td>j</td>
<td>juvenile</td>
</tr>
<tr>
<td>KIFAP3</td>
<td>kinesin-associated protein 3</td>
</tr>
<tr>
<td>LMN</td>
<td>lower motor neuron</td>
</tr>
<tr>
<td>MCP-1</td>
<td>chemokine (C-C motif) ligand 2</td>
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<tr>
<td>M-CSF</td>
<td>macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>med</td>
<td>median</td>
</tr>
<tr>
<td>miRNA</td>
<td>micro-RNA</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>macrophage inflammatory protein-1-alpha</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>macrophage inflammatory protein-1-beta</td>
</tr>
<tr>
<td>MND</td>
<td>motor neuron disease</td>
</tr>
<tr>
<td>mo</td>
<td>months</td>
</tr>
<tr>
<td>NCRI</td>
<td>Neurological Clinical Research Institute</td>
</tr>
<tr>
<td>NEALS</td>
<td>Northeast ALS Consortium</td>
</tr>
<tr>
<td>NEFH</td>
<td>neurofilament heavy subunit</td>
</tr>
<tr>
<td>NGS</td>
<td>next-generation sequencing</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-kappa B</td>
</tr>
<tr>
<td>Nrf2</td>
<td>nuclear factor E2-related factor 2</td>
</tr>
<tr>
<td>OPTN</td>
<td>optineurin</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>platelet derived growth factor (subunit composition BB)</td>
</tr>
<tr>
<td>PFN1</td>
<td>profilin 1</td>
</tr>
<tr>
<td>PLS</td>
<td>primary lateral sclerosis</td>
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<td>PMA</td>
<td>progressive muscular atrophy</td>
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<td>polyQ</td>
<td>poly-glutamine</td>
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<td>Pro-Hep</td>
<td>pro-hepcidin</td>
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<td>polyvinylidene fluoride</td>
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<td>chemokine (C-C motif) ligand 5</td>
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<td>RBP</td>
<td>RNA binding protein</td>
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<td>RCT</td>
<td>randomized controlled trial</td>
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<tr>
<td>RNase</td>
<td>ribonuclease</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SALS</td>
<td>sporadic ALS</td>
</tr>
<tr>
<td>SCA2</td>
<td>spinocerebellar ataxia type 2</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
</tbody>
</table>
SETX senataxin
SOD1 Cu/Zn-supeoxide dismutase
SPG11 spatacsin
SQSTM1 sequestosome 1
statin HMG-CoA reductase inhibitor
TAF15 TATA box binding protein associated factor
TARDBP TAR DNA-binding protein 43
TBS tris-buffered saline
TBS-T tris-buffered saline with Tween 20
TCH346 omigapil
Tim23 translocase of the mitochondrial inner membrane subunit 23
TLR Toll-like receptor
TNFα tumor necrosis factor alpha
TSAT transferrin saturation
UBI ubiquitinated protein inclusion
UBQLN2 ubiquilin-2
UMN upper motor neuron
UNC13A UNC13A protein
UPR unfolded protein response
VAPB vesicle-associated membrane protein B
VCP valosin-containing protein
VDAC voltage-dependent anion channel 1
VEGF vascular endothelial growth factor
WT wild-type
yrs years
ACKNOWLEDGMENTS

I dedicate this thesis to my loving parents, Ping Su and Xiaohong He. Their generous guidance, support and encouragement shaped who I am, and sustained me through many hardships. They have given me life, not once, but many times.

I also dedicate this thesis to my dearest wife, Wint Nandar. Her abundant kindness, intelligence and patience are an inspiration, and provide a model for the kind of person I strive to be. The light of her love has filled my life with joy and warmth.

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Lastly, I would like to acknowledge and thank the patients with amyotrophic lateral sclerosis who agreed to our research and for whom we continue to work towards a cure.
EPIGRAPH

“I will now try to summarize the features of amyotrophic lateral sclerosis: 1) Paralysis without loss of sensation of the upper limbs… 2) The legs are affected in turn… 3) Bulbar symptoms appear… Death follows in 2 or 3 years, on average, from the onset of bulbar symptoms. This is the rule but there are a few anomalies… At present, the prognosis is grave. As far as I know, there is no case in which all the symptoms occurred and a cure followed. Is this an absolute block? Only the future will tell.”

Jean-Martin Charcot, 1874
Chapter 1

Genetic Heterogeneity of ALS: Implications for Clinical Practice and Research

1.1 Abstract

Genetic insights into the pathophysiology of amyotrophic lateral sclerosis (ALS) are untangling the clinical heterogeneity that may contribute to poor clinical trial outcomes and thus to a lack of effective treatments. Mutations in a large number of genes, including SOD1, C9ORF72, TARDBP, FUS, VAPB, VCP, UBQLN2, ALS2, SETX, OPTN, ANG, and SPG11, are thought to cause ALS, whereas others, including ATAXN2, GRN, HFE, NEFH, UNC13A, and VEGF, appear to be disease-modifying genes. Epigenetic influences may also play important roles. An improved understanding of ALS genetics should lead to better trial designs, insights into common molecular pathways, and better characterization of preclinical models. New genetic sequencing techniques, which use high-throughput methods to assess variants across the genome or exome, may facilitate rational patient stratification for clinical trials and permit more individualized prognostic information and treatment decisions in clinical care.
1.2 Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease with a worldwide incidence of 1.7-2.3 per 100,000 population per year (1) and prevalence of 4-6 per 100,000 population (2). It affects lower motor neurons (LMNs) in the brainstem and spinal cord, upper motor neurons (UMNs) in the motor cortex and the corticospinal tract, resulting in progressive weakness and atrophy of skeletal muscles. However, ALS is not solely confined to motor neurons and should be thought of as a broader neurodegenerative disorder (see 1.3 The ALS Syndrome).

ALS is devastating. The average age of onset is 61.8 years (3). Prognosis is poor, with a median survival of 27.5 months from symptom onset and a four-year survival rate of 40% (4). Death results from respiratory failure. Our insight into pathogenesis is limited, resulting in frustration for the clinician and patient when the affected individual inevitably asks “why did I get this?” Lack of knowledge regarding pathogenesis also prevents physicians from providing accurate prognostic information for individuals despite attempts to construct prognostic algorithms (5). Disease course is highly variable, with survival ranging from months to decades (6).

Insight into the genetics of both familial and apparently sporadic ALS may enhance understanding of ALS pathogenesis and pave the way for more accurate prognosis, improved stratification of subjects for clinical trials, and ultimately more effective pharmacotherapy. It also provides background for the pathophysiological processes discussed in this dissertation, which are modified by factors influencing disease progression. This review explores what is currently known about ALS genetics, with a focus on relevance to clinical practice and treatment trials.
1.3 The ALS Syndrome

The situation confronting the clinician evaluating a patient for possible ALS illustrates how genetics may inform diagnosis and prognosis. The disorder is defined by clinical, electrophysiological, and neuropathological criteria (7, 8). However, these diagnostic criteria do not capture the heterogeneity of ALS. Although a combination of UMN and LMN findings characterizes classic ALS, the continuum of disease ranges from exclusively UMN (primary lateral sclerosis, PLS) to exclusively LMN involvement (progressive muscular atrophy, PMA). Clinical overlap between PLS and hereditary spastic paraplegia, and between PMA and distal hereditary motor neuropathy, coupled with a better average prognosis for PLS and PMA than for classical ALS, makes diagnosis and counseling of individual patients challenging (7, 8). The clinician also faces “ALS-Plus” syndromes, in which patients demonstrate extrapyramidal signs, cerebellar findings, autonomic nervous involvement, sensory abnormalities, oculomotor abnormalities, or other signs indicating involvement beyond motor neurons (8).

Adding to this complexity, ALS overlaps with frontotemporal dementia (FTD) clinically and neuropathologically. Approximately 15% of patients with pathologically confirmed FTD have findings of motor neuron disease (MND), while an additional 25% or more may have minor motor system dysfunction (9, 10). In 1 study of ALS patients, 20% had cognitive impairment severe enough for a diagnosis of dementia, usually FTD, while an additional 30% had milder cognitive impairment (11). Meta-analysis suggests deficits in psychomotor speed, fluency, language, visual memory, immediate verbal memory, and executive functioning are often present in ALS patients without overt dementia (12).

From the clinician’s perspective, patients with ALS share the unifying feature of motor neuron dysfunction but may demonstrate varying degrees of UMN versus LMN involvement and
abnormalities beyond motor neurons. This heterogeneity makes understanding pathogenesis, designing clinical trials, and developing therapies challenging. Insight into the genetics of ALS may enable progress in these areas, especially if common themes emerge that point to therapeutic targets or mechanisms of disease pathogenesis.
1.4 ALS Clinical Trials

Numerous clinical treatment trials for patients with ALS have been conducted (Table 1-1). However, almost 2 decades after randomized controlled trials (RCTs) demonstrated that riluzole provides a modest benefit (13, 14), it remains the only drug with proven efficacy. The failure of clinical trials likely results from the heterogeneity of ALS and the lack of biomarkers that can distinguish subtypes. This greatly limits the ability to identify groups of patients within a “failed” clinical trial who may have responded to therapy.

In 2006, Traynor et al. rigorously reviewed a broad range of compounds for ALS neuroprotection (15). Unfortunately, none of the compounds on this list that completed testing showed clinical efficacy (16). In addition to lack of pharmacologic effect, other methodologic issues contributing to poor clinical trial outcomes have been described (17). Recently, a Phase III RCT of dexpramipexole did not show efficacy (18) despite rigorous design and positive Phase II results (19, 20).

Different molecular mechanisms responsible for ALS may drive clinical heterogeneity. Abnormalities in pathways that regulate oxidative stress (21), glutamate hyperexcitability (22), RNA processing (23, 24), protein degradation (25), endosomal trafficking (26), and mitochondrial function (27, 28), among others, occur in ALS, but the temporal and spatial relationships between these abnormalities are unclear. Much of our current knowledge of the molecular biology of ALS derives from studies of mutations that cause disease in humans, or ALS-like phenotypes in model systems. It is clear that no single gene, or set of genes, accounts for the pathophysiology (29). Understanding this genetic diversity may provide insights into ALS pathophysiology, ultimately leading to effective therapies.
Table 1-1. ALS Clinical Trials

<table>
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Table 1-1 (Continued). ALS Clinical Trials

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<tr>
<td>Xaliproden</td>
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<td>Xaliproden</td>
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<td>2004b</td>
<td>(109)</td>
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</table>
Notes: *modified from Beghi et al., 2011 (16) with permission of the publisher; †total number of patients in study.
1.5 The Genetics of ALS

Genes that cause hereditary diseases are traditionally identified using “positional cloning” (110). The locations of candidate genes are mapped to chromosome intervals by linkage studies, and the intervals surveyed to identify causative genes (111). However, most cases of ALS are sporadic and difficult to characterize using this technique. ALS is also relatively rare, and the number of individuals with a family history of ALS small. This limits positional cloning efforts, as sample sizes are insufficient to effectively narrow candidate regions for subsequent sequencing.

Next-generation high-throughput sequencing (NGS) of whole genomes or, more cost effectively, whole exomes, circumvents these limitations. Because protein-coding exonic regions harbor approximately 85% of the mutations affecting disease-related traits while comprising only 2% of the human genome (112), whole-exome sequencing yields rich datasets while saving resources. Whole-genome and whole-exome sequencing have streamlined efforts to identify novel mutations, resulting in a growing list of genes linked to familial ALS (FALS) (Table 1-2).

ALS is classified into sporadic ALS (SALS) and FALS. Meta-analysis using prospective population registries suggests that about 5% of ALS is familial (113). Clinically, SALS and FALS are indistinguishable (114). Screening for mutations in the genes currently linked to ALS identifies known genetic variants in approximately 60% of all patients with FALS, with chromosome 9 open reading frame 72 (C9ORF72) representing 40% (115), Cu/Zn-superoxide dismutase (SOD1) 20% (116), fused in sarcoma (FUS) 5% (117), and TAR DNA-binding protein 43 (TARDBP) 3%. All genes found to be mutated in FALS can also be found to be mutated in SALS, albeit at low frequencies (118). Even in cases with known genetic etiology, it is unclear
how the genetic insult, present in all somatic cells, induces selective vulnerability in motor neurons (119, 120).

An understanding of genes involved in ALS may be essential towards developing effective therapeutics. A discussion of specific genes linked to ALS follows. This discussion is not exhaustive, but rather focuses on genes more commonly linked to ALS in epidemiological studies and also includes rare genetic alterations if they shed light on novel pathways or phenotypes. Genetic mutations that are very rare or whose contributions to ALS are less well-documented, are included and referenced in the tables of causative genes (Table 1-2) or modifiers of disease risk or progression (Table 1-3), but are not discussed in detail.
Table 1-2. Genes Likely Causing ALS

<table>
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<tr>
<th>Gene (Ref)</th>
<th>Function</th>
<th>Clinical phenotype</th>
<th>Epidemiology</th>
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<td>Cellular transport(^*)</td>
<td>j-ALS, j-PLS, HSP</td>
<td>&lt;1% FALS</td>
</tr>
<tr>
<td>ANG (122)</td>
<td>RNA metabolism</td>
<td>ALS, PD</td>
<td>&lt;1% ALS overall</td>
</tr>
<tr>
<td>C9ORF72 (120)</td>
<td>RNA metabolism(^*)</td>
<td>ALS, FTD</td>
<td>40% FALS, 5-6% SALS</td>
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<td>CHMP2B (123)</td>
<td>Cellular transport</td>
<td>ALS, FTD</td>
<td>Unknown</td>
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<tr>
<td>DAO (22)</td>
<td>Glutamatergic signaling</td>
<td>ALS</td>
<td>&lt;1% FALS</td>
</tr>
<tr>
<td>FUS (24)</td>
<td>DNA/RNA metabolism</td>
<td>ALS, j-ALS, FTD</td>
<td>5% FALS, &lt;1% SALS</td>
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<tr>
<td>hnRNPA1 (124)</td>
<td>RNA metabolism</td>
<td>ALS, FTD, IBMPFD</td>
<td>Unknown</td>
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<td>hnRNPA2B1 (124)</td>
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<tr>
<td>OPTN (125)</td>
<td>Protein metabolism</td>
<td>ALS</td>
<td>&lt;1% FALS</td>
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<tr>
<td>PFN1 (126)</td>
<td>Axonal outgrowth</td>
<td>ALS</td>
<td>&lt;1% FALS</td>
</tr>
<tr>
<td>SETX (127)</td>
<td>DNA/RNA metabolism</td>
<td>j-ALS</td>
<td>&lt;1% FALS</td>
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<tr>
<td>SOD1 (21)</td>
<td>Prevent oxidative damage</td>
<td>ALS, FTD, PMA</td>
<td>20% FALS, 3% SALS</td>
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<td>SPG11 (128)</td>
<td>Neuronal maturation(^*)</td>
<td>j-ALS, HSP</td>
<td>Unknown</td>
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<td>SQSTM1 (129)</td>
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<td>TARDBP (23)</td>
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<td>UBQLN2 (25)</td>
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<td>VAPB (26)</td>
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<td>&lt;1% FALS</td>
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<td>VCP (131)</td>
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<td>ALS, FTD, IBMPFD</td>
<td>1-2% FALS</td>
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Note: \(^*\)proposed function.
Table 1-3. Genes Modifying ALS Risk or Progression

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Note: *proposed function.
1.5.1 Cu/Zn-superoxide dismutase (SOD1)

Modern genetic studies of ALS began with the identification of 11 missense mutations in SOD1 in 13 distinct FALS families (21). The discovery was the first to pinpoint a genetic cause for ALS, paving the way for in vivo models of FALS that enabled analysis of disease mechanisms (144, 145) and large-scale ALS bioinformatics databases such as the ALS Online Database (ALSoD) (146). Mutations in SOD1 account for approximately 20% of FALS, and are generally autosomal dominant (116, 147). Patients with SOD1-linked FALS exhibit varied clinical phenotypes. The majority are limb-onset, of which 75% have lower limb onset. Median age of onset is slightly less than 60 years, and median disease duration is 2 to 4 years (148).

Founder effects and SOD1 mutation-dependent phenotypes impact treatment and clinical trials. The D90A SOD1 mutation is recessive, and patients homozygous for this variant are mildly affected, with survival usually extending beyond 10 years. The allele, which is found in higher frequencies among Scandinavian families, has been linked to a single founder and may be tightly linked with a protective, disease modifying-factor (149). In contrast, the A4V SOD1 mutation causes a dominant, rapidly progressive form of ALS. This allele accounts for 50% of SOD1-linked FALS in North America but is rare in Europe. A4V SOD1 has been traced to 2 founder mutations, 1 in Europe and the other in Amerindians. Modern databases have no records of Amerindian cases of A4V SOD1-induced FALS, raising the intriguing possibility, among others, that a protective genetic factor cosegregates with the allele in this ethnic group (150).

Over 170 genetic alterations in SOD1 are listed in ALSoD (146), with the majority being missense mutations. SOD1 protein catalyzes the conversion of toxic superoxide anions into hydrogen peroxide, and in doing so, also prevents superoxide reactions that generate highly toxic hydroxyl radicals. However, disease pathology is not correlated with reduced enzyme activity.
Mutant SOD1 is neurotoxic via gain-of-function pathways that likely involve protein misfolding and aggregation (152). Mutant SOD1 misfolds and is targeted to the proteasome through oligoubiquitination; however, it bypasses proper clearance pathways (153). It also appears to be toxic to critical components of the cell’s degradation machinery, including proteasomes and autophagic pathways (154, 155). Although SOD1 is abundant in the cytosol of all cells, its half-life is longest in motor neurons. Oxidative damage, which accumulates over time, preferentially induces misfolding of mutant SOD1. This may explain partially the selective vulnerability of motor neurons in SOD1-linked FALS (156).

Non-cell-autonomous pathways are implicated in studies of mutant SOD1, suggesting therapies for ALS must target pathways that act between cells in addition to those within cells. Glia that express wild type (WT) SOD1 are neuroprotective in chimeric mice harboring both WT- and mutant SOD1-expressing motor neurons and glia (157). Expression of mutant human SOD1 in mouse primary spinal motor neurons is insufficient to induce motor neuron degeneration in vitro; however, astrocytes that express mutant SOD1 are toxic to mouse motor neurons with WT SOD1 (158). Astrocytes derived from post-mortem FALS and SALS patient tissue are toxic to mouse embryonic stem cell-derived motor neurons, whereas mutant SOD1 knockdown in these astrocytes significantly attenuates this effect (159).

1.5.2 Chromosome 9 open reading frame 72 (C9ORF72)

Recently, expansion of GGGGCC hexanucleotide repeats within the C9ORF72 gene were implicated in ALS-FTD spectrum disorders (120). Repeat lengths greater than 30 units are linked to disease and may reach 700-1,600 units in affected individuals. C9ORF72 expansion is found in approximately 5-6% of patients with SALS and up to 40% of those with FALS
worldwide, and is the most common known genetic cause of SALS and FALS (115, 119). The expansion is not penetrant in individuals younger than 35 years, 50% penetrant at 58 years in men and 63 years in women, and fully penetrant at 86 years in men, while a minority of women do not develop symptoms past 80 years (160). The C9ORF72 expansion is implicated in patients with ALS, those with FTD, and those with both, suggesting potential shared pathogenic mechanisms.

C9ORF72-linked ALS is distinct clinically, on average, from SALS and other forms of FALS. Onset is more commonly bulbar (40% versus 25%). Cognitive dysfunction, especially FTD, is much more common (45-50% versus 10%), as are psychotic symptoms, although these remain rare. Children of patients with C9ORF72 ALS develop disease an average of 7 years earlier than their parents, possibly representing anticipation due to expansion instability (161, 162). Survival appears to be slightly decreased in patients with the C9ORF72 expansion (162-164). Reported effects on age of onset have varied (163-165).

C9ORF72 expansion likely induces neurodegeneration via toxic gain-of-function, rather than loss-of-function. NGS-based analysis of the C9ORF72 region in 389 ALS patients failed to identify traditional loss-of-function mutations (166). A patient homozygous for the expansion had a phenotype within the usual disease spectrum (167), whereas homozygosity for a loss-of-function mutation would be expected to generate a more severe clinical phenotype. The mRNA transcript of the GGGGCC repeat sequesters RNA binding proteins (RBPs), diminishing the pool of RBPs available for normal regulation of RNA metabolism (168). Antisense oligonucleotides directed to the C9ORF72 transcript or repeat expansion ameliorate expansion-induced gene dysregulation and vulnerability to excitotoxicity in vitro (169). This supports the concept that ALS is a ribonucleopathy, particularly because mutations in the genes that encode TDP-43 and
fused in sarcoma (FUS), which are both RBPs, also cause disease (117, 170). Non-ATG-initiated translation of the \textit{C9ORF72} GGGGCC repeat region has been reported, which produces extremely hydrophobic dipeptide repeat proteins that aggregate and localize to regions affected in ALS-FTD, including hippocampus and frontotemporal neocortex (171, 172). Epigenetic alterations, including hypermethylation of cytosine residues adjacent to guanines (CpG dinucleotide-enriched islands) near the GGGGCC repeat, may be present in \textit{C9ORF72}-linked disease. Increased methylation correlates with shorter survival, possibly representing a prognostic biomarker (173).

1.5.3 TAR DNA-binding protein 43 (\textit{TARDBP})

Tau- and \(\alpha\)-synuclein-negative ubiquitinated protein inclusions (UBIs) that aggregate in the cytoplasm, nucleus, and neuritic tangles are characteristic of SALS and FALS, and a subset of FTD. The identification of TDP-43 as the ubiquitinated protein in these UBIs, and the subsequent discovery that mutations in \textit{TARDBP}, which codes for TDP-43, are a rare cause of autosomal dominant ALS, established the importance of RNA metabolism in the disease (23, 170). Mutations in \textit{TARDBP} occur in up to 5\% of patients with FALS without dementia and 3\% of FALS patients overall (174). Sixty percent of patients with limb onset \textit{TARDBP}-linked ALS initially demonstrate upper extremity involvement. The mean age of onset is approximately 50 years in 4 out of the 5 most common \textit{TARDBP} mutations, and median disease duration is 5 years (175).

TDP-43 is an RNA- and DNA-binding protein that regulates transcription and mRNA splicing, transport, and stability. It may also regulate micro-RNA (miRNA) generation, the cell cycle, and apoptosis (176). It is a ubiquitously expressed ribonucleoprotein normally located in
the nucleus that targets over 6,300 distinct mRNAs. Depletion of TDP-43 alters levels of more than 600 mRNAs, including those that encode the ALS-related proteins FUS (117) and progranulin (138); and it induces more than 960 alternate splicing events. TDP-43 depletion downregulated genes that affect synaptic transmission, ion transport and channel activity, and passive transmembrane transporters; it upregulated genes that affect inflammation and cellular defense responses (177).

Mutant TDP-43 may induce neurotoxicity by disrupting cytoplasmic stress granules. TDP-43 harbors nuclear localization and export signals and interacts with cytoplasmic granules containing translationally silenced RNA that are transported to target sites, such as dendritic spines or presynaptic boutons, where translation is resumed. Under conditions of cellular stress, this enables the cell to prioritize protein synthesis. However, mutant TDP-43 alters stress granule dynamics and is incorporated earlier into granules that become significantly larger and are, paradoxically, immature and dysregulated (178, 179). Mutant TDP-43 may be toxic via both gain-of-function and loss-of-function activities. The former results from mutant TDP-43-induced disruption of stress granules, which aggregate and cause the cytoplasmic UBIs seen in ALS. The latter results from the subsequent depletion of TDP-43 from the nucleus, where it is no longer available to properly regulate mRNA splicing and other events during RNA metabolism (180).

1.5.4 Fused in sarcoma / translated in liposarcoma (FUS)

FUS is a ubiquitously expressed nucleoprotein that regulates RNA and DNA binding, gene expression, and mRNA splicing. It may also affect miRNA processing, genomic maintenance, and, much like TDP-43, cytoplasmic stress granule dynamics. FUS mutations are found in up to 5% of FALS patients, and they cause a clinical phenotype similar to TARDBP-
linked ALS (24, 117). The mean age of onset is 45 years, and mean survival is 30 months (148). LMN dysfunction predominates, with variable UMN involvement. A few cases of mutant FUS-associated ALS-FTD have been reported. Reduced disease penetrance may be present (181). Interestingly, FUS proteinopathy is associated with a juvenile-onset form of ALS characterized pathologically by basophilic inclusions distinct from those found in classic SALS. In these patients, motor symptoms begin between ages 17-22 years, disease progression is rapid, and FTD is not seen (182).

FUS contains nuclear localization and export signals, and it cycles between cytoplasm and nucleus. Normally, FUS colocalizes with TDP-43 to stress granules in motor neurons subjected to cellular injury (183). FUS targets more than 5,500 mRNAs. Depletion of FUS alters the expression of approximately 640 mRNAs and the splicing of more than 300 additional mRNAs. Most of the genes regulated by FUS are distinct from those regulated by TDP-43, and the small subset of genes regulated by both FUS and TDP-43 do not show greater expression changes when both proteins are depleted, suggesting parallel rather than synergistic functions (184).

The mechanism underlying mutant FUS-induced toxicity remains unknown. Gain-of-function toxicity via cytoplasmic aggregation in stress granules and loss-of-function, via depletion of nuclear RNA regulation capacity, are implicated. Although mutant FUS and TDP-43 appear to behave similarly, localizing to cytoplasmic stress granules that aggregate, they do so through distinct pathways and require different binding partners, suggesting distinct neurotoxic pathways (185). Mutant FUS- and TDP-43-expressing astrocytes are deleterious to WT motor neurons in model systems, suggesting both mutations also act non-cell-autonomously (186, 187).
FUS mutations are also linked to defects in DNA repair pathways, and patients with FUS-linked ALS harbor increased DNA damage (188).

1.5.5 Vesicle-associated membrane protein B (VAPB)

VAPB mutations cause varied forms of ALS, including ALS with essential tremor, late-onset PMA, and classical ALS with rapid progression (26). Three mutations are known. The P56S VAPB, which is most common, and T46I VAPB missense mutations cause ALS, whereas the ΔS160 VAPB deletion does not (189, 190). VAPB protein associates with intracellular membranes, such as those of the endoplasmic reticulum (ER). The protein is found at the junction of intracellular vesicles and cytoskeletal structures, and it regulates ER-Golgi transport and secretion (26).

Mutant VAPB is believed to cause neurotoxicity via both cell-autonomous and non-cell-autonomous pathways. Functionally, P56S VAPB is a null mutation. Mutant VAPB sequesters normal VAPB into cytosolic aggregates, attenuating its function. Aggregation also engages the unfolded protein response (UPR) and increases cellular vulnerability to ER stress (190, 191). This cell-autonomous mechanism is exacerbated by non-cell-autonomous effects. Amino-terminal domains on the VAPB protein are cleaved and secreted as ligands for ephrin (Eph) receptors. Proper ephrin signaling is required for neuronal maturation. Ephrin receptors prevent glutamate excitotoxicity via interactions with N-methyl-D-aspartate (NMDA) receptors. Trapping of normal VAPB protein by mutant VAPB interferes with VAPB-dependent, Eph receptor-mediated signaling (192). Mutant VAPB also potentiates mutant TDP-43-induced neurotoxicity (193).
1.5.6 Valosin-containing protein (VCP)

Exome sequencing has identified VCP mutations in European families with autosomal dominant ALS. VCP mutations were previously identified in patients found to have a combination of inclusion body myopathy, Paget disease, and FTD (IBMPFD). Mutations in VCP account for approximately 1-2% of FALS patients (131, 194) and less than 1 percent of those with SALS (195). Patients with this mutation may have FTD. Symptoms of myopathy or Paget disease may also be present. Severe motor neuron degeneration with rapid progression may occur (131).

VCP is an ATPases Associated with diverse cellular Activities (AAA)-type ATPase that regulates ubiquitin-dependent degradation, cell signaling, organelle biogenesis, autophagy, proteostasis, and the cell cycle (131). VCP-linked ALS is characterized by P62-, ubiquitin-, and TDP-43-positive inclusions (196). In mice, overexpression of mutant VCP produces ubiquitin- and TDP-43-positive aggregates in stress granules, suggesting TDP-43 plays a role in VCP-induced disease (197). Mutant VCP also impacts mitochondria. Decreased mitochondrial membrane potential and increased mitochondrial uncoupling are associated with VCP mutations and lead to significantly decreased ATP production and cellular ATP depletion (198).

1.5.7 Ubiquilin-2 (UBQLN2)

Mutations in UBQLN2 cause dominantly inherited, X-linked ALS and ALS-FTD. UBQLN2 mutations account for up to 2% of FALS patients. Disease onset occurs from the late teens to 70 years and is earlier in men than women. Disease duration averages slightly less than 4 years. FTD may precede motor symptoms. UBQLN2 mutations affect proline residues in a unique 12-unit PXX tandem-repeat domain within the ubiquilin 2 protein, although mutations
outside of this region occur and are associated with more FTD-predominant phenotypes (25, 199, 200).

Ubiquilins mediate proteasome-dependent protein degradation, and ubiquilin 2 mutations may disrupt the ubiquitin-proteasome system. Proper clearance of misfolded or damaged proteins is essential for cellular functioning, especially in long-lived motor neurons that are vulnerable to protein accumulation. Ubiquilin 2-positive inclusions are also observed in non-UBQLN2-linked ALS. They may accumulate in P62-positive aggregates with variable TDP-43 colocalization (25), and in the setting of FUS mutation (201). Dysfunctional ubiquitin-dependent degradation may represent a convergent pathway partly responsible for UBQLN2, TARDBP, and FUS-linked ALS.

Staining for ubiquilin 2 in cases of C9ORF72-linked disease reveals dystrophic neurites with focal swellings, dot-like stippled, and irregular aggregates in the molecular layer and CA1-CA4 region of the hippocampus; neuronal inclusions in the granular layer of the cerebellum; and dystrophic neurites and aggregates in the neocortex. This pathology is absent in non-expansion cases (202). These findings hint at common downstream pathways, possibly involving the ubiquitin-proteasome system, in UBQLN2- and C9ORF72-linked ALS.

1.5.8 Alsin (ALS2)

ALS2 mutations are associated with recessive, juvenile forms of ALS, PLS, and hereditary spastic paraplegia (121, 203). There are 2 main ALS2 splice variants, corresponding to short and long forms of the alsin protein. Mutations affecting both forms of the protein cause juvenile ALS. In contrast, mutations that affect only the long form affect UMN predominantly and cause juvenile PLS (121, 204). This suggests the functional short form of alsin may be
neuroprotective for LMNs, explaining the spectrum of clinical phenotypes, although subsequent reports have challenged this simple genotype-phenotype relationship (203).

Loss-of-function of WT alsin activity is believed to underlie mutant alsin-induced neurotoxicity. WT alsin interacts directly with mutant SOD1 and attenuates toxicity caused by reactive oxygen species (205, 206). Alsin-deficient neurons have disrupted AMPA receptor trafficking, increasing vulnerability to glutamate-induced excitotoxicity (207). Alsin deficiency causes accumulation of early endosomes, reduced endosome motility, increased conversion of endosomes to lysosomes, and increased lysosomal-dependent degradation (208). Knockdown of alsin activity selectively decreases growth and survival of spinal motor neurons via a GTPase-dependent mechanism (209). Astrocytes can protect spinal, but not cortical, motor neurons from alsin-induced axonal growth cone defects and decreased survival (210).

1.5.9 Senataxin (SETX)

Mutations in the SETX gene, which encodes a DNA/RNA helicase, are associated with a rare, autosomal dominant form of juvenile ALS. Missense mutations in SETX are most common. Also known as “distal hereditary motor neuronopathy”, this ALS variant is characterized by symptom onset during childhood or adolescence, slow disease progression, limb weakness with severe muscle wasting, and pyramidal features. Bulbar and respiratory muscles are generally spared (127). Ataxia may be observed (211).

Mutant senataxin may cause neurotoxicity through DNA damage and genomic instability, which is induced by topological strain as the cell coordinates DNA transcription and replication. Endogenous senataxin relieves topological strain caused by replication forks encountering transcription units. Senataxin associates with forks near regions of active transcription and
promotes replication across these genes. Mutant senataxin induces aberrant, cytotoxic DNA structures and DNA-RNA hybrids arising from replication defects (212). Silencing of senataxin decreases neuritogenesis, induces aberrant neurite growth, and inhibits neuronal differentiation. SETX-linked abnormalities are believed to result from haploinsufficiency (213).

1.5.10 Optineurin (OPTN)

OPTN mutations occur in approximately 1% of patients with FALS. Mutations may be missense, frameshift or truncation, and can be autosomal dominant or recessive. OPTN-linked ALS usually presents with lower limb onset, UMN signs, absence of FTD, age of onset ranging from 25 to 70 years, and disease duration ranging from 1 to 10 years (125, 214, 215). In some cases of non-SOD1-linked ALS, optineurin colocalizes with TDP-43 and FUS in cytoplasmic aggregates, linking OPTN mutation with TDP-43 and FUS-induced toxicity (216).

Two major mechanisms of OPTN mutation-induced neurotoxicity are proposed: a recessive pathway involving loss-of-function of WT optineurin regulatory activities, particularly inhibition of the transcription factor nuclear factor-kappa B (NF-κB); and a dominant pathway involving gain-of-function of toxic protein aggregation induced by dysregulated autophagy. Optineurin indirectly inhibits NF-κB activity via interactions with NF-κB regulatory binding partners that sequester the transcription factor in the cytoplasm. Optineurin expression is itself upregulated by NF-κB activation, resulting in a negative feedback loop (217). Mutant optineurin is unable to suppress NF-κB activity, resulting in cell death associated with mitochondria-induced apoptosis (218). WT optineurin also associates with protein aggregates in an ubiquitin-independent manner, regulating their clearance by autophagy. Optineurin depletion induces protein aggregation and causes motor neuron axonopathy in model systems (219).
1.5.11 Angiogenin (ANG)

Approximately 20 distinct mutations in ANG, whose product is a member of the pancreatic ribonuclease A (RNase A) superfamily, may be associated with ALS (122). Nine ANG mutations are also reported in Parkinson disease (PD), and 2 mutations are common to both diseases. ANG mutations occur in approximately 0.5% of patients with ALS and the same percentage of patients with PD, a 10-fold increase compared to the frequency of these mutations in the general population (220). Patients with ANG-linked ALS have a typical range of clinical features and variability, with the exception of a higher, approximately 60% rate of bulbar onset (122). However, the link between ALS and ANG remains controversial, with reports suggesting it is not associated with unique neuropathology (221).

ANG mutations may cause disease through loss-of-function of endogenous angiogenin activity. ANG variants associated with ALS generally lack RNase activity. WT angiogenin is induced by hypoxia; it regulates the angiogenic activity of vascular endothelial growth factor (VEGF) (122). Administration of exogenous angiogenin protects motor neurons, improves motor function, and increases lifespan in G93A SOD1 mice (222). WT angiogenin also promotes motor neuron neurite extension and pathfinding in vitro and has neuroprotective activities under hypoxic conditions. In contrast, mutant angiogenin variants lack these properties (223).

1.5.12 Spatacsin (SPG11)

The clinical overlap between ALS and hereditary spastic paraplegia led to identification of SPG11 mutations in patients with a rare form of autosomal recessive juvenile ALS with long survival. The mean age of onset is 16 years, and the mean survival is 34 years. Phenotypes vary widely, with motor involvement ranging from mild LMN and UMN symptoms to pronounced
weakness. A majority of patients have bulbar symptoms. Pyramidal signs are common, whereas cognitive deficits are absent (128, 224).

The function of spatacsin is unknown. Mutations in SGPL1 are classically associated with spastic paraplegia with a thin corpus callosum (225). In zebrafish, endogenous spatacsin is required for proper spinal motor neuron axon outgrowth and neuromuscular junction connectivity (226).

1.5.13 Genes that Modify Disease Risk or Progression

A number of genes that do not directly cause ALS modify disease risk or progression (Table 3). These include ATXN2, GRN, HFE, NEFH, UNC13A, and VEGF.

Expansions in the intermediate-length CAG-repeat, which code for poly-glutamine (polyQ) tracts, in the ATXN2 gene are associated with increased ALS risk. Normally, the ataxin-2 polyQ tract length is 22-23 units. Expansions larger than 34 units cause spinocerebellar ataxia type 2 (SCA2). PolyQ expansions numbering 27-33 repeats are associated with ALS and are found in up to 5% of patients with FALS and SALS combined. Intermediate-length ataxin-2 polyQ tracts are also found in healthy subjects, albeit at a lower frequency, suggesting that ATXN2 expansion modifies ALS disease risk, rather than directly causing disease (132, 227-229). Patients with ATXN2 expansion have classic ALS (230).

granules, increasing their cytoplasmic distribution, while decreasing nuclear abundance (231). Expansion-positive ataxin-2 enhances mutant FUS-induced ER stress, promotes fragmentation of the Golgi apparatus, and triggers mitochondria-induced apoptosis (232).

Mutations in the GRN gene, which encodes progranulin, modify ALS disease course. GRN mutations cause tau-negative, ubiquitin-positive FTD (233), are identified in up to 10% of patients with FTD overall, and are found in up to 25% of patients with familial FTD (234). The presence of GRN mutations in patients with ALS is associated with an earlier age of onset (mean 51 years) and shorter duration (median 2.5 years) than non-GRN-linked ALS (138). Aphasia, executive dysfunction and mild parkinsonism may be present (235). Progranulin is a secreted growth factor regulating cell cycle progression, cell motility, inflammation, tissue repair, and tumorigenesis (236). Progranulin knockdown induces caspase-dependent cleavage of TDP-43. The cleavage products exhibit subcellular redistribution, decreased solubility, and increased aggregation, suggesting GRN mutation influences ALS-FTD spectrum disorders via TDP-43 dependent pathways (237).

Dysregulated iron metabolism may contribute to neurodegenerative disorders, including AD, PD, and ALS (238). Increased frequency of the H63D polymorphism in the HFE iron regulatory gene, which encodes the human hemochromatosis protein, is reported in ALS patients (139, 239-242). Although the link between H63D HFE and ALS has been questioned (243-245), all studies find that H63D HFE occurs in 25-30% of in patients with ALS. A meta-analysis examining the prevalence of HFE polymorphisms suggests H63D HFE is found at a baseline frequency of under 15% in numerous geographic regions and ethnic groups worldwide (246). Together, these results support an association between H63D HFE and ALS. The presence of H63D HFE may increase ALS risk as much as 4-fold (247). H63D HFE is known to alter iron
profiles and increase metabolic and oxidative stress in CNS (248), effects that may impact ALS pathophysiology.

Abnormalities in neurofilaments may contribute to ALS. Early in the disease, neurofilaments accumulate in proximal axons and perikarya of LMNs (249). Polymorphisms in the NEFH gene, which encodes the neurofilament heavy subunit, are found in patients with ALS (141, 250). They are relatively rare, have low penetrance, and are associated with a typical range of clinical features and variability. Mutations affect a 43-unit repeat region harboring phosphorylation sites required for neurofilament crosslinking. Disrupted crosslinks may be toxic to neurons by inducing neurofilament disorganization and aggregation. Neurofilament-dependent slow axonal transport may also be affected. High CSF levels of neurofilament protein are found in patients with ALS, especially those with UMN-dominant disease or more rapid progression (251). Blood levels of neurofilament protein are higher in ALS patients and correlate with faster rates of functional decline as measured by ALSFRS-R scores (252).

A genome-wide association study of approximately 5,000 ALS patients and 15,000 control subjects identified the UNC13A gene as a susceptibility factor for ALS (142). Subsequently, the minor UNC13A allele was associated with approximately 1-year shorter survival in ALS patients (253, 254). UNC13A protein is located in the presynaptic terminals of central and neuromuscular synapses, where it regulates neurotransmitter release. UNC13A primes neurotransmitter vesicles, readying them for neurotransmitter release. Mice that lack UNC13A have disrupted glutamate signaling due to arrested synaptic vesicle maturation and morphological abnormalities in spinal cord neurons and neuromuscular junctions (255).

Alterations in the VEGF gene promoter in mice were found to produce adult-onset, progressive motor neuron degeneration (143). Subsequently, analysis of 750 ALS patients and
more than 1,200 control subjects indicated that homozygosity for select polymorphisms in the VEGF promoter sequence, which decreases VEGF expression, is linked to 1.8-fold greater risk of ALS (256). Additional reports suggested VEGF promoter polymorphisms were associated with a 4-9 year earlier age of onset (257), although later studies failed to replicate these findings (258). Meta-analysis demonstrates that only the -2578AA genotype, which causes low VEGF expression, is associated with 1.25-fold increased disease risk in men, and a 1.37-fold increased risk when comparing male to female ALS patients (259).
1.6 Apparently sporadic ALS

Although many causative alleles are linked to FALS, most cases of ALS are sporadic rather than hereditary, at least by history. Many factors may cause sporadic neurological disease, including apparently sporadic disease due to ascertainment bias or low-penetrance mutations in genes responsible for hereditary disease; *de novo* mutations; variations in disease-susceptibility genes; and epigenetic events (260). Evidence suggests all of these processes impact SALS.

A significant number of patients with apparently SALS carry an ALS-causing gene variant found in FALS patients. Among major mutations, *C9ORF72* expansions are found in 5-6% of SALS patients (115), *SOD1* in 3% (116), *TARDBP* in 2% (261), and *FUS* in less than 1% (262). This suggests that FALS-associated gene variants are present in up to 10% of patients with apparently SALS. Accordingly, it is increasingly recognized that the barriers between sporadic and familial disease are porous, and the distinction between the 2 is artificial. This will increasingly impact clinicians’ discussions with patients regarding disease etiology and implications for inheritance. The overlap between FALS and SALS has already begun to impact how clinician-researchers think about designing clinical trials, specifically with regard to the possibility of stratifying patients on the basis of genotype and disease mechanism rather than on traditional phenotypic features (see 1.9 Applying Genetic Insights to Clinical Trials).

Ascertainment bias due to small family sizes may cause patients with FALS to be misclassified as having SALS. Specifically, as the penetrance of a causative variant decreases, there is an increasing likelihood that it will manifest as sporadic disease, especially in smaller families (263). Additionally, low-penetrance monogenic variants, for example select *SOD1* mutations, are difficult to detect and may result in misclassification of patients as SALS, even in
large families. Whole exome sequencing of the patient and close relatives may identify these cases.

SALS may also result from de novo mutations. Previously, traditional, non-NGS techniques identified de novo mutations in known FALS genes, including SOD1 and FUS (264, 265). NGS techniques may streamline these efforts and uncover new genes linked to ALS. Recently, whole exome sequencing was applied to SALS patients and both parents of affected patients (134). In a significant number of cases, investigators could identify a de novo mutation in the patient’s genome that likely affected the function of a gene, which must have occurred as a spontaneous parental germline mutation. However, these mutations arose in a different set of genes than those identified as causative in FALS. The functional significance of these de novo mutations remain unknown.
1.7 Epigenetic and Other Factors Influencing ALS

Epigenetic factors may influence ALS pathophysiology. Epigenetics refers to heritable, DNA sequence-independent changes in gene expression. The most well-characterized epigenetic modifications are DNA methylation of CpG dinucleotides, which is associated with transcriptional repression, and acetylation of lysine residues on histone tails, which is associated with transcriptional activation (266). Since these modifications are lineage- and environment-dependent, and are unique to each cell, they may contribute to differences in vulnerability across varied tissue and cell types, such as motor neurons. Comparisons of post-mortem brains of patients with ALS versus control subjects indicate altered methylation patterns in genes influencing calcium dynamics, excitotoxicity, oxidative stress, neuroinflammation, neuronal exocytosis, brain development, ubiquitin-dependent protein degradation, cell survival, and apoptosis (267, 268). Increased DNA methyltransferase enzyme levels and DNA methylation patterns, which are associated with neurodegeneration involving apoptosis, are observed in motor neurons of ALS patients (269). WT FUS inhibits the histone acetyltransferase activities of cyclin D1, which regulates the cell cycle, implicating dysregulated epigenetic pathways in FUS-linked ALS (270).

Prion-like protein interactions that propagate neurotoxicity are implicated in ALS (271). Extracellular aggregates of mutant SOD1 penetrate cells via macropinocytosis in vitro and rapidly exit the macropinocytic compartment. In the cytoplasm, these aggregates nucleate the aggregation of soluble mutant SOD1 protein. Affected cells subsequently secrete mutant SOD1 aggregates, propagating further aggregation to neighboring cells (272). Insoluble TDP-43 aggregates derived from diseased brains are capable of seeding TDP-43 aggregation in vitro, causing characteristic phosphorylated, ubiquitinated TDP-43 accumulations that induce
proteasome dysfunction. TDP-43 aggregates are secreted and are capable of inducing aggregation in adjacent cells (273). These findings raise the possibility that ALS neurotoxicity may act through a progressive propagative mechanism involving contiguous spread between neighboring cells, or noncontiguous trans-synaptic or non-synaptic spread between distant CNS sites (271). Mutations in prion-like domains of heterogeneous nuclear ribonucleoproteins that cause self-polymerization and aggregation in stress granules have been identified in FALS (124).
1.8 Applying Genetic Insights to Clinical Practice

1.8.1 Prognostic Uses of NGS Data

Patients with ALS and their caregivers face many decisions regarding resource allocation and advance care planning. Accurate prognosis would aid these decisions. There are prognostic categories based on age and region of onset, diagnostic delay, El Escorial classification, riluzole use, and gender (5). However, each category has broad ranges for disease progression rates and survival times which confound attempts to predict prognosis in individuals. Biomarkers may have prognostic value, but their use remains preliminary (274, 275). Genetic information may aid prognosis. For example, the D90A SOD1 variant is associated with slow progression and long survival (149), whereas the A4V SOD1 variant is associated with particularly rapid disease progression (150). Future research may identify more precise relationships between genetic information and specific clinical phenotypes. This could improve care and maximize quality of life.

1.8.2 Genetic Testing of Symptomatic Individuals

One clinical situation in which the question of genetic testing commonly arises is that of patients with ALS who have first- or second-degree relatives with ALS or FTD. A recent review recommended offering genetic testing to such individuals, but not to other patients with ALS (276). For the latter group, testing may be considered upon the patient’s request. Discussion of the uncertainties of genetic testing should be reviewed with patients who are offered or desire such testing. These uncertainties include incomplete understanding of the pathogenicity and penetrance of select mutations; the possibility for the presence of mutations in multiple genes in
the same individual; poor correlation between genotype and phenotype in the majority of ALS genes; and issues of phenotypic pleiotropy (one gene influencing multiple phenotypic traits which are seemingly unrelated). For the clinician, the “take home message” is that identification of a genetic mutation known to be associated with FALS has uncertain significance in a patient with SALS and thus uncertain implications for his or her family members. As our understanding of ALS genetics grows, this will presumably change, and recommendations will likely evolve in response.

1.8.3 Pre-symptomatic Genetic Testing

The other common clinical situation associated with genetic testing involves asymptomatic at-risk subjects. Providing genetic information to asymptomatic relatives of individuals with FALS poses complex ethical issues because of the potential psychological effects of identifying a mutation likely to cause a fatal disease that cannot be prevented. Additionally, significant psychological repercussions are not limited to individuals who are found to carry a mutation linked to ALS, but also those found not to have the pathologic mutation (277). For these reasons, and because of uncertainties surrounding ALS genetics as currently understood (see above), the same authors recommended against proposing genetic testing to pre-symptomatic individuals unless testing is requested specifically or would be relevant for a research program in which the individual wishes to enroll (276). For the patient who requests testing in a clinical setting, the results of a large research study that offered genetic screening for first-degree relatives of individuals with FALS offers guidance (277, 278). The authors recommended counseling prior to pre-symptomatic genetic testing to determine whether the involved individuals “really wish to learn the results” (277). Flexibility in permitting
individuals to change their minds about learning the results of genetic testing was also recommended, because patient decisions are not static. Although these recommendations arose from a research study, they are relevant to the clinical setting in which genetic testing may be requested by pre-symptomatic relatives of individuals with FALS.

1.8.4 Broad Ethical Issues

Multidisciplinary counseling for symptomatic and pre-symptomatic genetic testing must take into account a number of important ethical concepts, including confidentiality and the disclosure of results to family members. Patient autonomy must always be respected, but non-maleficence (“do no harm”) is also an important clinical consideration, particularly in light of the psychological consequences of discovery or non-discovery of a mutation known to be associated with ALS. Issues involving potential discrimination or social stigmata based on genetic status should also be considered (276). As increasing numbers of genetic variants are linked to apparent SALS and FALS, and the boundaries between SALS and FALS continue to blur, ethical issues surrounding genetic testing will arise with increasing frequency. Additional complexity will arise if and when treatments for pre-symptomatic individuals become available. Additional guidelines should be developed, reviewed, and regularly updated so that clinicians have clear recommendations to aid decision-making.
1.9 Applying Genetic Insights to Clinical Trials

The information gained from genomic analysis may facilitate personalized medicine for the treatment of ALS. Personalized medicine uses genetic information to tailor therapies to an individual patient. Increased use of NGS strategies to identify genetic variants in ALS will clarify molecular mechanisms underlying neurodegeneration. Data on whether individual genetic variants act through unique molecular mechanisms, or whether different variants converge onto common pathways, will be especially informative. If each gene or variant acts through a distinct mechanism, developing pharmacotherapies will be very challenging. If NGS-based strategies identify common pathways, developing novel therapies that target these pathways may prove more feasible. Determining genetically-defined subtypes of ALS will improve understanding of pathophysiology and allow genetics-based patient stratification during clinical trials. Identifying “enriched cohorts” who are likely to respond to therapy based on genetically-determined biological pathways will permit more efficient clinical trials. This approach has advantages over current phenotype-based inclusion and exclusion criteria, which may fail to identify responsive subgroups.

Clinical heterogeneity in ALS is problematic and may be a major contributor to poor trial outcomes. Oncology has been at the vanguard of genomic approaches to personalized medicine and clinical trials, and experiences gained from treating cancer may be relevant to neurodegenerative disorders, such as ALS (279). Personalized cancer genomics is driven by the genetic diversity among different patients and within tumors. Analysis of founder mutations, which exist at the earliest stages of carcinogenesis and are found in many subclones and metastases, implicated disruptions in biologically relevant pathways. Subsequently, genetically-
based therapies targeting specific mutations enabled wide application of personalized approaches to cancer therapy and clinical trials.

Selection of cohorts based on genotype may improve ALS clinical trial outcomes. Small trials devoted to patients with a single mutation, such as \textit{C9ORF72} expansion repeat, or one or more of a group of interrelated mutations, such as \textit{TARDBP, FUS}, and \textit{ATXN2}, may improve the chances for a response due to decreased biological heterogeneity and better matching of pre-clinical efficacy with patient selection. Many pre-clinical studies involving novel agents use transgenic rodent models expressing 1 or a few mutations (e.g., G93A SOD1). The agent is then tested on a cohort of patients in which a very limited number of individuals harbor an analogous mutation. Small trials devoted to patients who share a specific mutation, or a set of mutations, affecting a common biological pathway, are likely to yield improved results. Such trials are currently challenging because of genetic heterogeneity and the difficulty in identifying relevant mutations and predominating pathogenic processes. In the future, NGS technologies may mitigate barriers to these trial designs. Because of the low prevalence of ALS, multi-center collaborations will be critical to the success of these targeted trials.

Subset effects have significant implications for past studies. It is entirely possible that drugs which “failed” ALS trials were actually effective in meaningfully altering disease course in a subset of patients, but that this beneficial effect was overlooked because investigators lacked the genetic tools to stratify individuals. As genetically-determined biochemical pathways are identified, analyzing the relationships between these pathways and the mechanisms of action of “failed” as well as novel ALS drugs may prove valuable.

Improved understanding of ALS genetics has resulted in novel and promising treatment avenues, including antisense oligonucleotide therapy. By binding to a specific target mRNA and
facilitating its degradation, antisense oligonucleotides may decrease levels of the resultant protein. In G93A SOD1 rats, intrathecal delivery of an antisense oligonucleotide was found to decrease spinal cord SOD1 mRNA and protein concentrations, and to prolong survival (280). Intrathecal infusions have also been conducted safely in human subjects (281), raising the hope that such genetically-directed therapy may be possible in clinical treatment trials.

As large-scale databases of genetic information become available, it may become feasible to identify individuals at risk for ALS using genetic screening and initiate trials before symptoms appear. Such a strategy poses ethical challenges, not only because the penetrance of causative mutations remains uncertain, but because of the constellation of issues surrounding the genetic testing of pre-symptomatic individuals, including informed consent and patient autonomy, as discussed earlier. However, trials of presymptomatic individuals may greatly benefit preventive approaches to the disease. Likewise, genetic studies of relatives of individuals with FALS, who carry the disease allele but are disease-free, may identify suppressor mutations that are protective. Ultimately, the development of pharmacotherapies that are effective at preventing or treating ALS may require a multi-faceted understanding of pathogenesis that accounts for numerous and varied potential genetic insults, epigenetic and environmental factors resulting in incomplete penetrance, and the particular vulnerability of motor neurons and select cognitive neurons responsible for ALS-FTD.
1.10 Conclusions

There has been steady progress in understanding the genetic basis of ALS in the past 2 decades. Recognition of common pathogenic mechanisms is emerging, thus identifying potential pathways that can be targeted by novel therapies. Further research is needed to determine how these insults induce selective vulnerability in motor neurons and, in some patients, other neurons such as those associated with cognition. Further understanding is also needed of the mechanisms whereby phenotypes are expressed in a time-dependent fashion, usually manifesting as adult-onset disease.

Ultimately, insights into genetics and disease pathways must be translated into clinical applications if they are to improve patient well-being. For the clinician, until such time as more effective treatments are devised, genetic information about individual patients with ALS will likely be used for prognosis and for discussion of genetic testing of family members. For the clinical researcher, genetics-based stratification will likely be one element of successful ALS clinical trials. Novel techniques, including NGS technologies, are capable of generating vast quantities of raw data, which can be processed efficiently by sophisticated computational algorithms that harness powerful bioinformatics platforms. However, this information must be grounded in a biologically-relevant framework stemming from rigorous basic and translational research. Proper clinical trial designs, such as multi-stage trials and smaller, mutation- or pathogenic pathway-focused ones, will be greatly facilitated by genetic insights available from current and future technologies. For all who are involved in the diagnosis and care of individuals with ALS, as well as for the patients and their caregivers, insights into the genetics of the disease offer hope for better care and more effective treatments.
1.11 Acknowledgment

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Chapter 2

H63D HFE Polymorphisms are Associated with Increased Disease Duration and Decreased Muscle SOD1 Expression in Amyotrophic Lateral Sclerosis Patients

2.1 Abstract

Introduction: H63D HFE polymorphisms increase risk of neurodegenerative disorders and, specifically, may increase ALS risk. Investigating the physiological alterations induced by H63D polymorphisms in ALS patients may elucidate mechanisms by which this genotype alters disease. Materials: Clinical measures and muscle biopsies were available from patients previously diagnosed with ALS who underwent HFE genotyping. Clinical outcomes and SOD1 protein expression were analyzed using standard statistical analyses. Results: ALS patients harboring H63D HFE (n=16) had 28.1 months longer average disease duration and 39.3% lower muscle SOD1 protein than ALS patients with wild type HFE (n=22). Discussion: Combined with previous reports suggesting the H63D polymorphism is associated with ALS, these results support a model wherein the H63D polymorphism is involved in ALS via pathways involving SOD1 but may limit cellular damage in individuals who develop disease. The association between HFE genotype and disease duration has important implications for clinical care and treatment trials.
2.2 Introduction

Genetic polymorphisms are an example of an intrinsic factor modifying disease progression that is relatively stable throughout life. Among these, the H63D polymorphism in the \textit{HFE} iron regulatory gene is relevant to amyotrophic lateral sclerosis (ALS).

The H63D \textit{HFE} polymorphism is a common genetic alteration in ALS patients, with a prevalence of approximately 30\% reported in a number of patient populations globally (1-5). The \textit{HFE} gene product plays a major role in iron homeostasis, and there are two major \textit{HFE} allelic variants in humans: H63D and C282Y (6). The C282Y \textit{HFE} allelic variant is strongly associated with the hereditary iron overload disease hemochromatosis as well as cancers such as hepatocellular carcinoma (HCC), breast cancer, colorectal cancer, and childhood acute lymphoblastic leukemia (7-9). In contrast, the H63D \textit{HFE} polymorphism is mainly associated with neurodegenerative diseases such as ALS (1-5) and Alzheimer disease (10, 11).

Missense mutations in the \textit{SOD1} gene, whose product enzyme functions to eliminate superoxide free radicals, account for 10-20\% of cases of familial ALS and were the first genetic alterations linked to ALS (6, 12, 13). Furthermore, expression of wild type (WT) SOD1 protein is significantly decreased in lymphocytes and affected brain areas in sporadic ALS patients (14, 15). Therefore, altered expression of both WT and mutant forms of SOD1 is associated with ALS pathophysiology. Here we determine whether the H63D polymorphism is associated with altered expression of SOD1 in muscle samples of ALS patients. Additionally, we assess whether the H63D genotype influences disease duration and site of onset, and whether SOD1 expression levels are associated with clinical outcomes.
2.3 Materials and Methods

2.3.1 Patients and Samples

Records from the ALS clinic at Penn State Hershey Medical Center from January 2004 through December 2008 were reviewed to identify patients diagnosed with clinically definite, probable, probable laboratory-supported, or possible ALS who met revised El-Escorial research criteria (16) and underwent testing for the *HFE* H63D genotype as part of a previous IRB-approved study and who had undergone muscle biopsies as part of their diagnostic workup. Muscle samples were stored at -80°C prior to experiments. Subjects were separated into 2 groups by genotype: wild type (WT) *HFE* or presence of the H63D allele (homozygous H63D/H63D and heterozygous WT/H63D treated as 1 bin). The compound heterozygous *HFE* genotype (H63D/C282Y) was excluded. Additional evaluation of muscle tissue was performed after obtaining informed consent from those patients still living. The study was approved by the Penn State Hershey Medical Center Institutional Review Board.

2.3.2 Patient Characteristics

Clinical measures were obtained and included patient age, gender, region of disease onset, and time of onset, muscle biopsy, and death.

2.3.3 *HFE* Genotyping

Genomic DNA was purified from ALS patient muscle biopsy samples using the DNeasy Tissue kit (Qiagen, Valencia, California). PCR followed by restriction fragment length analysis and confirmation DNA sequencing as previously reported was used to analyze H63D and C282Y
HFE polymorphism status (2). Only patients with WT HFE or the H63D polymorphism were included.

2.3.4 Slot Blot

SOD1 expression in ALS muscle samples was determined by slot blot. Protein was extracted from biopsies from ALS patients with WT HFE or the H63D polymorphism using ProteoExtract Complete Mammalian Proteome Extraction kit (Calbiochem, La Jolla, CA), and soluble protein concentration was determined by RC-DC Protein Assay (Bio-Rad, Hercules, CA). Approximately 2.0 μg protein was immobilized onto a Polyvinylidene fluoride (PVDF) membrane. Protein expression in control cell lysate (sc-24-767, Santa Cruz Biotechnology, Inc.) was used to normalize between membranes. Membranes were probed with SOD1 antibody (BD Biosciences PharMingen, San Diego, CA) at 1:500 dilution, and optical density was measured by ImageJ and Fuji Imaging analysis software. Data were expressed as the ratio of SOD1 in WT or H63D, respectively, normalized to expression levels in the control cell lysate.

2.3.5 Statistical Analysis

GraphPad Prism 4.03 (GraphPad Software, San Diego, California) was used for statistical analysis. The Fisher exact test was used to evaluate categorical data. A two-sample t-test was used to compare group means, and the Wilcoxon rank sum test was used when data were skewed. Pearson correlations were used to evaluate the association between variables. All tests were conducted at the $P < 0.05$ statistical significance level.
2.4 Results

2.4.1 Clinical Results

Clinical variables from 22 ALS patients with WT *HFE* and 16 ALS patients either homozygous or heterozygous for H63D *HFE* were obtained. All were classified as sporadic ALS, because there was no family history of ALS. No testing for SOD1 mutations was performed. All subjects were Caucasian. A majority of ALS patients (15 of 16) in the H63D *HFE* group were homozygous for the H63D polymorphism. Clinical characteristics including gender, age at disease onset, time from onset to muscle biopsy, and site of onset were not different between groups (Table 2-1).
Table 2-1. Patient Characteristics

<table>
<thead>
<tr>
<th>Clinical variable</th>
<th>WT HFE</th>
<th>H63D HFE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (% male)</td>
<td>68.2</td>
<td>75.0</td>
<td>0.729*</td>
</tr>
<tr>
<td>Age at onset (yrs), mean (range)</td>
<td>56.7 (34.0-78.5)</td>
<td>56.5 (30.3-82.3)</td>
<td>0.973†</td>
</tr>
<tr>
<td>Onset to biopsy time (mo), mean (range)</td>
<td>23.2 (4.9-122.3)</td>
<td>21.9 (2.9-65.0)</td>
<td>0.861†</td>
</tr>
<tr>
<td>Site of onset (% bulbar)</td>
<td>13.6</td>
<td>12.5</td>
<td>1.000*</td>
</tr>
</tbody>
</table>

Notes: * – Fisher exact test, † – Student t-test
Total disease duration, defined as the time from symptom onset to death or tracheostomy/mechanical ventilation, was available for 21 out of 22 patients in the WT HFE group (1 patient lost to follow-up) and 16 out of 16 patients in the H63D HFE group. One patient in each of the WT and H63D groups was still living, and they were excluded from total disease duration calculations, yielding sample sizes of n = 20 in the WT and n = 15 in the H63D HFE groups. Patients with H63D HFE had 28.1 months longer mean disease duration than patients with WT HFE (duration = 75.3 ± 12.7 mo, H63D HFE group; duration = 47.2 ± 11.0 mo, WT HFE group; data expressed as mean ± SEM; Wilcoxon rank sum P = 0.017; Figure 2-1). Stratification by gender did not affect results.
Figure 2-1. Longer total disease duration in ALS patients harboring H63D HFE. Patients homozygous or heterozygous for the H63D HFE polymorphism (n = 15) had a mean disease duration 28.1 months longer than patients with WT HFE (n = 20, P = 0.017). Bar lines denote average values.
2.4.2 Molecular Results

Muscle samples from 22 ALS patients with WT \textit{HFE} and 16 ALS patients either homozygous or heterozygous for H63D \textit{HFE} were obtained. SOD1 expression was decreased by 39.3\% in ALS patients who harbored the H63D polymorphism compared to ALS patients with WT \textit{HFE} (SOD1 relative expression level = 2.74 ± 0.32, H63D \textit{HFE} group; SOD1 relative expression = 4.51 ± 0.38, WT \textit{HFE} group; data expressed as mean ± SEM; two-sample \textit{t}-test \( P = 0.0018 \); Figure 2-2). Stratification by gender did not affect results.
Figure 2-2. Reduced SOD1 protein expression in muscle biopsy samples of ALS patients with H63D HFE. ALS patients either homozygous or heterozygous for the H63D HFE polymorphism (n = 16) have 39.3% lower muscle tissue SOD1 protein expression compared to ALS patients with wild type (WT) HFE (n = 22, p = 0.0018). Bar lines denote average values.
2.4.3 Tests of Association

Pearson correlation was performed on the 20 patients with H63D \textit{HFE} and 15 patients with WT \textit{HFE} for whom both total disease duration and muscle SOD1 relative expression data were available. For all patients combined there was a significant negative correlation between muscle SOD1 expression levels and total disease duration (Pearson \( r = -0.360, P = 0.034, R^2 = 0.130; n = 35 \); Figure 2-3).
Figure 2-3. SOD1 protein expression is negatively associated with total disease duration in ALS patients. When data for H63D and WT HFE patients were combined, there was a significant negative correlation between muscle SOD1 expression levels and total disease duration (Pearson $r = -0.360$, $p = 0.034$, $R^2 = 0.130$).
2.5 Discussion

Previously, we reported an association between H63D polymorphism and ALS, a finding later observed by other groups (1-5). Although the association of the H63D polymorphism was recently challenged (17, 18) it is noteworthy that all studies involving Caucasian populations report frequencies of approximately 30% for the combination of heterozygous and homozygous H63D HFE genotypes in ALS patients. This frequency of H63D HFE genotype in ALS is a compelling reason to investigate the impact of the HFE genotype on the disease. Here, we show that presence of the H63D HFE allele increases total disease duration in ALS patients by an average of approximately 2 years. This is in contrast to a previous study by Sutedja et al (3).

However, only 55.7% of ALS patients were deceased at the time of measurement in that study, which only included 13 patients homozygous for H63D HFE. Thus, survival data for homozygotes may have been available for only 7 patients in that study (actual numbers were not reported). By contrast, our study includes a larger number of patients, the majority of whom were homozygous for H63D HFE, resulting in increased statistical power to detect differences in this genotype.

The magnitude of the homozygous H63D HFE effect on survival in ALS is highly significant clinically, given the average median survival of 2-4 years reported in most epidemiological studies (19). Notably, this effect may partly explain the increased frequency of the H63D polymorphism observed in the sporadic ALS (SALS) population. Specifically, the frequency of the H63D polymorphism in the general population is relatively unchanged over time and SALS is not inherited. Therefore, if H63D HFE increases disease duration by 2 years, or almost twice the median survival length, then due to the censoring effects of death the frequency of H63D polymorphism would be approximately double in the ALS versus general
population as a result. This is consistent with the frequencies observed in our initial report on the association between the H63D polymorphism and ALS (proportion of control group harboring the H63D polymorphism = 14.29%, proportion of ALS group harboring the H63D polymorphism = 30.58%) (1).

Here we demonstrate an association between the H63D polymorphism and decreased SOD1 protein expression in ALS patient muscle samples, which is consistent with our previous finding that the H63D polymorphism decreases SOD1 expression in human neuroblastoma cells transfected with H63D HFE (1). Decreased native SOD1 in the setting of the H63D polymorphism, together with findings which show decreased SOD1 protein expression in brain and lymphocytes of ALS (14, 15) patients, implicates the H63D polymorphism in ALS disease processes. Decreased SOD1 expression may reflect a number of processes, such as increased SOD1 misfolding and aggregation leading to decreased soluble pools of native SOD1. Research has suggested a role for misfolded non-mutant SOD1 protein in sporadic ALS via abnormal inclusion bodies localized with lysosomes (20).

Additionally, in familial forms of ALS mediated by SOD1 mutation, misfolded SOD1 contributes to cellular injury through a number of mechanisms, including mitochondrial dysfunction (21, 22) and disruption of proteasome-dependent degradation (23). Decreased native SOD1 may also cause oxidative stress, which is enhanced by the H63D polymorphism in our cell culture models (24), further contributing to ALS disease processes. Alternatively, the H63D polymorphism may promote protein misfolding through increased endoplasmic reticulum (ER) stress, leading to decreased native SOD1. The latter is intriguing considering our report that suggested increased ER stress in both cell culture and animal models of the H63D polymorphism
(25). These mechanisms may also act in tandem, resulting in a cycle of enhanced ER stress coupled with lower native SOD1 protein levels.

In light of our finding that the H63D polymorphism is associated with increased disease duration, these results may suggest that in the setting of WT SOD1, the H63D polymorphism causes a degree of SOD1 aggregation leading to low levels of ER stress, which may increase the risk for ALS. However, because misfolded WT SOD1 may be adequately cleared by unfolded protein response mechanisms compared to misfolded mutant SOD1 in familial forms, the long-term effect of the H63D polymorphism in a SALS patient with WT SOD1 may be an induction of low levels of ER stress promoting adaptive pathways that are protective against further cellular injury. This effect may result in the increased disease duration observed in this study, although this mechanism remains speculative and warrants further investigation.

In this study, the H63D polymorphism was associated with decreased soluble SOD1 expression, and SOD1 protein expression levels were negatively correlated with total disease duration when all genotypes were combined. This finding may support the model proposed above, wherein the H63D polymorphism induces a low level of ER stress via WT SOD1 misfolding, which may increase the risk for developing ALS while at the same time protecting against cellular damage and increasing disease duration in those individuals who do develop ALS. Notably, the results also suggest soluble WT SOD1 expression levels in muscle may represent a crude biomarker for total disease duration in ALS patients regardless of HFE genotype.

This study was limited by small sample sizes. Nonetheless, the number of ALS patients homozygous for H63D HFE remained adequately statistically powered compared to a previous report that analyzed genotype effects on survival (3). Another consideration is selection bias;
many of the patients selected for muscle biopsy underwent this procedure because their clinical and/or electrodiagnostic findings were in some way atypical. However, all patients met revised El-Escorial research criteria as noted, and the ages and gender distributions were within the expected ranges. SOD1 genotyping was not performed on these patients who are no longer available due to death or lack of ongoing follow-up. However, none had a family history of ALS.

The magnitude of the H63D HFE effect on disease duration, if confirmed in other, larger studies, could provide clinicians with an important test to aid in prognosis. HFE genotyping likely would also be of value in stratifying individuals into subgroups for assessing the efficacy of new drugs in clinical trials. Follow-up studies aimed at increasing sample sizes as well as studies investigating the mechanisms underlying the effect of H63D polymorphism on disease duration are planned. Additionally, studies are underway to determine which mechanism(s), such as direct protein interactions or indirect signal transduction events, cause decreased native SOD1 expression in the setting of an H63D polymorphism.
2.6 Acknowledgment

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Chapter 3

Biomarker-Based Predictive Models for Prognosis in Amyotrophic Lateral Sclerosis

3.1 Abstract

Importance: Although median survival in amyotrophic lateral sclerosis (ALS) is 2-4 years, survival ranges from months to decades, creating prognostic uncertainty. Strategies to predict prognosis would benefit clinical management and outcomes assessments of clinical trials. Objective: To identify biomarkers in plasma and cerebrospinal fluid (CSF) of ALS patients that can predict prognosis. Design: We conducted a retrospective study of plasma (n=29) and CSF (n=33) biomarkers identified in samples collected between March 2005 and August 2007 from patients with ALS. Clinical information extended from initial presentation to death. Genotyping for HFE gene status was performed. Multiplex and immuno-assay analysis of plasma and CSF was used to measure levels of 35 biomarkers. Statistical modeling was used to identify biomarkers panels that could predict total disease duration. Setting: An academic, tertiary care center. Participants: Patients who were undergoing diagnostic evaluation in the Neurology outpatient clinic, and who were eventually identified as having definite, probable, laboratory-supported probable, or possible ALS as defined by revised El-Escorial criteria. All were Caucasian and none had a family history of ALS. Exposures: None. Main Outcome Measures: Total disease duration, defined as the time from symptom onset to death. The hypothesis being tested was formulated after data collection. Results: Multivariable models for total disease duration using biomarkers from plasma, CSF, and plasma and CSF combined incorporated seven, six and six biomarkers, respectively, to achieve goodness-of-fit R-squared values of 0.769, 0.617 and 0.962. After classification into prognostic categories, actual and predicted
values achieved moderate-to-good agreement, with Cohen’s Kappa values of 0.526, 0.515 and 0.930 for plasma, CSF, and plasma and CSF combined. Inflammatory biomarkers, including select interleukins, growth factors such as granulocyte-colony stimulating factor, and L-ferritin, had predictive value. **Conclusions and Relevance:** This study provides proof-of-concept for a novel multivariable modeling strategy to predict ALS prognosis. These results support unbiased biomarker discovery efforts in larger patient cohorts with detailed longitudinal follow-up.
3.2 Introduction

Intermediate factors influencing disease progression, which have the characteristics of both intrinsic and extrinsic factors, depend on and reflect the individuals innate genetic and metabolic profiles as well as environment. Proteins that can serve as biomarkers are an example, and have the potential to improve clinical management and the development of new treatments.

Biomarkers are objective physiological measures reflecting biological processes or treatment effects (1, 2). By improving prognostic determination, they could aid clinical care planning, including discussions of gastrostomy tube placement, non-invasive ventilation, and end-of-life decisions. Prognostic biomarkers also could have a meaningful impact on the conduct of clinical trials. It is now often impossible to determine if a potential therapeutic agent in a failed clinical trial is wholly ineffective, or may have benefited a subgroup of patients who could be stratified by as-yet-unidentified biomarkers. Biomarker subgroup analyses in clinical trials has the potential to permit the stratification of clinical response results according to predicted prognosis.

A number of amyotrophic lateral sclerosis (ALS) biomarker studies have been conducted or are ongoing. In blood, amino acids (3-5), inflammatory cytokines (6-10), growth factors (11-13), and metabolites (14, 15) have been studied. In cerebrospinal fluid (CSF), researchers have analyzed similar classes of biomarkers (5, 16-18), with special attention to neurofilament protein (19-22), tau (23), S100-β (23, 24), and cystatin C (25, 26). Previously, we demonstrated that protein biomarkers in both plasma and CSF may aid the diagnosis and stratification of ALS patients (27, 28). However, these studies focused primarily on diagnosis, rather than clinically relevant prognostic endpoints, and generally followed a targeted, rather than unbiased, discovery
approach. As such, biomarker studies utilizing unbiased screens of candidate targets for disease progression and prognosis are limited.

This study attempted to identify biomarkers relevant for ALS prognosis. Study design, subject recruitment, sample collection and biobanking, quality control, and data analysis proceeded with attention to recent ALS biomarker research guidelines (29). The results support the utility of biomarker-based approaches to analyze ALS disease progression and prognosis.
3.3 Methods

3.3.1 Patients and Samples

We conducted a retrospective study of plasma and CSF biomarkers identified in samples collected between March 2005 and August 2007 from patients who were undergoing diagnostic evaluation in the Neurology outpatient clinic, and who were eventually identified as having definite, probable, laboratory-supported probable, or possible ALS (30). Clinical information extended from initial presentation to death, and spanned June 1989 through March 2013. Blood samples were collected by venipuncture, and centrifuged immediately to isolate plasma. CSF samples were obtained after written informed consent by standard lumbar puncture under sterile conditions with local anesthetic. Plasma and CSF were obtained between 8 AM and 12 PM to limit circadian effects. Samples were frozen after collection, then later thawed on ice and centrifuged to remove particulate matter. Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, Missouri) was added, and samples were refrozen at -80°C in 200 µl aliquots until use. All patients provided written informed consent. The study was approved by the Penn State Hershey Medical Center Institutional Review Board.

3.3.2 Patient Characteristics

Clinical variables recorded included date of birth, gender, site and time of symptom onset, ALS Functional Rating Scale-Revised (ALSFRS-R) scores (31), and time of death. Total disease duration was defined as time from symptom onset to death. None of the patients underwent tracheostomy and mechanical ventilation. Because of the suggested association
between ALS and *HFE* polymorphisms (32-36), H63D and C282Y *HFE* genotyping was performed.

### 3.3.3 *HFE* Genotyping

Genomic DNA was purified from leukocytes using the QIAamp DNA Mini kit (Qiagen, Valencia, California). PCR followed by restriction fragment length analysis and confirmation DNA sequencing as previously reported was used to analyze H63D and C282Y *HFE* status (34).

### 3.3.4 Multiplex Assay

Multiplex biomarker analysis of plasma and CSF was performed using the Bio-Plex Pro Human Cytokine 27-Plex assay system (Bio-Rad Laboratories, Hercules, California). Briefly, 50 µl of plasma at a 1:3 dilution or undiluted CSF was added to 50 µl of antibody-conjugated beads on respective assay wells, followed by 25 µl of detection antibody. Fifty µl of streptavidin-phycoerythrin was added and the reaction proceeded to completion. Washes between steps were performed using an automated Bio-Plex Pro wash-station (Bio-Rad Laboratories). Assay plates were read using a Bio-Plex 200 multiplex system (Bio-Rad Laboratories), and data analyzed using Bio-Plex Manager software (Bio-Rad laboratories). Analyte concentration was calculated based on the standard curve for each cytokine. Each sample was analyzed in duplicate, and the coefficient of variance was less than 10% for each sample included in the final analysis. Levels of all analytes were measured using the multiplex assay system, except those related to iron metabolism, which are not available on the multiplex panel. These biomarkers were analyzed by enzyme-linked immunosorbent assay (ELISA), immunoradiometric assay or atomic absorption spectrometry as detailed below.
3.3.5 Immunoassays

Plasma and CSF levels of β-2 microglobulin (US Biological, Swampscott, Massachusetts) and transferrin (Bethyl Laboratories, Montgomery, Texas) were analyzed ELISA. L-ferritin levels were measured by immunoradiometric assay (Siemens Medical Solutions, Malvern, Pennsylvania), while H-ferritin levels were measured by ELISA as previously reported (37). Plasma levels of C-reactive protein (R&D Systems, Minneapolis, Minnesota) and pro-hepcidin (DRG International, Mountainside, New Jersey) were analyzed by ELISA. Assays were conducted per manufacturer’s protocols.

3.3.6 Iron Measurement and Calculation of Transferrin Saturation

Total iron content in plasma and CSF was determined by digestion in ultrapure nitric acid (JT Baker, 9598-00; Phillipsburg, New Jersey), 1:4 v/v, followed by incubation at 60°C for 24 hours. Samples were diluted 1:100 in ddH2O, and then analyzed on a Perkin Elmer Atomic Absorption Spectrometer 600 series (Waltham, Massachusetts). Transferrin saturation (TSAT) in plasma was calculated as TSAT (%) = plasma iron (mol/L) / [2 × Transferrin (mol / L)] × 100. Replicate sample variation was less than 5%, and an external standard was included in each set of analyses.

3.3.7 Statistical Analysis and Multivariable Modeling

Total disease duration was calculated from clinical data and treated as the dependent variable. Descriptive statistics were calculated for biomarker and clinical values. Multiple linear regression incorporating stepwise-forward, main-effects only analyses was conducted. Variable selection algorithms used McHenry’s method (38). Briefly, the variable with highest R-squared
was entered as an independent variable, followed by the variable that next most increased predictive likelihood. Switching was integral to the algorithm, such that with each successive variable added to the model, all other variables were checked for increases in the likelihood function, until the set of variables for an n-variable model was stable. The process was then repeated for n + 1 variables, until no further significant variables remained.

Models were validated using the original biomarker and clinical values of the plasma- and CSF-discovery cohorts; limitations of this approach are covered in the discussion. Confidence bands were constructed for model-based predictions at the individual-subject level. Actual and predicted values for total disease duration were grouped into prognostic categories, and agreement analyzed using Cohen’s Kappa statistics. SAS 9.3 (SAS Institute, Cary, North Carolina) or NCSS 8 (NCSS LLC, Kaysville, Utah) was used for statistical analyses. Significance tests were two-sided with significance set at the p < 0.05 level.
3.4 Results

3.4.1 Clinical Results

Plasma and CSF samples were available from 29 and 33 ALS patients, respectively. Of these, 18 patients provided both plasma and CSF samples. All were Caucasian and none had a family history of ALS. Descriptive statistics of patient characteristics are provided in Table 3-1.
Table 3-1. ALS Patient Characteristics

<table>
<thead>
<tr>
<th>Clinical variable</th>
<th>Plasma Cohort</th>
<th>CSF Cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>29</td>
<td>33</td>
</tr>
<tr>
<td>Gender, number of men (% men)</td>
<td>19 (65.5)</td>
<td>23 (69.7)</td>
</tr>
<tr>
<td>Age at onset (yrs.), med. (range)</td>
<td>57.6 (35.0-83.6)</td>
<td>58.3 (35.0-79.5)</td>
</tr>
<tr>
<td>Onset to sample time (mo.), med. (range)</td>
<td>17.7 (4.1-189.5)</td>
<td>10.4 (3.2-64.1)</td>
</tr>
<tr>
<td>Site of onset, number bulbar, (% bulbar)</td>
<td>10 (34.5)</td>
<td>10 (30.3)</td>
</tr>
<tr>
<td>ALSFRS-R score at sample collection, med. (range)</td>
<td>37 (16-48)</td>
<td>41 (16-48)</td>
</tr>
<tr>
<td>H63D HFE, number harboring variant, (% harboring)</td>
<td>11 (37.9)</td>
<td>12 (36.4)</td>
</tr>
<tr>
<td>C282Y HFE, number harboring variant, (% harboring)</td>
<td>1 (3.4)</td>
<td>5 (15.2)</td>
</tr>
<tr>
<td>Total disease duration (mo.), med. (range)</td>
<td>33.4 (9.0-193.6)</td>
<td>42.0 (9.0-149.9)</td>
</tr>
</tbody>
</table>

**HFE status:** all subjects harboring variants were heterozygous for H63D or C282Y HFE; none were double heterozygous for both alleles.
3.4.2 Biomarkers Results

Biomarkers analyzed are listed in Table 2. Five biomarkers were associated with greater than 10% variability between measurements and were excluded from subsequent analyses: IL-4, IL-8, IL-15 and IL-17 in plasma and IL-1β in CSF. C282Y HFE status was excluded because of the limited number of patients harboring this allele. A total of 31 biomarkers and three categorical clinical variables (gender, H63D HFE status and site of symptom onset) were entered into individual plasma or CSF multivariable model algorithms. For the combined plasma and CSF model, each available plasma and CSF biomarker was entered into the algorithm, as well as the ratio of plasma to CSF levels for each biomarker that was measured in both plasma and CSF. The ranges of biomarkers assayed are given in Table 3-2.
Table 3-2. Biomarkers Analyzed with Ranges

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Plasma Median</th>
<th>Plasma IQR</th>
<th>CSF Median</th>
<th>CSF IQR</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2M (ng/ml)</td>
<td>979.4</td>
<td>826.5-1188.1</td>
<td>735.5</td>
<td>630.7-890.7</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>1.36</td>
<td>0.92-2.670</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>53.05</td>
<td>38.93-88.48</td>
<td>11.91</td>
<td>9.76-13.28</td>
</tr>
<tr>
<td>FGF Basic</td>
<td>17.16</td>
<td>0.00-33.74</td>
<td>75.52</td>
<td>54.96-95.58</td>
</tr>
<tr>
<td>G-CSF</td>
<td>37.12</td>
<td>32.74-41.54</td>
<td>5.91</td>
<td>4.24-9.57</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>11.51</td>
<td>2.48-21.98</td>
<td>64.67</td>
<td>51.99-76.54</td>
</tr>
<tr>
<td>H-Ferritin (ng/ml)</td>
<td>0.367</td>
<td>0.244-0.537</td>
<td>0.387</td>
<td>0.093-0.831</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>130.0</td>
<td>107.4-163.9</td>
<td>42.03</td>
<td>34.51-47.58</td>
</tr>
<tr>
<td>IL-1β</td>
<td>3.85</td>
<td>2.74-4.40</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-1RA</td>
<td>178.0</td>
<td>133.8-260.5</td>
<td>57.42</td>
<td>50.54-73.45</td>
</tr>
<tr>
<td>IL-2</td>
<td>6.62</td>
<td>0.00-13.40</td>
<td>53.94</td>
<td>46.31-61.54</td>
</tr>
<tr>
<td>IL-4</td>
<td>-</td>
<td>-</td>
<td>13.47</td>
<td>11.13-14.96</td>
</tr>
<tr>
<td>IL-5</td>
<td>3.05</td>
<td>2.05-5.14</td>
<td>3.97</td>
<td>3.19-4.54</td>
</tr>
<tr>
<td>IL-6</td>
<td>10.82</td>
<td>7.06-14.41</td>
<td>28.82</td>
<td>25.75-31.90</td>
</tr>
<tr>
<td>IL-7</td>
<td>3.15</td>
<td>2.63-4.09</td>
<td>55.18</td>
<td>48.68-64.87</td>
</tr>
<tr>
<td>IL-8</td>
<td>-</td>
<td>-</td>
<td>4.23</td>
<td>3.53-4.64</td>
</tr>
<tr>
<td>IL-9</td>
<td>20.00</td>
<td>8.71-30.48</td>
<td>88.17</td>
<td>74.52-105.97</td>
</tr>
<tr>
<td>IL-10</td>
<td>3.69</td>
<td>2.18-7.60</td>
<td>11.46</td>
<td>9.90-12.67</td>
</tr>
<tr>
<td>IL-12</td>
<td>7.49</td>
<td>5.26-11.35</td>
<td>26.43</td>
<td>24.02-29.80</td>
</tr>
<tr>
<td>IL-15</td>
<td>-</td>
<td>-</td>
<td>23.77</td>
<td>21.00-27.86</td>
</tr>
<tr>
<td>IL-17</td>
<td>-</td>
<td>-</td>
<td>77.95</td>
<td>62.04-92.40</td>
</tr>
<tr>
<td>IP-10</td>
<td>510.1</td>
<td>344.7-694.9</td>
<td>252.5</td>
<td>166.1-344.8</td>
</tr>
<tr>
<td>Iron (µg/dl)</td>
<td>73.10</td>
<td>59.05-99.25</td>
<td>8.00</td>
<td>6.10-12.20</td>
</tr>
<tr>
<td>L-Ferritin (ng/ml)</td>
<td>72.33</td>
<td>29.74-140.60</td>
<td>1.28</td>
<td>0.80-2.57</td>
</tr>
<tr>
<td>MCP-1</td>
<td>93.70</td>
<td>66.53-145.95</td>
<td>137.0</td>
<td>113.0-145.95</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>10.12</td>
<td>8.65-11.76</td>
<td>8.61</td>
<td>7.89-9.44</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>47.86</td>
<td>33.59-64.57</td>
<td>17.38</td>
<td>15.07-20.08</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>349.3</td>
<td>177.1-605.8</td>
<td>34.98</td>
<td>20.66-47.82</td>
</tr>
<tr>
<td>Pro-Hep (ng/ml)</td>
<td>374.5</td>
<td>186.5-592.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RANTES</td>
<td>6510.7</td>
<td>4891.8-9300.7</td>
<td>10.09</td>
<td>8.77-12.15</td>
</tr>
<tr>
<td>TNFα</td>
<td>25.42</td>
<td>0.00-63.71</td>
<td>30.95</td>
<td>24.95-35.60</td>
</tr>
<tr>
<td>Transferrin (mg/L)</td>
<td>2364.3</td>
<td>1862.5-3627.3</td>
<td>25.60</td>
<td>10.10-36.08</td>
</tr>
<tr>
<td>TSAT (%)</td>
<td>23.72</td>
<td>12.67-30.47</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Units: all biomarkers are expressed in pg/ml unless otherwise specified.
3.4.3 Multivariable Models

The plasma duration model is presented in Figure 3-1, with regression coefficient confidence intervals, model selection iteration summaries and analysis of variance tables presented in Table 3-3. The predictive model achieved an R-squared = 0.769 and took the form:

disease duration = 0.128 [IP-10] + 12.825 [IL-10] – 22.472 [IL-1β] – 0.248 [IL-1RA] – 5.467 [IL-12] – 0.00463 [RANTES] + .380 [Eotaxin] + 114.654. The agreement between actual and predicted total duration was categorized, with predictions more than 50% above or 33% below actual (a geometrically symmetric error range) classified as “high” or “low” predictions, respectively, and those within this range classified as a “match”. Prediction matched actual in 14 cases, was high in 9 cases, and was low in 6 cases.
Figure 3-1

Total Disease Duration - Plasma Model

- **Category**
  - Match
  - High
  - Low
  - 95% CI

- Duration 50% above actual
- Duration 33% below actual

R-squared = 0.769
Figure 3-1. Multivariable modeling and goodness-of-fit validation results using plasma biomarkers. Dash-dotted lines delimit predictions that are 50% above or 33% below actual total disease duration. Dots represent predictions that fall within this range (“match”), up-arrowheads predictions that are above this range (“high”), and down-arrowheads predictions that are below this range (“low”). Shaded bands represents prediction limit 95% confidence interval (CI).
Table 3-3. Plasma Model Statistics

Regression Coefficients

<table>
<thead>
<tr>
<th>Independent Variable</th>
<th>Regression Coefficient</th>
<th>Standard Error</th>
<th>Lower 95% C.L.</th>
<th>Upper 95% C.L.</th>
<th>Standardized Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>114.6544</td>
<td>22.1257</td>
<td>68.6415</td>
<td>160.6673</td>
<td>0.0000</td>
</tr>
<tr>
<td>IP-10</td>
<td>0.1275</td>
<td>0.0205</td>
<td>0.0848</td>
<td>0.1703</td>
<td>0.8008</td>
</tr>
<tr>
<td>IL-10</td>
<td>12.8249</td>
<td>2.5339</td>
<td>7.5553</td>
<td>18.0945</td>
<td>1.1556</td>
</tr>
<tr>
<td>IL-1β</td>
<td>-22.4724</td>
<td>5.0494</td>
<td>-32.9732</td>
<td>-11.9716</td>
<td>-0.5738</td>
</tr>
<tr>
<td>IL-1RA</td>
<td>-0.2483</td>
<td>0.0650</td>
<td>-0.3835</td>
<td>-0.1132</td>
<td>-0.6093</td>
</tr>
<tr>
<td>IL-12</td>
<td>-5.4671</td>
<td>1.4422</td>
<td>-8.4664</td>
<td>-2.4679</td>
<td>-0.7316</td>
</tr>
<tr>
<td>RANTES</td>
<td>-0.0046</td>
<td>0.0014</td>
<td>-0.0075</td>
<td>-0.0018</td>
<td>-0.4280</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>0.3799</td>
<td>0.1625</td>
<td>0.0419</td>
<td>0.7178</td>
<td>0.3274</td>
</tr>
</tbody>
</table>

Note: The T-value used to calculate these confidence limits was 2.080

Subset Selection Detail

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
<th>No. of Terms</th>
<th>No. of X’s</th>
<th>R-squared</th>
<th>Term Entered</th>
<th>Term Removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Add</td>
<td>0</td>
<td>0</td>
<td>0.0000</td>
<td>Intercept</td>
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<tr>
<td>1</td>
<td>Add</td>
<td>1</td>
<td>1</td>
<td>0.1963</td>
<td>IP-10</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Add</td>
<td>2</td>
<td>2</td>
<td>0.3398</td>
<td>Onset-Age</td>
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</tr>
<tr>
<td>3</td>
<td>Add</td>
<td>3</td>
<td>3</td>
<td>0.3800</td>
<td>MIP-1β</td>
<td></td>
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<tr>
<td>4</td>
<td>Add</td>
<td>4</td>
<td>4</td>
<td>0.4369</td>
<td>IL-1RA</td>
<td></td>
</tr>
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<td>5</td>
<td>Switch</td>
<td>4</td>
<td>4</td>
<td>0.4682</td>
<td>IL-10</td>
<td>MIP-1β</td>
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<td>6</td>
<td>Switch</td>
<td>4</td>
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<td>Onset-Age</td>
</tr>
<tr>
<td>7</td>
<td>Switch</td>
<td>4</td>
<td>4</td>
<td>0.4799</td>
<td>IL-1β</td>
<td>IL-12</td>
</tr>
<tr>
<td>8</td>
<td>Add</td>
<td>5</td>
<td>5</td>
<td>0.5931</td>
<td>IL-12</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Add</td>
<td>6</td>
<td>6</td>
<td>0.7089</td>
<td>RANTES</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Add</td>
<td>7</td>
<td>7</td>
<td>0.7690</td>
<td>Eotaxin</td>
<td></td>
</tr>
</tbody>
</table>

Analysis of Variance Detail

<table>
<thead>
<tr>
<th>Model Term</th>
<th>DF</th>
<th>R-squared</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F-Ratio</th>
<th>Probability Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
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<td>77687.62</td>
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<td>0.0000</td>
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<tr>
<td>Model</td>
<td>7</td>
<td>0.7690</td>
<td>38540.38</td>
<td>5505.77</td>
<td>9.988</td>
<td>0.0000</td>
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<tr>
<td>IP-10</td>
<td>1</td>
<td>0.4237</td>
<td>21232.60</td>
<td>21232.60</td>
<td>38.516</td>
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<tr>
<td>IL-10</td>
<td>1</td>
<td>0.2818</td>
<td>14121.39</td>
<td>14121.39</td>
<td>25.617</td>
<td>0.0001</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1</td>
<td>0.2179</td>
<td>10918.77</td>
<td>10918.77</td>
<td>19.807</td>
<td>0.0002</td>
</tr>
<tr>
<td>IL-1RA</td>
<td>1</td>
<td>0.1607</td>
<td>8051.93</td>
<td>8051.93</td>
<td>14.606</td>
<td>0.0010</td>
</tr>
<tr>
<td>IL-12</td>
<td>1</td>
<td>0.1581</td>
<td>7921.76</td>
<td>7921.76</td>
<td>14.370</td>
<td>0.0011</td>
</tr>
<tr>
<td>RANTES</td>
<td>1</td>
<td>0.1289</td>
<td>6457.63</td>
<td>6457.63</td>
<td>11.714</td>
<td>0.0026</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>1</td>
<td>0.0601</td>
<td>3012.50</td>
<td>3012.50</td>
<td>5.465</td>
<td>0.0294</td>
</tr>
<tr>
<td>Error</td>
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<td>0.2310</td>
<td>11576.49</td>
<td>551.26</td>
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</tr>
<tr>
<td>Total (Adjusted)</td>
<td>28</td>
<td>1.0000</td>
<td>50116.87</td>
<td>1789.89</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The CSF duration model is presented in Figure 3-2, with additional statistical information presented in Table 3-4. The predictive model achieved an R-squared = 0.617 and took the form:

Figure 3-2

Total Disease Duration - CSF Model

- Category: Match, High, Low
- 95% CI
- Duration 50% above actual
- Duration 33% below actual

Predicted Duration (Months)

Actual Duration (Months)

R-squared = 0.617
Figure 3-2. Multivariable modeling and goodness-of-fit validation results using CSF biomarkers. Dash-dotted lines delimit predictions that are 50% above or 33% below actual total disease duration. Dots represent predictions that fall within this range (“match”), up-arrowheads predictions that are above this range (“high”), and down-arrowheads predictions that are below this range (“low”). Shaded bands represents prediction limit 95% confidence interval (CI).
Table 3-4. CSF Model Statistics

<table>
<thead>
<tr>
<th>Independent Variable</th>
<th>Regression Coefficient</th>
<th>Standard Error</th>
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<th>Upper 95% C.L.</th>
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<td>1.1856</td>
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Note: The T-value used to calculate these confidence limits was 2.056

Subset Selection Detail

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Analysis of Variance Detail

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<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F-Ratio</th>
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The combined plasma and CSF duration model is presented in Figure 3-3, with additional statistical information presented in Table 3-5. The predictive model achieved an R-squared = 0.962 and took the form: total disease duration = 0.1323 [Plasma IP-10] – 18.004 [CSF IL-8] + 10.871 [Plasma IL-5] + 0.338 [Plasma L-Ferritin] + 0.176 [CSF MCP-1] – 7.480 [Plasma / CSF IFN-γ] – 12.058. After categorization as above, prediction matched actual in 16 cases, was high in 1 case, and was low in 1 case.
Figure 3-3

Total Disease Duration - CSF & Plasma Model

Duration 50% above actual
Duration 33% below actual

R-squared = 0.962
Figure 3-3. Multivariable modeling and goodness-of-fit validation results using combined plasma and CSF biomarkers as well as plasma-to-CSF ratios. Dash-dotted lines delimit predictions that are 50% above or 33% below actual total disease duration. Dots represent predictions that fall within this range ("match"), up-arrowheads predictions that are above this range ("high"), and down-arrowheads predictions that are below this range ("low"). Shaded band represents prediction limit 95% CI.
Table 3-5. CSF & Plasma Model Statistics

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<tr>
<th>Independent Variable</th>
<th>Regression Coefficient</th>
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Note: The T-value used to calculate these confidence limits was 2.201

Subset Selection Detail

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Analysis of Variance Detail

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<th>DF</th>
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<th>Sum of Squares</th>
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Actual and predicted disease duration were classified into prognostic categories using a separate scheme according to the following criteria: “rapid” progression – disease duration less than two years; “average” progression – disease duration two to four years; “slow” progression – disease duration more than four years. Agreement was measured using weighted Cohen’s Kappa statistics on the resultant contingency tables (Table 3-6). The combined plasma and CSF model resulted in agreement between actual and the predicted prognostic categories in 17 of 18 patients. Actual and predicted prognoses achieved Kappa = 0.526 for the plasma model, Kappa = 0.515 for the CSF model, and Kappa = 0.930 for the combined plasma and CSF model.
Table 3-6. Comparison of Actual and Predicted Prognostic Categories

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3.5 Discussion

This study employed a novel modeling strategy to predict ALS prognosis using panels of plasma and CSF biomarkers, with two significant findings. First, the methods identified several biomarkers with predictive value that are biologically relevant to ALS, including inflammatory cytokines, growth factors and markers of iron metabolism, suggesting new directions for research on disease pathophysiology. Second, multivariable modeling techniques serve as proof-of-concept for a novel strategy aimed at predicting prognosis. Mathematically, the models incorporated a manageable number of predictive factors to achieve reasonable goodness-of-fit. Although the particular models obtained in this study may not be generalizable to larger cohorts, the results argue for the utility of multivariable modeling in biomarker-based ALS research.

Plasma models had reasonable ability to predict total disease duration using seven biomarkers; in order of descending predictive value (as measured by R-squared) these were IP-10, IL-10, IL-1β, IL-1RA, IL-12, RANTES and eotaxin. IL-1β and IL-12 predict shorter disease duration in the model. These cytokines are secreted by activated macrophages to stimulate T-cell-based inflammatory responses, and may reflect deleterious chronic inflammation. In contrast, IL-10 predicts longer disease duration. This immunoregulator mediates a number of anti-inflammatory effects, via suppression of macrophages and antigen-presenting cells, inhibition of several inflammatory cytokines including IL-1β and IL-12, and prevention of overwhelming immune responses leading to tissue damage (39). These results suggest that, in periphery, a lower level of inflammation is associated with longer disease duration. Although speculative, the plasma cytokine profile is suggestive of M2 versus M1 macrophage activation (40). In this context, the inverse relationship between IL-1RA, which inhibits the effects of IL-1β, and total disease duration suggests IL-1RA levels may be more important as a marker of
systemic inflammation than a direct indicator of an anti-inflammatory response. Moreover, the expression of IL-1RA may indicate chronic inflammation in ALS. We also show that RANTES, which has been previously associated with ALS (41) and is a chemotactic molecule for T-cells, eosinophils and basophils, predicts shorter disease duration.

The CSF model incorporated six biomarkers; in order of descending predictive value these were IL-9, age of onset, IL-5, IL-12, MIP-1β and G-CSF. Consistent with clinical observations, increasing age of onset predicts shorter disease duration, arguing for the validity of results. As in plasma, IL-12 predicts shorter disease duration, supporting the argument for a negative impact of inflammation in both the central nervous system (CNS) and periphery on disease duration. Levels of G-CSF, a growth factor previously shown to have neuroprotective effects on motor neurons in ALS (17), predicts longer disease duration in the CSF model. MIP-1β, a chemoattractant for macrophages and microglia, was also a positive predictor in CSF. These results support the concept that balanced immune modulation in CNS, possibly mediated by distinct classes of immune regulators (such as M2 versus M1 macrophages), is required to prevent neurotoxicity (40, 42). IL-9 and IL-5 in CSF, together with eotaxin in plasma, mediate eosinophil-based immune responses and may be connected to findings suggesting elevated eosinophil-derived neurotoxin levels in CSF of ALS patients (43).

The combined plasma and CSF model, which achieved high R-squared and Cohen’s Kappa, was based on six biomarkers; in order of descending predictive value these were plasma IP-10, CSF IL-8, plasma IL-5, plasma L-ferritin, CSF MCP-1, and the ratio of plasma-to-CSF IFN-γ. Plasma L-ferritin predicts longer disease duration in the model, implicating iron status in ALS disease course. Other markers of iron metabolism were not significant. A low plasma-to-CSF IFN-γ ratio predicts longer disease duration, whereas absolute levels did not impact disease
duration. This may reflect a situation in which low levels of systemic inflammation, coupled with moderate levels of CNS immune activation, promotes neuroprotection. The positive correlation between plasma IP-10 and total disease duration suggests that a specific induction of this cytokine by IFN-γ, versus the direct pro-inflammatory effects of IFN-γ, is beneficial. In CSF, levels of IL-8, a pro-inflammatory cytokine that activates neutrophils, predicts shorter duration, whereas MCP-1, which attracts monocytes, predicts longer duration. This may again implicate differing immune cells, or even subclasses of the same cell, in CNS immune modulation that affords neuroprotection. Results also suggest that a combination of plasma and CSF biomarkers have greater predictive power than their levels in plasma or CSF alone.

With regard to prediction accuracy, R-squared best reflects goodness-of-fit; however, it is not likely to be immediately intuitive to the clinician. Classification by error bands best accounts for continuous error between actual and predicted duration. Less error is acceptable when durations are short versus when they are long: a prediction error of 6 months is unacceptable if the actual duration is 2 months; however, it is reasonable if the actual duration is 10 years. However, this method allows greater error with longer duration times. Classification by prognostic categories is potentially the most clinically relevant. However, this method is artificially stringent at the boundaries between categories: an overestimate of only 2 months when the actual duration is 23 months yields a mismatch, for example. Practical application of the results requires attention to these strengths and limitations.

This study provides proof-of-concept for a novel multivariable modeling strategy to predict ALS prognosis. Statistical methods used relatively simple models and coefficients because it was assumed that, all else being equal, less complex formulae requiring fewer independent factors provide more valid predictions. Models only incorporated main effects and
basic interaction terms (biomarker plasma-to-CSF ratios), maximizing the generalizability of equations by decreasing the potential for detecting false-positive higher-order relationships.

A limitation of this study is its cross-sectional nature, utilizing samples obtained at a single point in time for each patient. It is possible that levels of biomarkers change both in absolute value and relative to one another during disease course. Better knowledge of these variations may improve the precision of models and elucidate underlying disease mechanisms. A longitudinal follow-up study utilizing measurements of biomarkers at more than one point in patients’ disease trajectories is planned.

The small sample sizes precluded independent discovery and validation cohorts for predictive modeling. This is another limitation of this study, potentially resulting in artificially inflated goodness-of-fit. However, the present results provide a compelling starting point for the use of this methodology in larger cohorts. More generally, survival time prediction using statistical models suffers from inherent shortcomings independent of any specific strategy. Individual variations in survival times are large enough that the best clinical models provide only approximate indications of prognosis. Point predictions are most error-prone, with rates of “serious error” (predictions less than half or more than twice of the actual values) often exceeding 50% (44). Predictions using prognostic categories (Table 3-6) may be more appropriate.

The present results suggest multivariable models incorporating plasma biomarker panels may have prognostic value in ALS. Future studies should employ unbiased discovery methodologies in large patient cohorts, with detailed longitudinal follow-up.
3.6 Acknowledgment

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3.7 References


Chapter 4

Serum Ferritin is Elevated in Amyotrophic Lateral Sclerosis Patients

4.1 Abstract

**Objective:** To measure serum ferritin, which reflects iron metabolism, in ALS patients versus healthy and disease controls, and determine if ferritin affects survival. **Methods:** We retrospectively analyzed data from 138 ALS patients, 152 healthy controls, and 82 disease controls. Gender, age, site of onset, and dates of symptom onset and death were recorded. Survival was defined as the time from symptom onset to death. Ferritin was measured using immunoassay. ANOVA and Pearson’s correlation was used to compare ferritin between groups and test the association between ferritin versus age and survival. Ferritin was categorized into high and low groups, and Kaplan-Meier analysis performed. **Results:** Age differed between ALS patients versus healthy and disease controls. However, age did not affect ferritin levels. Gender proportions differed between ALS patients versus healthy and disease controls, and gender affected serum ferritin levels. Ferritin comparisons were stratified for gender. In both men and women, ferritin was higher in ALS patients versus healthy and disease controls. However, ferritin did not affect survival in either gender, by tests of association or survival analysis. **Conclusions:** ALS patients have altered iron metabolism not simply due to the presence of neurological disease. Serum ferritin alone may be insufficient to predict survival.
4.2 Introduction

Chapter 3 introduced the concept of panels of protein biomarkers to aid in predicting amyotrophic lateral sclerosis prognosis. Inflammatory cytokines, growth factors, and markers of iron metabolism had predictive power (1). A biomarker of particular interest is ferritin, which is related to iron metabolism.

Dysregulated iron metabolism is a pathogenic pathway that has received attention in the study of amyotrophic lateral sclerosis (ALS) pathophysiology (2). HFE iron regulatory gene variants are present with an increased frequency in patients with ALS, and may also increase disease risk (3-8). Iron dyshomeostasis affects pathways implicated in ALS, including oxidative stress (9, 10). Increased serum levels of the iron storage protein ferritin are associated with ALS diagnosis (11, 12), accelerated disease progression (13), and decreased survival (14). Ferritin also may be a potential ALS biomarker, as levels of L-ferritin, which primarily stores iron and lacks ferroxidase activity, have been used successfully in both diagnostic (15) and prognostic models (1). Expression of mutant superoxide dismutase (SOD1), implicated in familial ALS, as well as overexpression of wild type SOD1, are known to alter iron metabolism and increase serum ferritin levels (16).

Despite evidence correlating ferritin with measures of ALS, including diagnosis, progression and survival, it remains uncertain whether elevated ferritin is specific to ALS, or indicative of neurological disease in general. Previous studies linking serum ferritin levels to ALS compared patients with ALS to healthy controls. We extend these studies by analyzing serum ferritin in patients with ALS, healthy controls, and patients with non-ALS neurological disease. We also determined whether ferritin correlates with survival in patients with ALS.
4.3 Patients and Methods

We retrospectively analyzed (IRB #20473) data from patients with possible, probable, laboratory-supported probable, or definite ALS (17); healthy controls; and patients with other neurological diseases (disease controls) seen at a university-based multidisciplinary ALS clinic or who provided samples to the Northeast ALS Consortium (NEALS) / Neurological Clinical Research Institute (NCRI) ALS Biofluid Repository (http://www.alsconsortium.org/neals_samples.php). Other neurological diseases were varied, and included chronic migraine, peripheral neuropathy, meningitis, dementia, Parkinson’s disease and Alzheimer’s disease. Gender, ethnicity and age were recorded for all subjects. For those with ALS, we also noted site of onset, pattern of ALS inheritance, time of patient-identified symptom onset and time of death. Total disease duration (survival) was defined as the time from patient-identified symptom onset to death. Familial cases were determined by family history. No testing for mutations known to be associated with ALS was performed. All patients provided informed consent.

Analysis of ferritin was performed in the clinical laboratory at Penn State Hershey Medical Center. In brief, ferritin was bound to biotinylated antibody, captured by streptavidin and detected using horseradish peroxidase-labeled monoclonal antibody using the Vitros 5600 automated immunoassay system (Ortho Clinical Diagnostics, Rochester, NY). Ferritin levels between groups were compared using one-way ANOVA. Pearson’s correlation was used to analyze the associations between ferritin versus age and ferritin versus survival. Ferritin levels were categorized into low (below the median) and high (equal to or above the median) groups and Kaplan-Meier survival analysis with logrank analysis was conducted. All tests were two-sided with significance set at the $p < 0.05$ level.
4.4 Results

Clinical variables from 138 patients with ALS, 152 healthy controls and 82 disease controls were obtained. The majority of subjects were white. Gender proportions were different between groups, and thus subsequent results were stratified by gender. Age at serum sample collection was different between groups; however, Pearson’s correlation test indicated no association between serum ferritin and age in this population. Thus results were not stratified by age (Table 4-1). The vast majority of patients with ALS were classified as having sporadic ALS. Clinical characteristics for patients with ALS are presented in Table 4-2.

Serum ferritin levels were elevated in men (Figure 4-1A) and women (Figure 4-1B) with ALS versus healthy controls or disease controls. Kaplan-Meier survival analysis, stratified by gender, was performed. Serum ferritin category did not affect survival in either gender (Figure 4-2). Pearson’s correlation using raw values also did not indicate an association between ferritin levels and survival in either gender (Figure 4-3).
Table 4-1. Clinical Characteristics of All Study Subjects

<table>
<thead>
<tr>
<th>Clinical variable</th>
<th>ALS (N=138)</th>
<th>Healthy Controls (N=152)</th>
<th>Disease Controls (N=82)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, number of men (% men)</td>
<td>87 (63.0)</td>
<td>49 (32.2)</td>
<td>36 (43.9)</td>
</tr>
<tr>
<td>Ethnicity, number non-white (% non-white)</td>
<td>1 (0.7)</td>
<td>5 (3.2)</td>
<td>2 (2.4)</td>
</tr>
<tr>
<td>Age at sample (yrs.), med. (range)</td>
<td>62.1 (30.8-82.7)</td>
<td>39.9 (20.0-81.0)</td>
<td>54.5 (23.1-81.5)</td>
</tr>
</tbody>
</table>

Note: Age and ferritin values were not correlated (Pearson’s $r = 0.10$, $p$-value > 0.05)
Table 4-2. ALS Patient Characteristics

<table>
<thead>
<tr>
<th>Clinical variable</th>
<th>Men (N=87)</th>
<th>Women (N=51)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at onset (yrs.), med. (range)</td>
<td>58.2 (30.2-81.3)</td>
<td>60.4 (23.3-79.6)</td>
</tr>
<tr>
<td>Onset to sample time (mo.), med. (range)</td>
<td>23.6 (3.3-246.7)</td>
<td>20.5 (9.0-166.3)</td>
</tr>
<tr>
<td>ALS type, number familial, (% familial)</td>
<td>1 (1.1)</td>
<td>3 (5.9)</td>
</tr>
<tr>
<td>Site of onset, number bulbar, (% bulbar)</td>
<td>26 (29.9)</td>
<td>23 (45.1)</td>
</tr>
<tr>
<td>Total disease duration (mo.), med. (range)</td>
<td>45.3 (7.3-193.7)</td>
<td>35.2 (18.6-206.3)</td>
</tr>
</tbody>
</table>
Figure 4-1

A) Serum Ferritin (Men)

B) Serum Ferritin (Women)
Figure 4-1. Serum ferritin is elevated in patients with ALS. A) Men with ALS have higher levels of serum ferritin (mean 286.6 ng/ml) versus healthy controls (mean 160.8 ng/ml, \( p < 0.001 \)) or disease controls (mean 164.5 ng/ml, \( p = 0.003 \)). B) Women with ALS have higher levels of serum ferritin (mean 142.6 ng/ml) versus healthy controls (mean 69.3 ng/ml, \( p < 0.001 \)) or disease controls (mean 77.5 ng/ml, \( p < 0.001 \)). Box plot with interquartile range (left), dot plot (middle) and bar graph with means showing standard error of the mean (right) depicted for each group.
Figure 4-2

A) Survival (Men)

B) Survival (Women)

Serum Ferritin
- Low
- High
Figure 4-2. Serum ferritin categories do not affect survival in patients with ALS. A) Men with serum ferritin levels below the median value (202.0 ng/ml) have similar survival to those with levels equal to or above the median value (median 45.3 versus 44.0 months, \( p = 0.778 \)). B) Women with serum ferritin levels below the median value (123.0 ng/ml) have similar survival to those with levels equal to or above the median value (median 30.4 versus 36.0 months, \( p = 0.360 \)).
Figure 4-3

A) Ferritin vs. Survival (Men)

B) Ferritin vs. Survival (Women)
Figure 4-3. Serum ferritin levels are not associated with survival in patients with ALS. Pearson’s correlation indicates no association between serum ferritin levels and survival in men (A; Pearson’s $r = 0.011$, $p = 0.933$) or women (B; Pearson’s $r = 0.197$, $p = 0.237$).
4.5 Discussion

This study found that serum ferritin in patients with ALS was elevated versus healthy controls, consistent with previous research (11, 12, 14); as well as versus patients with other neurological diseases, suggesting the effect is not simply due to neurological disease. In the circulation, ferritin is an acute phase reactant that reflects systemic inflammation (18), as well as a well-studied indicator of stored iron (19). Previous research demonstrated levels of C-reactive protein and other acute phase reactants were not correlated with serum ferritin in patients with ALS (12), suggesting that ferritin was specific to ALS rather than being indicative of an inflammatory reaction associated with neurological disease in general. Our results provide stronger evidence for a specific role of elevated serum ferritin in ALS versus other neurological diseases; mean ferritin levels in patients with ALS were approximately twice those found in both healthy and disease controls.

Although our data do not investigate a direct relationship between the serum biomarker and the biology underlying the disease, the relationship of ferritin to iron status is well established. Iron dyshomeostasis induces oxidative stress by generating reactive oxygen species via the Fenton reaction, and oxidative damage is a hallmark of ALS (20). Ferritin binds to and sequesters iron and other trivalent metals, preventing oxidative damage (21). In transgenic SOD1 G93A mice, expression of both H- and L-ferritin decreased iron catalyzed free radical formation, limiting oxidative damage to lipids, proteins, and nucleic acids possibly caused by mutant SOD1-induced mitochondrial dysfunction (22). These results suggest elevated serum ferritin may be an adaptive response to increased levels of oxidative stress in ALS. Intriguingly, multivariable prognostic models suggest higher levels of plasma L-ferritin predict longer disease duration in patients with ALS (1). Therefore, high levels of ferritin may indicate an enhanced
capacity to detoxify iron and prevent further oxidative damage, which may promote neuronal survival in response to oxidative stress.

Ferritin may also play a role in the complex inflammatory and immune responses seen in ALS. It has been suggested that a peripheral cytokine profile favoring M2 versus M1 macrophage activation is beneficial in ALS (1). M1 and M2 macrophages have distinct iron handling profiles, in which pro-inflammatory M1 macrophages have limited iron recycling capacity characterized by lower iron-responsive element-binding protein (IRP) activity; higher H-ferritin levels; lower transferrin receptor, ferroprotein, and hemeoxygenase-1 expression; a smaller labile iron pool; and limited iron internalization and release, whereas immunoregulatory M2 macrophages have effective iron recycling capacity characterized by the opposite trends in iron handling (23). Macrophages are a predominant source of ferritin, and specifically L-ferritin, in the blood (24). Evidence suggests that in the context of ALS, a deleterious M1 macrophage profile characterized by poor iron recycling is associated with low levels of ferritin release, whereas a beneficial M2 macrophage profile characterized by effective iron recycling is associated with higher release and elevated serum ferritin levels. In this framework, serum ferritin could be a marker of improved immunomodulation and decreased oxidative stress (22). Importantly, M2 macrophages are required for the repair of injured and deteriorating skeletal muscles, which requires iron for myofiber formation (25), a process highly relevant to ALS. In addition, microglia release of H-ferritin promotes myelinogenesis, which may be necessary for CNS tissue repair in ALS (26, 27).

Our second main finding is that serum ferritin levels were not associated with disease duration in patients with ALS. This is in contrast to previous reports suggesting higher ferritin levels correlated with accelerated disease progression (13) and decreased survival (14). Because
serum ferritin reflects body iron stores, and increased iron is believed to induce oxidative stress in ALS, it has been hypothesized that elevated ferritin correlates with ALS disease course because it reflects elevated levels of iron and glutathione peroxidase in lumbar spinal cord of diseased patients (28). However, body iron homeostasis is not a closed system; rather, it is significantly affected by environmental factors, including diet. For example, dysphagia affects up to 80% of all patients with ALS, and as a consequence a large number of patients receive enteral nutrition (29), which may exacerbate iron overload. The recommended daily iron intake for healthy individuals is 10 mg/day; however, the iron content of typical enteral formulas ranges from 13 to 24 mg/L, and most patients with ALS receiving home enteral nutrition consume at least one liter of polymeric formula per day (30). Studies evaluating the role of serum ferritin in ALS should consider enteral nutrition as a possible confound, one which may even worsen iron overload in select individuals with ALS; for example in the approximately 30% of patients with ALS that carry the HFE gene variant associated with elevated cellular iron uptake (3-7, 31). Although the percentage of patients receiving enteral nutrition and the iron content of enteral formulas were not reported in previous studies or available in our study, iron overload exacerbated by enteral nutrition may play a role in the divergent disease course results.

In addition to possible differences in enteral nutrition and iron content, Danzeisen et al. hypothesized that ALS cells have increased iron uptake due to alterations in the IRP/transferrin receptor system. In this framework, cells respond to the subsequent iron overload by increasing ferritin through an IRP-iron response element independent fashion, thereby sequestering and storing excess iron (16). This may account for increased ferritin levels in patients with ALS versus healthy or disease controls. If this ferritin-dependent iron buffering capacity is perturbed, perhaps in the setting of greater cellular damage corresponding to increased disease severity,
then ferritin levels may not adequately respond to excess iron, which would remain unbuffered and able to induce oxidative damage. This may be one reason why serum ferritin does not correlate with survival in our study.

There are a number of limitations to our study. Serum ferritin was the only marker of iron metabolism analyzed in this study. Other studies have demonstrated significant differences in transferrin and the transferrin saturation coefficient in patients with ALS versus healthy controls (14). However, elevated serum ferritin is the most robust finding across a number of studies (11, 12, 14), with conflicting results reported for other markers of iron metabolism, including transferrin and transferrin saturation (12). Thus, we chose to study serum ferritin. Our study was cross-sectional, and did not follow serum ferritin levels over time. This has been a limitation of all studies investigating ferritin levels in ALS, and ALS biomarkers research in general. Longitudinal analysis may uncover the temporal dynamics of ferritin in ALS, allowing more precise delineation of its role in pathophysiology. This is an especially important analysis if serum ferritin levels reflect microglia polarization status.

In summary, our finding of increased serum ferritin in patients with ALS may reflect iron overload, or, more specifically, upregulation of adaptive mechanisms for buffering iron and other trivalent trace metals (21). The lack of correlation between ferritin and survival in our study may reflect an environmental interaction influenced by body iron status, or a loss of ferritin-dependent iron buffering capacity seen with increasingly severe disease. Uncovering the precise role of ferritin in ALS, and specifically whether it is associated with improved or worsened outcomes, may improve biomarkers research. Ferritin is part of complex homeostatic pathways related to iron metabolism and immunomodulation. In ALS, it is likely that ferritin has additional functions beyond its role as an iron storage protein, and is more than a downstream indicator of
inflammatory status. Serum ferritin may reflect immunomodulation by classically or alternatively activated macrophages, which drives pathophysiological and neuroprotective processes.
4.6 Acknowledgment

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4.7 References


Chapter 5

HMG-CoA Reductase Inhibitors and HFE Polymorphism Accelerate Disease Progression and Shorten Survival in the SOD1 G93A ALS Mouse Model

5.1 Abstract

**Background:** Both HMG-CoA reductase inhibitors (statins) and the H63D polymorphism in the HFE iron regulatory gene reportedly impact ALS risk or disease progression. We hypothesized that statins accelerate disease progression and decrease survival in ALS mouse models; HFE genotype influences these effects; and statin-induced mitochondrial dysfunction mediates these effects. **Methods:** We analyzed double transgenic mice harboring SOD1 G93A and heterozygous for H67D HFE (homologous to human H63D HFE), their single transgenic SOD1 G93A or heterozygous H67D HFE littermates; as well as their WT littermates. For the survival study, animals were administered simvastatin or vehicle beginning at disease onset, and allowed to reach endstage. Disease progression was measured by grip strength. Plasma ferritin levels were measured by ELISA. Mitochondrial fractions from gastrocnemius muscle and lumbar spine were analyzed. For the mechanism study, animals were administered simvastatin in a similar fashion, gastrocnemius muscle and lumbar spine were collected at the symptomatic 120-day timepoint, and mitochondrial fractions analyzed. For the rescue study, animals were administered daily simvastatin, coenzyme Q10, or both, beginning at disease onset and allowed to reach endstage. **Results:** SOD1 mice had increased plasma ferritin levels compared to WT mice. Simvastatin administration and H67D HFE accelerated disease progression. Simvastatin administration adversely impacted survival. Coenzyme Q10 administration did not rescue the statin-induced decrease in survival in SOD1 or double transgenic mice. Statins did not alter
levels of mitochondrial proteins in lumbar spine or gastrocnemius muscle. **Conclusions:** Our results suggest statins accelerate disease progression and decrease survival in SOD1 mutant mice. H67D *HFE* worsens the statin effect on disease progression. Mitochondrial dysfunction does not mediate these effects. These findings suggest that patients with ALS receiving statins, especially those harboring H63D *HFE*, should be monitored for changes in disease progression. Studies of the effects of statins on disease trajectory in patients with ALS harboring H63D versus WT *HFE* may further guide clinician use of statins in patients with ALS.
5.2 Introduction

Extrinsic factors modifying amyotrophic lateral sclerosis (ALS) disease progression include pharmacotherapies, which often interact with genetic components. An example of such a pharmacotherapy that may be influenced by an individual’s genetic profile is HMG-CoA reductase inhibitors (statins), which may have different consequences in patients with ALS harboring H63D HFE. Statins may accelerate ALS disease progression (1, 2); and H63D HFE, which occurs in approximately 30% of all patients with ALS (3-9), may modify disease risk.

Statins decrease lipid levels and are widely prescribed to promote cardiovascular health. In the United States, approximately 25 million people currently take statins. New cholesterol management guidelines published in 2013 by the American College of Cardiology and the American Heart Association (10) would increase the number of people eligible for statin therapy from approximately 43 to 56 million (11). The incidence of cardiovascular disease is approximately 2,000 per 100,000 population per year between the ages of 50-70 (12), when ALS incidence is highest (13). Because many ALS patients receive or will receive statins, there is a need to investigate the effects that statins may have on ALS disease course.

There is increasing recognition of the effects of statins on ALS risk, disease progression, and survival (14). The data to date are inconclusive. Statins may induce muscle damage, including rhabdomyolysis, and are linked to an “ALS-like syndrome” (15), although causation has not been established for the latter cases. In patients with ALS, statins may accelerate disease progression as well as increase the frequency and severity of muscle cramping (1). Analysis of results from two clinical trials suggests greater functional decline in women with ALS taking statins (2). However, other studies have shown no association between statins and ALS risk (16, 17) or survival (18). Recent meta-analysis suggests that no strong association exists between
statins and accelerated ALS disease progression, but that there is insufficient evidence for
definitive recommendations regarding statin therapy in patients with ALS (14).

Existing analyses of statin-induced effects in ALS have not stratified patients based on
factors relevant to the mechanisms implicated in the adverse effects of statins. The H63D
polymorphism in the HFE iron regulatory gene, which has been linked to neurodegenerative
diseases (19), may modify or unmask statin-induced adverse effects in ALS. Disease pathways
implicated in ALS, including iron dyshomeostasis, oxidative damage, ER stress, mitochondrial
dysfunction, and cholesterol disruption are present in animal and cellular models of HFE
polymorphism (20-23). It is possible that this genotype induces sub-threshold vulnerability in
ALS patients, which, when combined with statin therapy, may trigger adverse effects that are
more pronounced than in those ALS patients without H63D HFE.

Mitochondrial dysfunction may contribute to the adverse effects of statins (24), and may
be a converging mechanism underlying the potential H63D HFE-induced exacerbation of these
effects. Statins decrease levels of coenzyme Q10, an essential redox component of the
mitochondrial electron transport chain (ETC) (25), and also decrease synthesis of heme A, which
impairs complex IV of the ETC (26). Statin-induced myotoxicity is linked to impaired
mitochondrial fatty acid oxidation (27). Both the prevalence of mutations linked to mitochondrial
dysfunction and biochemical abnormalities in mitochondrial metabolism are increased in patients
with statin-induced myopathies (28). A small cohort of patients were studied in whom statin-
induced symptoms were eventually diagnosed as being due to ALS. Discontinuation of statins
improved ALS symptoms whereas increased statin dosage or rechallenge exacerbated symptoms;
and coenzyme Q10 supplementation ameliorated symptoms (29). Separately, mitochondrial
dysfunction occurs in cellular models of H63D \textit{HFE} (22), suggesting a converging pathway through which statins and \textit{HFE} polymorphism modifies ALS.

Statin and \textit{HFE} polymorphism-induced mitochondrial dysfunction is relevant to ALS pathophysiology, because disrupted mitochondria are known to contribute to ALS (30). In humans, over-oxidized wild-type (WT) superoxide dismutase (SOD1) is linked to sporadic ALS and forms toxic complexes with mitochondrial Bcl-2 (31). Mutant SOD1 alters mitochondrial protein composition and trafficking (32), and impairs ETC electron transfer efficiency and complex IV-mediated respiration (33). Tar DNA-binding protein 43 (TDP-43) linked ALS is associated with mitochondrial aggregation and altered mitochondrial fusion and fission (34).

Analysis of statin-induced effects in animal models of ALS, with stratification for \textit{HFE} genotype, may clarify the adverse effects of a commonly prescribed pharmacotherapy in patients with ALS, and serve as a model system that can be used to investigate the mechanisms underlying such effects, particularly mitochondrial dysfunction. With these goals, we used double transgenic mice harboring SOD1 G93A and heterozygous for H67D \textit{HFE} (homologous to human H63D \textit{HFE}), to investigate the effects of statins on ALS disease progression and the mechanisms underlying these effects.
5.3 Materials and Methods

5.3.1 Animals and Drug Administration

SOD1 G93A male mice (strain B6SJL-Tg(SOD1*G93A)1Gur/J; stock #002726; Jackson Laboratory, Bar Harbor, ME) were crossed with female mice harboring WT HFE or female mice homozygous for H67D HFE, as previously described (20). The resultant male and female double transgenic mice carrying SOD1 G93A and heterozygous for H67D HFE; their single transgenic SOD1 G93A or heterozygous H67D HFE littermates; as well as their WT littermates, were used in subsequent experiments. Animals had ad libitum access to food and water and were housed under standard conditions. DNA was extracted from tail biopsies using DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA). SOD1 G93A genotyping was performed using polymerase chain reaction primers (PCR) amplifying a 236-bp DNA fragment harboring G93A mutation. The forward and reverse primers were 5’CATCAGCCCTAATCCATCTGA-3’ and 5’-CGCGACTAACAATCAAAGTGA-3’. H67D HFE genotyping used 5’-AGGACTCACTCTCTGCGCAGGAGGTAACCA-3’ as the forward and 5’-TTTCTTTTACAAAGCTATATCCCCAGGGT-3’ as the reverse primers, followed by BspHI restriction enzyme digestion for 2 hours at 37°C. DNA was visualized by agarose gel electrophoresis. Experiments adhered to the NIH Guide for the Care and Use of Laboratory Animals and were subject to Institutional Animal Care and Use Committee approval.

For the survival and mechanism studies, animals were administered simvastatin (Calbiochem, San Diego, CA) 2 mg/kg or vehicle daily via intraperitoneal (i.p.) injection starting at disease onset, defined below. Simvastatin was metabolically activated prior to administration. Briefly, 16 mg of simvastatin was dissolved in 400 µl ethanol. Then, 600 µl of 0.1 N sodium
hydroxide was added, and the solution incubated at 50°C for 2 hours. Next, 25 µl of 1.0 N hydrochloric acid was added to adjust the pH to 7.4. Finally, 3 ml of sterile saline was added to dilute the solution to 4 mg/ml simvastatin. Vehicle solution comprised 400 µl ethanol in 3.6 ml sterile saline. Stock simvastatin and vehicle solutions were stored at 4°C for a maximum of 2 weeks, and diluted at a 1:20 ratio in sterile saline prior to use. For the rescue study, animals were administered simvastatin 2 mg/kg, coenzyme Q10 (Sigma-Aldrich Corp., St. Louis, MO) 10 mg/kg, or a combination of simvastatin and coenzyme Q10, dissolved in 0.5% ethanol and 1% Tween 80 (Sigma-Aldrich Corp.) in sterile saline, daily via i.p. injection starting at disease onset.

5.3.2 Behavioral Paradigms and Survival

Beginning at 60 days of age, motor performance was assessed via rotarod (Columbus Instruments, Columbus, OH) twice a week. Disease onset was determined by measuring the amount of time a mouse remained on the rod rotating at 15 rpm prior to first fall, with a maximum test time of 180 seconds. Onset was defined as the age when a mouse failed to stay on the rod for more than one standard error of the mean (>1 SEM) below the mean time it remained on the rod during previous trials, which was defined as the presymptomatic phase.

Limb strength was assessed via gripstrength (Columbus Instruments) to determine disease progression. Mice were gently pulled horizontally by the base of the tail as they grasped a horizontal bar attached to the gripstrength meter with their hindlimbs or forelimbs. The maximum exerted force was recorded for each trial, with the value from three trials averaged for each animal. Gripstrength was measured once a week from 70 to 133 days.

For the mechanism study, animals were sacrificed at 120 days, the timepoint corresponding to the biggest differences in gripstrength between groups. For the survival and
rescue studies, animals were sacrificed at disease endstage, defined as the inability of an animal to right itself within 30 seconds of being placed on its side. Disease duration was defined as the time from disease onset to endstage.

5.3.3 Cholesterol Determination

Blood was collected from SOD1 and double transgenic mice at endstage by cardiac puncture. A WT or H67D HFE mouse was randomly yoked to each SOD1 or double transgenic mouse, and sacrificed with blood collection via cardiac puncture when the appropriate disease yoke reached endstage. Blood was centrifuged at 10,000 g for 15 minutes in the presence of heparin to isolate plasma. Cholesterol was assessed using the cholesterol esterase-linked Cholesterol Fluorometric Assay kit (Cayman Chemical, Ann Arbor, MI). Briefly, 50 µl of sample at a 1:400 dilution of plasma to buffer was added in duplicate to successive wells on a 96-well microplate. An equal volume of assay cocktail containing cholesterol oxidase, cholesterol esterase, horseradish peroxidase (HRP), cholesterol detector, and buffer was added, and the plate incubated for 30 minutes at 37°C away from light. Fluorescence was measured using a Spectra Max Gemini EM platereader (Molecular Devices, Sunnyvale, CA) with excitation at 540 nm and emission at 590 nm. Measurements were calibrated to known standards.

5.3.4 Ferritin Determination

Ferritin was assessed in plasma collected at endstage via enzyme-linked immunoassay (ELISA) using Mouse Ferritin ELISA kit (Immunology Consultants Laboratory, Portland, OR). Briefly, 100 µl of sample at a 1:40 dilution of plasma to buffer was added in duplicate to an anti-mouse ferritin coated microplate. The plate was incubated for 1 hour at room temperature (RT),
and wells were washed 4 times. Next, 100 µl of enzyme-antibody conjugate was added to each well, and the plate was incubated for 10 minutes at RT away from light. Wells were washed 4 times, 100 µl of chromogen-substrate solution was added, and the plate was incubated for 10 minutes at RT away from light. Next, 100 µl of stop solution was added, and absorbance measured using a Spectra Max 340 PC 384 platereader (Molecular Devices) at 450 nm. A 4-parameter logistic curve was created using known standards, and sample concentrations fitted using the curve.

5.3.5 Mitochondria Isolation

Lumbar spinal cord and gastrocnemius muscle were harvested, and mitochondria isolated via differential centrifugation using Mitochondria Isolation Kit for Tissue (Thermo Fisher Scientific, Waltham, MA). In brief, 130-170 mg of tissue was washed with phosphate buffered saline (PBS). Spinal cord tissue was transferred to a 2 ml homogenizer tube, whereas muscle tissue was first minced with a razor blade prior to being transferred to the homogenizer tube. Next, 800 µl of homogenization buffer supplemented with bovine serum albumin (BSA) was added, and the mixture homogenized using a Potter-Elvehjem tissue grinder (Wheaton Science Products, Millville, NJ) attached to an overhead stirrer. An equal volume of centrifugation buffer was added, and the solution centrifuged at 700 g for 10 minutes. Next, supernatant containing mitochondria and cytosol was removed and centrifuged at 3,000 g for 15 minutes. Supernatant containing the cytosolic fraction was removed, and the mitochondrial pellet was washed by centrifugation at 12,000 g for 5 min in 500 µl of centrifugation buffer. Next, each pellet was dissolved in 150 µl of 1% Triton-X in tris-buffered saline (TBS) to rupture mitochondrial membranes. The solution was centrifuged at 12,000 g for 2 minutes, and supernatant containing
solubilized mitochondrial proteins was stored at -80°C for downstream analysis. All samples and reagents were maintained at 4°C in the presence of protease inhibitor cocktail (Sigma-Aldrich Corp.) throughout the procedure to minimize protein degradation.

5.3.6 Protein Analysis

Western blots were performed to analyze mitochondrial protein levels. Briefly, solubilized mitochondrial proteins (10 µg total protein) were separated on 4-20% TGX polyacrylamide Tris-HCl gels (Bio-Rad, Hercules, CA) by electrophoresis and transferred at 4°C onto nitrocellulose membrane. Membranes were blocked with 5% nonfat dry milk in TBS with Tween 20 (TBS-T). Next, membranes were incubated with primary antibody overnight at 4°C followed by anti-host HRP-linked secondary antibody (Amersham Bioscience, Piscataway, NJ) for one hour. Primary antibodies comprised subunit NDUFS1 of complex I of the ETC (1:500 spinal cord, 1:1000 muscle; Abcam, Cambridge, MA), complex IV of the ETC (1:1000 spinal cord and muscle; Cell Signaling Technology, Danvers, MA), cytochrome c (1:250 spinal cord, 1:500 muscle; Abcam), voltage-dependent anion channel 1 (VDAC1) of the mitochondrial outer membrane (1:1000 spinal cord, 1:2000 muscle; Abcam), and translocase of the mitochondrial inner membrane subunit 23 (Tim23) (1:250 spinal cord and muscle; BD Biosciences, East Rutherford, NJ). Chemiluminescence was visualized with Enhanced Chemiluminescent System (Perkin Elmer, Waltham, MA) on a Fujifilm LAS-3000 (Fujifilm, Tokyo, Japan). Densitometry was performed using Multigauge 3.0 software (Fujifilm).
5.3.7 Statistical Analysis

Data were expressed as mean ± SEM. Kaplan-Meier survival curves and Cox proportional hazards regression were used to analyze survival. Gripstrength data for disease progression was analyzed with repeated measures two-way analysis of variance (ANOVA) with Tukey-Kramer post-hoc tests using mixed models. Analysis was conducted on data from disease relevant mice harboring SOD1 mutation over the period from 98 days, slightly after when the first SOD1 mice had disease onset, to 133 days, the last timepoint when SOD1 mice applied sufficient force for reliable readings. Experimental groups were compared by two-way ANOVA with Tukey-Kramer post-hoc tests. All tests were two-sided with significance set at the $P < 0.05$ level. SAS 9.3 (SAS Institute, Cary, NC) and NCSS 9 (NCSS LLC, Kaysville, UT) were used for statistical analyses.
5.4 Results

5.4.1 Plasma levels

WT mice administered simvastatin had decreased cholesterol versus WT mice administered vehicle. Both vehicle- and statin-treated SOD1 mice had similar, low cholesterol levels, possibly reflecting a floor effect (Figure 5-1A). SOD1 mice had increased plasma ferritin compared to WT mice, independent of statins (Figure 5-1B).
Figure 5-1

A) Cholesterol

- Group: Control (black) vs. Statin (gray)
- p < 0.01
- p < 0.05

B) Ferritin

- Group: Control (black) vs. Statin (gray)
- p < 0.001
**Figure 5-1.** Endstage plasma levels.  

A) Plasma cholesterol was lower in statin- vs. vehicle-treated mice (control group cholesterol = 2.14 ± 0.15 mM, statin group cholesterol = 1.61 ± 0.15 mM, n = 7-9 per group; F(1,31) = 4.34, p = 0.0456). Cholesterol was lower in SOD1 vs. WT mice (WT cholesterol = 2.16 ± 0.14 mM, SOD1 cholesterol = 1.52 ± 0.15 mM; F(1,31) = 8.66, p = 0.006). There was an interaction between genotype and statin treatment, with statins associated with a greater decrease in cholesterol in WT vs. SOD1 mice (WT control cholesterol = 2.58 ± 0.20 mM, WT statin cholesterol = 1.70 ± 0.21 mM, SOD1 control cholesterol = 1.52 ± 0.23 mM, SOD1 statin cholesterol = 1.52 ± 0.21 mM; F(1,31) = 4.26, p = 0.047; WT control vs. WT statin p = 0.021, WT control vs. SOD1 control and SOD1 statin p = 0.008 and 0.004, respectively).  

B) Plasma ferritin was increased in SOD1 vs. WT mice (WT ferritin = 25.7 ± 6.2 ng/ml, SOD1 ferritin = 65.7 ± 7.0 ng/ml, n = 7-9 per group; F(1,31) = 19.76, p < 0.001).
5.4.2 Disease progression and survival

In hindlimb, there was a main effect of treatment, with mice administered simvastatin having greater declines in grip strength versus mice administered vehicle. In hindlimb and forelimb, there were main effects of HFE genotype and time, with double transgenic mice harboring H67D HFE having greater declines in grip strength versus SOD1 mice, and grip strength declining with time. In hindlimb, there was also an interaction between HFE genotype and time, with SOD1 animals having a greater rate of decline in grip strength over time versus double transgenic mice. (Figure 5-2).
Figure 5-2

A) Hindlimb Grip Strength

B) Forelimb Grip Strength
Figure 5-2. Disease progression. A) In disease relevant mice harboring SOD1 mutation, there was decreased hindlimb gripstrength in statin- vs. vehicle-treated mice (n = 8-13 per group, F(1,38) = 11.44, p = 0.002); decreased hindlimb gripstrength in H67D HFE versus WT HFE mice (F(1,38) = 13.67, p < 0.001); and decreased hindlimb gripstrength over time (F(5,172) = 22.48, p < 0.001). There was an interaction between HFE status and time, with gripstrength decreasing faster over time in SOD1 mice vs. double transgenic mice (F(5,172) = 4.64, p < 0.001). B) In disease relevant mice harboring SOD1 mutation, there was a trend towards decreased forelimb gripstrength in statin- vs. vehicle-treated mice (n = 8-13 per group, F(1,38) = 3.83, p = 0.058); decreased forelimb gripstrength in H67D HFE versus WT HFE mice (F(1,38) = 11.32, p = 0.002); and decreased forelimb gripstrength over time (F(5,172) = 29.98, p < 0.001).
Statin administration adversely impacted survival in mice harboring an SOD1 mutation, whereas the presence of H67D HFE benefited survival. In SOD1 mice, there was an 11-day difference in median between vehicle- and statin-treated mice. This represents approximately 8% of the median total life span or 28% of average disease duration in these animals. In double transgenic animals, there was a 10 day difference in median survival between vehicle- and statin-treated mice. This represents approximately 7% of the median total life span or 26% of average disease duration (Figure 5-3). Coenzyme Q10 administration, alone or in combination with simvastatin, did not affect survival in SOD1 or double transgenic mice (Figure 5-4).
Figure 5-3

Statin Administration Survival Curve

- **Group**
  - SOD1 Control
  - SOD1 Statin
  - Double Control
  - Double Statin

- **Percent Survival (%)**
  - 100%
  - 90%
  - 80%
  - 70%
  - 60%
  - 50%
  - 40%
  - 30%
  - 20%
  - 10%
  - 0%

- **Survival Time (Days)**
  - 110
  - 120
  - 130
  - 140
  - 150
  - 160
Figure 5-3. Statin administration survival. Statins adversely impacted survival, whereas presence of H67D HFE benefited survival, in SOD1 mice (risk ratio statin versus control = 1.97, 95% confidence limit (CL) = 1.35-2.86, p < 0.001; risk ratio double transgenic versus SOD1 = 0.66, 95% CL = 0.48-0.93, p = 0.016; n = 8-13 per group; SOD1 control median survival = 143 days, SOD1 statin median survival = 132 days, double transgenic control median survival = 146 days, double transgenic statin median survival = 136 days).
Figure 5-4

Statin & Coenzyme Q10 Survival Curve

Percent Survival (%)

Survival Time (Days)
Figure 5-4. Statin and coenzyme Q10 co-administration survival. Coenzyme Q10 administration, alone or together with simvastatin, did not influence survival in SOD1 or double transgenic animals (SOD1 coenzyme Q10 median survival = 138 days, SOD1 statin median survival = 134 days, SOD1 combination median survival = 134 days, double transgenic statin median survival = 138 days, double transgenic combination median survival = 137 days, n = 8-9 per group).
5.4.3 Mitochondrial proteins

In lumbar spine at 120-days, mice administered simvastatin had increased complex I of the ETC versus mice administered vehicle. However, the statin effect was similar in WT versus SOD1 animals (Figure 5-5A). SOD1 mice had decreased levels of complex IV of the ETC and cytochrome c versus WT mice (Figure 5-5B). Statin administration was associated with decreased cytochrome c levels in WT mice, whereas vehicle- and statin-treated SOD1 mice had similar, low levels of cytochrome c (Figure 5-5C). SOD1 mice had increased levels of VDAC1 versus WT mice. Mice administered statin had increased VDAC1 levels versus mice administered vehicle; however, the statin effect was similar in WT versus SOD1 animals (Figure 5-5D).
Figure 5-5. 120-day lumbar spine mitochondrial protein levels. A) Complex I was increased in mice treated with statins vs. control; however, statins induced similar effects in WT vs. SOD1 animals (control complex I = 1.01 ± 0.10, SOD1 complex I = 1.29 ± 0.09 ratio to WT control, n = 9-10 per group; F(1,35) = 4.20, p = 0.048). B) Complex IV was decreased in SOD1 vs. WT mice (WT complex IV = 1.07 ± 0.05, SOD1 complex IV = 0.83 ± 0.05 ratio to WT control; F(1,35) = 10.58, p = 0.003). C) Cytochrome c was decreased in SOD1 vs. WT mice (WT cytochrome c = 0.89 ± 0.03, SOD1 cytochrome c = 0.39 ± 0.03 ratio to WT control; F(1,35) = 107.28, p < 0.001). There was an interaction between genotype and statin administration, with statins associated with greater decreases in cytochrome c in WT vs. SOD1 mice (WT control cytochrome c = 1.00 ± 0.05, WT statin cytochrome c = 0.78 ± 0.05, SOD1 control cytochrome c = 0.36 ± 0.05, SOD1 statin cytochrome c = 0.41 ± 0.05 ratio to WT control; F(1,35) = 7.76, p = 0.009; WT control vs. WT statin p = 0.013, WT control vs. SOD1 control and SOD1 statin p < 0.001, WT statin vs. SOD1 control and SOD1 statin p < 0.001). D) VDAC1 was increased in SOD1 vs. WT mice (WT VDAC1 = 1.17 ± 0.07, SOD1 VDAC1 = 1.39 ± 0.07 ratio to WT control; F(1,35) = 4.53, p = 0.040). VDAC1 was increased in statin- vs. vehicle-treated mice; however statins induced similar effects in WT vs. SOD1 mice (control VDAC1 = 1.15 ± 0.07, statin VDAC1 = 1.41 ± 0.07 ratio to WT control; F(1,35) = 6.19, p = 0.178). Representative blots showing protein(s) of interest and Tim23 loading control shown above respective bar charts.
In gastrocnemius muscle at 120-days, SOD1 mice had decreased levels of complex I and IV of the ETC, increased levels of cytochrome c, and decreased levels of VDAC1 compared to WT mice. Simvastatin administration did not affect mitochondrial protein levels in gastrocnemius muscle at this timepoint (Figure 5-6).
Figure 5-6. 120-day gastrocnemius muscle mitochondrial protein levels. A) Complex I was decreased in SOD1 vs. WT mice (WT complex I = 1.00 ± 0.04, SOD1 complex I = 0.71 ± 0.04 ratio to WT control, n = 9-10 per group; F(1,35) = 21.88, p < 0.001). B) Complex IV was decreased in SOD1 vs. WT mice (WT complex IV = 1.03 ± 0.04, SOD1 complex IV = 0.85 ± 0.04 ratio to WT control; F(1,35) = 9.57, p = 0.004). C) Cytochrome c was increased in SOD1 vs. WT mice (WT cytochrome c = 0.84 ± 0.36, SOD1 cytochrome c = 2.94 ± 0.37 ratio to WT control; F(1,35) = 16.10, p < 0.001). D) VDAC1 was decreased in SOD1 vs. WT mice (WT VDAC1 = 1.02 ± 0.03, SOD1 VDAC1 = 0.72 ± 0.03 ratio to WT control; F(1,35) = 47.81, p < 0.001). Representative blots showing protein(s) of interest and Tim23 loading control shown above respective bar charts.
At endstage in lumbar spine, SOD1 mice had lower levels of complex I of the ETC compared to WT mice. WT mice administered simvastatin had increased complex I compared to WT mice administered vehicle, whereas vehicle- and statin-treated SOD1 mice had similar, low levels of complex I (Figure 5-7A). SOD1 mice had increased levels of complex IV of the ETC compared to WT mice. Mice administered statin had increased complex IV levels versus mice administered vehicle; however, the statin effect was similar in WT versus SOD1 mice (Figure 5-7B). SOD1 mice had decreased cytochrome c levels versus WT mice. WT mice administered statins had decreased cytochrome c versus WT mice administered vehicle, whereas vehicle- and statin-treated SOD1 had similar, low levels of cytochrome c (Figure 5-7C). Neither genotype nor statin administration affected VDAC1 levels (Figure 5-7D).
Figure 5-7

A) Complex I

B) Complex IV

C) Cytochrome C

D) VDAC1
**Figure 5-7.** Endstage lumbar spine mitochondrial protein levels. **A)** Complex I was decreased in SOD1 vs. WT mice (WT complex I = 1.18 ± 0.05, SOD1 complex I = 0.86 ± 0.05 ratio to WT control, n = 8-9 per group; F(1,31) = 23.37, p < 0.001). There was an interaction between genotype and statin treatment, with statins associated with increased complex I in WT mice and no change in SOD1 mice (WT control = 1.00 ± 0.07, WT statin = 1.36 ± 0.07, SOD1 control = 0.92 ± 0.07, SOD1 statin = 0.80 ± 0.07 ratio to WT control; F(1,31) = 13.34, p < 0.001; WT control vs. WT statin p = 0.002). **B)** Complex IV was decreased in SOD1 vs. WT mice (WT complex IV = 1.09 ± 0.04, SOD1 complex IV = 0.72 ± 0.04 ratio to WT control; F(1,31) = 50.14, p < 0.001). Complex IV was increased in statin- vs. vehicle-treated mice; however statins induced similar effects in WT vs. SOD1 mice (control complex IV = 0.85 ± 0.04, statin complex IV = 0.97 ± 0.04 ratio to WT control; F(1,31) = 5.86, p = 0.022). **C)** Cytochrome c was decreased in SOD1 vs. WT mice (WT cytochrome c = 0.93 ± 0.02, SOD1 cytochrome c = 0.38 ± 0.03 ratio to WT control; F(1,31) = 236.34, p < 0.001). There was an interaction between genotype and statin treatment, with statins associated with decreased cytochrome c in WT mice and no change in SOD1 mice (WT control cytochrome c = 1.00 ± 0.04, WT statin cytochrome c = 0.86 ± 0.04, SOD1 control = 0.33 ± 0.04, SOD1 statin = 0.43 ± 0.04 ratio to WT control; F(1,31) = 11.87, p = 0.002; WT control vs. WT statin p = 0.038). **D)** There was no effect of genotype or statin treatment on VDAC1. Representative blots showing protein(s) of interest and Tim23 loading control shown above respective bar charts.
At endstage in gastrocnemius muscle, SOD1 mice had decreased levels of complex I and IV of the ETC, cytochrome c, and VDAC1 compared to WT mice. Simvastatin administration did not affect mitochondrial protein levels in gastrocnemius muscle at this timepoint (Figure 5-8).
Figure 5-8

A) Complex I

B) Complex IV

C) Cytochrome C

D) VDAC1
Figure 5-8. Endstage gastrocnemius muscle mitochondrial protein levels. A) Complex I was decreased in SOD1 vs. WT mice (WT complex I = 1.10 ± 0.04, SOD1 complex I = 0.81 ± 0.04 ratio to WT control, n = 8-9 per group; F(1,31) = 24.57, p < 0.001). B) Complex IV was decreased in SOD1 vs. WT mice (WT complex IV = 1.08 ± 0.03, SOD1 complex IV = 0.76 ± 0.03 ratio to WT control; F(1,31) = 44.97, p < 0.001). C) Cytochrome c was decreased in SOD1 vs. WT mice (WT cytochrome c = 0.81 ± 0.08, SOD1 cytochrome c = 0.41 ± 0.08 ratio to WT control; F(1,31) = 11.28, p = 0.002). There was an interaction between genotype and statin treatment, with statins associated with a trend towards decreased cytochrome c in WT mice and a trend towards increase in SOD1 mice (WT control cytochrome c = 1.00 ± 0.11, WT statin cytochrome c = 0.60 ± 0.12, SOD1 control cytochrome c = 0.32 ± 0.12, SOD1 statin cytochrome c = 0.50 ± 0.12 ratio to WT control; F(1,31) = 6.30, p = 0.018; WT control vs. SOD1 control p = 0.001, WT control vs. SOD1 statin = 0.022). D) VDAC1 was decreased in SOD1 vs. WT mice (WT VDAC1 = 1.01 ± 0.06, SOD1 VDAC1 = 0.62 ± 0.06 ratio to WT control; F(1,31) = 20.74, p < 0.001). Representative blots showing protein(s) of interest and Tim23 loading control shown above respective bar charts.
5.5 Discussion

Our first finding suggested statins accelerated disease progression and decreased survival in both SOD1 and double transgenic mice. Our results represent the first study of statin-induced adverse effects in SOD1 mice, as well as SOD1 mice with H63D HFE polymorphism, and are consistent with reports of accelerated disease progression in patients with ALS (1, 2). In addition to providing further support for the need of larger epidemiological studies of the safety of statins in the ALS patient population, our study establishes a model that may be used to analyze mechanisms underlying statin-induced adverse events in ALS, as well as HFE genotype-dependent effects.

We began statin administration at disease onset, rather than at a presymptomatic age, for two reasons related to clinical relevance. First, the clinical effects of statins on disease progression are better supported by the literature than those on disease risk (1, 2), making analysis of progression and survival in animal models more clinically relevant. Second, even if statins were determined to have a large, detrimental impact to ALS risk, the impact on clinical guidelines for the general population remains uncertain, given the rarity of ALS and the common occurrence of cardiovascular disease.

Direct effects of statins on cholesterol and lipid homeostasis may underlie the statin-induced adverse effects, because elevated cholesterol and lipid levels may be protective in ALS. Patients with ALS who have a low LDL/HDL ratio are reported to have 35% increased risk of death compared to patients with a high LDL/HDL ratio, and have approximately 12 month shorter median survival (35). This may be related to the ALS hypermetabolic state (36, 37). Increased metabolic load also exists in SOD1 mice, which exhibit increased peripheral lipid utilization and clearance (38), and survive longer when fed a high-lipid, high-energy diet (39).
By decreasing peripheral lipid and cholesterol levels, statins may exacerbate malnutrition in hypermetabolic ALS patients, and limit LDL cholesterol availability to weakened skeletal muscle fibers. This may contribute to the accelerated disease progression and decreased survival in statin-treated SOD1 mice in this study, and to the accelerated disease progression reported in patients with ALS (1, 2). An additional finding was elevated plasma ferritin in SOD1 versus WT mice, consistent with reports suggesting high serum ferritin levels in patients with ALS (40-42). This further supports the validity of the animal models used in our study.

The type of statins used in human and animal studies is likely important. One study suggesting statins were not associated with decreased survival in patients with ALS did not distinguish between classes of statins (18). Simvastatin crosses the blood brain barrier, and exerts both peripheral and central effects on lipid metabolism (43). Systemic and central nervous system (CNS) cholesterol homeostasis are decoupled. The CNS accounts for approximately 25% of the unesterified cholesterol in the body, and the input of cholesterol into this compartment derives largely from de novo synthesis rather than transfer from the plasma (44). It has been shown that selected statins, including higher concentrations of simvastatin, have direct and detrimental effects on motor neuron morphology and viability (45).

Our second finding suggested double transgenic mice had consistently lower grip strength in both hindlimb and forelimb, and double transgenic mice administered statins had the lowest grip strength out of all groups tested. This effect is relevant because H63D HFE is found in up to 30% of patients with ALS (3-9). A number of mechanisms may mediate the HFE genotype-dependent effects on disease progression, as discussed earlier (20-23, 46-48). In particular, disruption of cholesterol induced by HFE polymorphism may exacerbate the adverse effects of statins in ALS. Single transgenic mice harboring H67D HFE have lower CNS cholesterol levels,
which correlate with increased caspase-3 and behavioral deficits (23). The significance of lower cholesterol levels on survival in ALS patients has been discussed above (35). Furthermore, decreased cholesterol and lipid content is observed in disorganized myelin in the CNS of SOD1 G93A rats (49).

Given the pathogenic mechanisms associated with HFE polymorphism, it is noteworthy that H63D HFE by itself prolonged survival in a mouse model of ALS. Clinically, the effects of H63D HFE on survival in patients with ALS remain inconclusive (9, 50). In the latter study, which suggested homozygosity for H63D HFE increases disease duration by approximately 2 years, a large effect size compared to the median survival of 28 months (51), we hypothesized that H63D HFE may induce basal levels of cellular stress. This cellular stress increases the risk for ALS, accounting for the increased prevalence of H63D HFE in patients with ALS, while also inducing adaptive response mechanisms that paradoxically promote survival in those patients who do develop ALS. The current animal results support this model, as longer survival in double transgenic versus SOD1 mice are consistent with the induction of adaptive responses.

Together, the HFE genotype-dependent disease progression and survival results suggest that H63D HFE accelerates disease progression, but stabilizes the advanced stage of the disease. Double transgenic mice have decreased grip strength, earlier in their lifespan, compared to SOD1 mice; however, they survive longer in this weakened state. If this occurs in patients with ALS, then H63D HFE may paradoxically be a detrimental genetic modifier, inducing adaptations that extend lifespan, but at the cost of a shift in disease trajectory such that the patient spends a longer period of time at an advanced disease stage. This supports the importance of HFE genotyping as a tool to help the clinician predict and discuss prognosis, and to manage care.
Our third finding suggested mitochondrial dysfunction does not underlie the adverse effects of statins in SOD1 mice. Research suggests decreased activity of complexes I and IV of the ETC in SOD1 mice (52). Complex I is an important source of reactive oxygen species in perturbed mitochondria (53), and complex IV and neuronal activities are correlated (54). The association of cytochrome c with the mitochondrial inner membrane is disrupted in the CNS of SOD1 mice, impairing respiration and promoting apoptosis (33). VDAC1, a porin of the mitochondrial outer membrane that is an important regulator of metabolite and ion flow, is disturbed by mutant SOD1 (55). Our results showing minimal statin effects on protein levels of important mitochondrial components contradict reports suggesting that statin-mediated adverse events, especially in ALS, are caused by mitochondrial dysfunction (24, 29). We did not measure biochemical activities, and cannot comment on the integrity and function of the mitochondrial components we analyzed, but our results support the hypothesis that statin-induced mitochondrial disruption is not a major driver of accelerated disease progression or decreased survival in SOD1 mice. The lack of response to coenzyme Q10, which is beneficial to mitochondria, on measures of survival in SOD1 and double transgenic animals further supports the conclusion that statins are detrimental to ALS via mitochondria-independent mechanisms.  

Although we did not find statin-induced mitochondrial dysfunction in our study, we observed changes to important mitochondrial components in gastrocnemius muscle of SOD1 versus WT mice, consistent with reports of respiratory ETC deficiencies in the muscle of patients with ALS (56). Our results support the idea that ALS involves degeneration beyond the CNS. Muscle and myofibril degeneration is marked by similar disease processes, including mitochondrial dysfunction, as those that characterize the CNS, in patients with ALS (56) and our animal model.
In conclusion, we show accelerated disease progression and decreased survival in ALS mice administered simvastatin. *HFE* polymorphism exacerbates statin-induced acceleration of disease progression. However, these effects were not associated with perturbations in mitochondrial proteins, and were not rescued by supplementation with coenzyme Q10, which is believed to be beneficial to mitochondria. Overall, our results suggest statins adversely impact ALS disease progression and survival, effects which are exacerbated by the H63D polymorphism in the *HFE* iron regulatory gene. Our data suggest clinical studies of disease progression is warranted in patients with ALS receiving statin therapy, especially those harboring H63D *HFE*. 
5.6 Acknowledgment

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5.7 References


Chapter 6
Factors Influencing ALS Disease Progression: Summary and Future Perspectives

6.1 Introduction

Amyotrophic lateral sclerosis (ALS) is a heterogeneous disorder not confined to motor neurons, and involves multiple disease pathways and variable phenotypes. The heterogeneity of the disorder contributes to poor clinical trial outcomes. Except for riluzole, every compound tested for ALS has failed to demonstrate sufficient efficacy to be approved for clinical use. Whether any patient subgroups benefited in these trials remains unknown, because it is not possible to accurately predict prognosis. However, in Chapter 3 we developed a biomarker strategy that is promising in this area. Improved patient outcomes depend on understanding the pathways responsible for disease, and uncovering novel targets for future therapies.

The pathogenesis of ALS begins well before a diagnosis can be made. In transgenic mouse models of ALS there is significant motor neuron loss prior to discernable symptom onset, with up to 50% loss prior to overt muscle weakness (1). Pinpointing the precise temporal and spatial progression of pathophysiological mechanisms underlying ALS, close to their initiation, would be ideal. However, this remains impractical, because of the difficulties in early clinical diagnosis, and the inability of animal models of ALS to fully recapitulate the pathophysiology and clinical response to treatment seen in patients. Given these challenges in understanding the events initiating ALS, analysis of the ongoing factors influencing disease progression becomes the next best strategy for elucidating disease mechanisms and uncovering novel drug targets.

This dissertation project investigated factors influencing ALS disease progression, using the framework that the interplay of a range of extrinsic to intrinsic factors determine phenotypes.
Intrinsic factors, such as a patient’s genetic makeup, are present at birth and remain relatively stable throughout life. Extrinsic factors, such as drug therapies, vary with time and environment. Intermediate factors, which have intrinsic and extrinsic characteristics, include proteins that can serve as biomarkers, and depend on and reflect the individual’s innate genetic and metabolic profiles as well as environment. The interplay of these factors determine the quality of life and disease duration of a patient with ALS.

Our analysis of intrinsic factors influencing disease progression focused on the H63D polymorphism in the HFE iron regulatory gene, which is implicated in neurodegenerative disorders (2) and is found at increased frequency in patients with ALS (3-7). We show that homozygosity for H63D HFE is associated with approximately 2-year longer disease duration in patients with ALS (8), even though research suggests H63D HFE homozygosity increases ALS disease risk (9). H63D HFE polymorphism was also associated with decreased levels of soluble, wild-type (WT) superoxide dismutase (SOD1) protein in muscle, suggesting increased SOD1 misfolding and aggregation leading to endoplasmic reticulum (ER) stress. We hypothesize that H63D HFE induces basal levels of ER stress, increasing ALS risk, but that adaptive responses to this stress promotes survival, paradoxically, in those individuals who develop ALS. For example, increased neuronal toxicity may promote an environment that preconditions immune effector cells, such as microglia and macrophages, to mount immunomodulatory responses that promote tissue remodeling and repair.

Studies directed at combined intrinsic and extrinsic factors influencing disease course investigated protein biomarkers, singly or in concert, in cerebrospinal fluid (CSF) and plasma of patients with ALS. We generated models that could predict prognosis with reasonable accuracy from panels of select biomarkers, using algorithms incorporating best subset selection and
multivariable regression. Predictive biomarker panels included inflammatory cytokines, growth factors, and markers of iron metabolism (10). Next, we demonstrated that serum ferritin, which has potential to predict disease progression as part of a biomarker panel, is increased in patients with ALS versus healthy controls as well as patients with non-ALS neurological disease. Although the variance in serum ferritin values makes its use in diagnosis difficult as a single biomarker, our results argue for a role of perturbed iron homeostasis in ALS disease progression. Our results introduce the concept of using biomarker panels, which may better reflect disease progression than single biomarkers, to aid prognosis; with subsequent analysis of relevant single biomarkers in the context of broader disease processes, such as immunomodulation.

We used the SOD1 G93A transgenic mouse model of ALS to study extrinsic factors influencing disease progression. We show that HMG-CoA reductase inhibitors (statins), which are commonly prescribed to manage lipid levels, accelerate disease duration and decrease survival in SOD1 mice. The effects were \textit{HFE} genotype dependent, as double transgenic mice harboring SOD1 G93A and H67D \textit{HFE}, homologous to human H63D \textit{HFE}, had the worst phenotype. This is consistent with clinical reports suggesting statins may accelerate disease progression (11, 12), and underscore the need for surveillance of disease progression in patients with ALS receiving statin therapy, especially those harboring H63D \textit{HFE}. There was little evidence of mitochondrial dysfunction in our animal models, in contrast to reports suggesting this pathway mediates the adverse effects of statins (13). We hypothesize statin-induced disruption of anti-inflammatory immunomodulation contributes, in part, to their adverse effects.

Evaluation of our results analyzing the interplay of intrinsic to extrinsic factors influencing ALS disease progression provide insight into the common mechanisms that contribute to ALS pathophysiology. Taken together, we propose the data in this thesis suggest
modulation of the polarization and function of microglia and macrophages represents a converging pathway through which these factors modify disease. M2 microglia and macrophages have anti-inflammatory, immunomodulatory characteristics, and are critical for limiting excessive inflammation and promoting tissue repair. This chapter suggests a common framework in which these cells mediate the downstream effects of the range of intrinsic to extrinsic factors, which act in concert to influence disease progression, studied in this dissertation (Figure 6-1).
Figure 6-1

Therapeutic strategies:
1) Immunomodulatory agents (pidotimod)
2) M2 potentiators (apoferitin delivery)
Figure 6-1. Model for interplay of intrinsic to extrinsic factors modifying ALS disease progression. ALS induces inflammation in the CNS and periphery, which recruits naïve microglia and macrophages to sites of inflammatory foci, via chemoattractant molecules such as IP-10 and MCP-1. Once at the site of injury, naïve microglia and macrophages may be polarized to one of two general states: M1, in response to IFN-γ, LPS, and GM-CSF; or M2, in response to IL-4, IL-13, M-CSF, or MCP-1. M1 responses drive an exacerbating feedback loop, wherein activated microglia and macrophages release pro-inflammatory effectors including IL-12 and ROS, worsening neuroinflammation that in turn further drives M1 polarization and function. M2 responses suppress disease processes, by causing alternatively activating microglia and macrophages to release immunomodulatory effectors including IL-10 and L-ferritin, and upregulate Nrf2-mediated activation of HO-1, limiting neuroinflammation. The balance of M1 versus M2 polarization and function contribute, in part, to ALS disease progression. H63D HFE prolongs disease duration by directly upregulating MCP-1, driving M2 polarization; upregulating Nrf2- and HO-1-based adaptive responses, preconditioning microglia and astrocytes towards M2 function; and promoting adaptive L-ferritin secretion. Statins accelerate ALS disease progression and decrease survival by inhibiting microglia and macrophage chemotaxis to sites of injury, blocking their immunomodulatory activity. Potential therapeutic strategies include, 1) immunomodulatory agents to drive M2 polarization and function, such as pidotimod; or 2) potentiation of M2 responses, such as targeted delivery of apoferritin to these cell subtypes, which efficiently handle and recycle iron.
6.2 M2 Microglia and Macrophages and Their Importance in ALS

Microglia and macrophages are important for both innate and adaptive immunity, and play a major role in host defense, inflammation, as well as tissue remodeling and repair. These immune cells can be reprogrammed to express a spectrum of functional phenotypes in response to signals from pathogens, regulatory lymphocytes, or damaged tissue. In general, microglia and macrophages are activated into two broad polarization states: M1, representing an inflammatory phenotype that mediates microbe clearance; and M2, representing an anti-inflammatory phenotype that favors immunomodulation and tissue repair (14, 15). M1 macrophages are induced by interferon-γ (IFN-γ), alone or in conjunction with LPS, tumor necrosis factor-α (TNF-α), or granulocyte-macrophage colony-stimulating factor (GM-CSF). In contrast, M2 macrophages are most potently induced by interleukin-4 (IL-4), IL-13, and macrophage colony-stimulating factor (M-CSF). These cells have blunted production of effector molecules, such as reactive oxygen and nitrogen species, used to clear pathogens; decreased production of inflammatory cytokines, such as IL-12 and IL-23; increased production of anti-inflammatory cytokines, such as IL-10; distinct iron, folate, and glucose metabolic profiles; and activities that promote tissue repair and immunomodulation (14).

M1 and M2 microglia and macrophages play important and distinct roles in ALS. Research supports the neuroprotective role of M2 polarized resident central nervous system (CNS) microglia in animal models and patients with ALS. In rodent models of ALS, CNS resident microglia have aberrant morphology, aggregate, and degenerate (16). Microglia undergo apoptosis in the spinal cord of SOD1 G93A mice, an effect that correlates with disease progression and CNS infiltration by pro-inflammatory, M1 splenic monocytes (17). CNS microglia from ALS mice at disease onset express markers consistent with M2 polarization, and
promote motor neuron survival and neurite extension \textit{in vitro}. In contrast, CNS microglia from end-stage ALS mice have an M1 phenotype, and are toxic to co-cultured motor neurons (18). Passive transfer of regulatory T\textsubscript{h}2 cells, which promote IL-4 expression and M2 microglia polarization, lengthens disease progression and prolongs survival in ALS mice. In patients with ALS, decreased T\textsubscript{h}2 counts correlate with accelerated disease progression, suggesting M2 microglia are neuroprotective and promote the stable phase of disease, defined as the slowly progressing stage following symptom onset characterized by relatively gradual declines in muscle tone and respiratory function (19). Together, these results support a framework where over-activation, or dysregulation of the time course of activation, of M1 microglia and macrophages is detrimental to ALS disease course, whereas improved M2-dependent immunomodulation is beneficial.
6.3 The Impact of H63D HFE on ALS Disease Progression and Macrophage Polarity

We demonstrate that patients with ALS homozygous for H63D HFE have an approximately 2 year longer disease duration (8), a large effect size compared to the average survival of 2-4 years. Cytokines associated with H63D HFE, such as MCP-1, may promote M2 macrophage and microglia polarization, and represents one pathway through which the polymorphism may increase survival in patients with ALS. Homozygosity, but not heterozygosity, for H63D HFE is associated with increased circulating levels of monocyte chemoattractant protein-1 (MCP-1) in humans (20), as well as increased MCP-1 secretion in neuroblastoma cell lines (21). Increased circulating and CSF levels of MCP-1 are reported in patients with ALS (22, 23). MCP-1 causes anti-inflammatory M2 macrophage polarization. MCP-1 promotes expression of M2 surface markers and secretion of the anti-inflammatory cytokine IL-10, whereas MCP-1 blockade enhances expression of M1-associated genes and cytokines. Deficiencies in CCR2, the receptor for MCP-1, induces M1 macrophage polarization, and increases expression of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α) and IL-6 (24). Loss of CCR2 from monocytes occurs in sporadic ALS (SALS) (25). Our own research suggests increased CSF MCP-1 levels predict longer disease duration (10). These results suggest elevated MCP-1 associated with H63D HFE homozygosity contributes to anti-inflammatory, M2 macrophage and microglia polarization, which is neuroprotective in ALS.

The association between H63D HFE and M2 macrophage polarity is further supported by data showing elevated hemeoxygenase-1 (HO-1) and transcription factor nuclear factor E2-related factor 2 (Nrf2), which are relevant for M2 macrophage function, in transgenic mice carrying HFE polymorphisms. The CNS is not protected from the effects of HFE polymorphisms (26). Mice carrying H67D HFE, homologous to human H63D HFE, have perturbed CNS iron
homeostasis, as indicated by increased H- and L-ferritin and decreased Tf; as well as increased HO-1 and Nrf2 (26). HO-1 and Nrf2 are part of the adaptive response to oxidative stress, and are preferentially expressed in M2 versus M1 polarized macrophages. Upregulation of HO-1 in this cell type potently stimulates secretion of IL-10, blunting inflammatory responses (27). Astrocytes are also able to induce HO-1 expression in microglia via an Nrf2-dependent mechanism, suppressing reactive oxygen species (ROS) production in response to interferon-γ (IFN-γ), and limiting excessive inflammation (28). These results suggest that the elevation in HO-1 and Nrf2 seen in mice carrying HFE polymorphism may reflect preferential, adaptive activation of M2 microglia and macrophages.

Thus far, evidence supports a model wherein H63D HFE-induced activation of M2 microglia and macrophages underlie the genotype-dependent benefit for ALS survival. However, H63D HFE is also found at increased frequency in patients with ALS (3-7), and may increase disease risk (9). These findings may be explained by the chronic, mild stress induced by H63D HFE. In addition to oxidative stress, which damages tissues and promotes inflammatory responses, H63D HFE also causes chronic activation of the unfolded protein response and persistent, intracellular ER stress, increasing neuronal vulnerability (29). Our results support these findings; decreased levels of soluble WT SOD1 in muscle of patients with ALS with homozygous H63D HFE versus WT HFE suggest increased SOD1 aggregation leading to ER stress (8), which contributes to ALS pathogenesis (30). HFE polymorphism likely induces cellular vulnerability which, at least initially, causes tissue damage. This initial damage, mediated by increased protein oxidation and ER stress, may increase the risk of developing ALS (9), and account for the increased prevalence of the allele in patients with ALS (3-7).
Despite this initial damage, however, the chronic stress induced by H63D \textit{HFE} upregulates adaptive mechanisms that limits further damage. Mice homo- and heterozygous for H67D \textit{HFE} have increased protein oxidation at 6 months; however, these increases are abolished at 12 months. Importantly, this reversal of increased protein oxidation is concomitant with marked increases in Nrf2, especially in homozygotes, reflecting the activation of adaptive mechanisms (26). Neuroinflammatory responses likely play a major role in the resolution of \textit{HFE} polymorphism-induced oxidative damage, given that the Nrf2-HO-1 system, which is activated in M2 macrophages, mediates the effect in animal models. Together, results suggest the mild stressors induced by H63D \textit{HFE} may have few adverse effects on survival, especially in homozygous individuals, because mild stress triggers adaptive responses that limit further damage. This refines our model, and suggests H63D \textit{HFE} induces chronic but mild stressors, such as ER stress via protein misfolding and protein oxidation via oxidative damage, to increase ALS risk. However, adaptive mechanisms, possibly mediated by preconditioning of cell types including M2 polarized macrophages, paradoxically promote neuronal survival in those individuals who develop ALS. These effects are greatest in individuals homozygous for H63D \textit{HFE}, as is consistent with our studying showing increased survival in this patient subgroup (8).

Numerous CNS cell types, including microglia and astrocytes, adapt to chronic, mild preconditioning. Persistent, mild activation of CNS microglia induces M2 polarization and anti-inflammatory responses in hippocampal slice cultures (31). Astrocytes preconditioned by proteasome inhibition are resilient in response to proteotoxic stress, and have increased survival, elevated levels of heat shock proteins and HO-1, and preservation of cellular glutathione levels compared to naïve astrocytes (32). Mild protein misfolding and ER stress induced by H63D \textit{HFE}, especially in homozygotes, may precondition astrocytes. This is important because
astrocytes are the most abundant cell-type in the CNS, play a vital role in metabolic support and neuroprotection under basal conditions, and contribute to the pathophysiology of ALS (33).

Together, these data suggest a dual mechanism through which H63D HFE may promote microglia and macrophage-dependent neuroprotection in ALS. First, H63D HFE promotes a cytokine profile, including increased MCP-1, which directly promotes M2 polarization. Second, H63D HFE induces chronic but mild stress that preconditions cells, including astrocytes, microglia, and macrophages, towards neuroprotective profiles, which for microglia and macrophages entails M2 polarization. Subsequently, M2 microglia and macrophages limit inflammation and promote tissue repair, which decreases neurotoxicity and increases neuroprotection in ALS.

Our proposed model suggests HFE genotype may influence the response to pharmacotherapy, especially anti-inflammatory agents, and that HFE genotyping is warranted in ALS clinical trials because of these effects. For example, minocycline, which has anti-apoptotic, anti-inflammatory, and neuroprotective effects in vitro and in animal models, may accelerate disease progression in patients with ALS (34). Minocycline blocks M1 microglia polarization in SOD1 mice, without affecting M2 polarization (35). Minocycline also has HFE genotype-dependent effects. It potently suppresses glutamate recycling and uptake in neuroblastoma cells harboring WT HFE, possibly contributing to glutamate toxicity; however, it does not affect glutamate uptake in cells harboring H63D HFE (36). Minocycline may benefit patients with ALS harboring the H63D HFE allele, by suppressing M1 macrophage polarization. However, it may harm patients harboring WT HFE, by exacerbating excitotoxicity. Genotype may have influenced the results of the minocycline clinical trial, with WT HFE individuals adversely
affected and H63D \textit{HFE} individuals showing a benefit. Because 30\% of patients should harbor H63D \textit{HFE}, the net apparent effect would be a detriment to disease progression.

As the minocycline data suggest, immunomodulation may prove beneficial in patients with ALS harboring H63D \textit{HFE}. Our data further suggest additional genotype-dependent treatment strategies that may be appropriate for ALS. Driving M2 macrophage and microglia polarization may prolong the stable phase of the disease in these individuals, as they harbor an immune profile characterized by upregulated MCP-1, Nrf2 and HO-1 that may be particularly responsive to immunomodulation. However, care must be taken not to overly suppress M1 macrophage-based activity, as these are important in the systemic response to infection as well as tumorigenesis. A number of immunomodulatory compounds exist, with varying degrees of potency and specificity for M2 microglia and macrophages. Among the compounds reported in the literature, pidotimod is especially promising. Pidotimod is a synthetic dipeptide that promotes M2 macrophage polarization and potentiates the function of these cells, improving migration and wound closure rate, while having little effect on M1 polarization or function (37). This compound is relevant for patients with ALS homozygous for H63D \textit{HFE}, who may express an immuno-protective, M2 macrophage and microglia profile at baseline. Currently little data exists on the ability of pidotimod to cross the blood brain barrier (BBB), and thus further research is needed to determine its influence on microglia, if given systemically. Nonetheless, it may prove valuable as an add-on therapy to riluzole, especially in patients with ALS harboring H63D \textit{HFE}. 
6.4 Protein Biomarkers and Macrophage Polarity in ALS

We used multiplex biomarker assays, coupled with multivariable regression algorithms, to construct models that predicted ALS prognosis with reasonable accuracy. Our predictive models suggest neuroinflammation contributes to ALS. The models do not support a simplistic mechanism wherein neuroinflammation is uniformly deleterious and immunosuppression beneficial. Instead, immunomodulation is required, as it appears that immune responses are neuroprotective in a time- and region-dependent manner, whereas excessive or deficient immune activation is neurotoxic in a similar, context-dependent fashion. This may explain, in part, the disappointing results of clinical trials testing anti-inflammatory agents, which may not have achieved the precise immunomodulation necessary for a benefit. In addition, the identities of the biomarkers themselves further support a role for M2 microglia and macrophage function in ALS.

A summary of our biomarker results, listing the contribution of relevant biomarkers from each compartment, CSF or plasma, to survival is presented in Table 6-1. The biomarker with highest $R^2$ in the combined model, which had the greatest predictive accuracy out of the three models, is plasma C-X-C motif chemokine 10 (IP-10). Levels of IP-10, which is classically considered a pro-inflammatory cytokine, are positively correlated with survival. IP-10 is secreted by a number of cell types, including monocytes, in response to IFN-γ, and is a chemoattractant for various cells of the immune system, including monocytes and macrophages. A high ratio of CSF-to-plasma IFN-γ, in turn, is also associated with increased survival. In the same model, higher levels of CSF MCP-1, which also serves as a monocyte and macrophage chemoattractant and drives M2 polarization, correlate with increased survival. This suggests adequate immune reserve involving functional monocytes, macrophages and microglia is neuroprotective. Lower plasma levels of IL-1β, which promotes M1 polarization, and lower plasma and CSF levels of
IL-12, which is secreted by M1 macrophages, combined with higher plasma levels of IL-10, an anti-inflammatory cytokine secreted by M2 macrophages, correlate with longer survival. Together, these results strongly support the neuroprotective role of M2 macrophages and microglia during ALS disease progression.
Table 6-1 Biomarkers Correlated with Disease Duration

<table>
<thead>
<tr>
<th>Predict shorter duration</th>
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<td><strong>Plasma</strong></td>
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<tr>
<td>IL-1β</td>
<td>IL-5</td>
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<tr>
<td>IL-1RA</td>
<td>IL-8</td>
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<td>IL-12</td>
<td>IL-12</td>
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<tr>
<td>RANTES</td>
<td>Onset age</td>
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<tr>
<td>High plasma-to-CSF IFN-γ ratio</td>
<td>L-ferritin</td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
<td><strong>CSF</strong></td>
</tr>
<tr>
<td>Eotaxin</td>
<td>IL-9</td>
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<tr>
<td>IL-5</td>
<td>G-CSF</td>
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<tr>
<td>IL-10</td>
<td>MCP-1</td>
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<tr>
<td>IP-10</td>
<td>MIP-1β</td>
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Low plasma-to-CSF IFN-γ ratio
The iron storage protein ferritin was another biomarker that predicted disease duration in our model. Higher plasma levels of L-ferritin, which sequesters and stores iron in a soluble, nontoxic ferric state, correlate with longer disease duration. High levels of serum ferritin are also seen in patients with ALS versus healthy controls (38, 39). Our research suggests the elevation in serum ferritin seen in ALS is not seen in healthy controls or patients with non-ALS neurological diseases, further implicating ferritin in disease. In that study, we suggest elevated serum ferritin may serve as an adaptation to increased levels of oxidative stress in ALS. This stems from the ability of ferritin to detoxify iron, which generates free radicals via the Fenton reaction, and other trivalent metals in the CNS. Additionally, we propose elevated ferritin may derive from M2 polarized macrophages, which exhibit efficient iron recycling (40), and are known to be an important source of the protein in the blood (41).

Together, these results further refine the model proposed in Section 6.3 of this dissertation. Previously, we suggested a cytokine profile favoring M2 microglia and macrophage polarization and function decreases neurotoxicity in ALS. By analyzing compartment specific effects using predictive biomarkers, we can propose region-dependent mechanisms of action. High plasma IP-10 correlates with longer survival in our models, as does a high CSF-to-plasma ratio of IFN-γ. This suggests, in plasma, adequate chemoattractant signals for monocytes and macrophages promote longer survival, possibly by recruiting them to sites of neuromuscular degeneration, where M2 macrophages are essential for proper myofiber repair (42). At the same time, low plasma IFN-γ levels limit the degree of peripheral M1 activation, preventing excessive systemic inflammation. In the CNS compartment, high CSF levels of IFN-γ reflect detrimental neuroinflammatory processes characterizing ALS, which initiate neuroprotective M2-based responses. In this context, if the CNS compartment is able to achieve sufficient M2 microglia-
mediated immunomodulation, as reflected by high CSF levels of MCP-1 and IL-10, and low levels of IL-1β and IL-12, then neuroprotective mechanisms are triggered, prolonging survival, consistent with our results (10).

H63D HFE modifies these events, first by directly contributing to cytokine profiles driving M2 polarization, and second by triggering chronic, mild stress that preconditions microglia and macrophages to preferentially mount M2-based responses. Lastly, H63D HFE also modifies macrophage-dependent ferritin responses. In general, macrophages are iron abundant. However, HFE polymorphism renders macrophages relatively iron poor because there is decreased circulating levels of hepcidin. Thus, the degree of iron saturation of ferritin found in H63D HFE macrophages should be decreased. L-ferritin is increased in the CNS of mice harboring HFE polymorphism (26), and M2 macrophages have improved iron recycling and greater ferritin release (40). Together, these data indicate that in the setting of ALS, H63D HFE microglia and macrophages secrete more L-ferritin, because a greater proportion are M2 polarized due to genotype effects; and the secreted L-ferritin is more iron-poor, due to the effects of HFE polymorphism on hepcidin and macrophage iron abundance. This would increase the availability of a form of ferritin that is particularly suited to sequester and detoxify free iron in the CNS, which may benefit survival in those patients with ALS harboring H63D HFE, consistent with our observations (8).

If these hypotheses are valid, then a potential therapeutic avenue would be CNS delivery of apoferritin in conjunction with an immunomodulatory strategy that drives M2 microglia and macrophage polarization. Apoferritin may be continuously infused via an intracerebroventricular (ICV) route, in conjunction with a cocktail of cytokines, including IL-4, IL-10, M-CSF, and MCP-1, to drive M2 polarization and promote immunomodulation. Apoferritin could also be
delivered ICV in liposomes coated with IL-4, in order to target microglia as well as naïve helper T cells. IL-4 ligand would drive immunomodulatory, M2 microglia polarization, while promoting the differentiation of naïve helper T cells into regulatory T_{H}2 cells. Subsequent delivery of apoferritin to M2 microglia, which efficiently recycle iron and release ferritin, may increase local levels of ferritin in areas of active microglia immunomodulation. This may augment the body’s own adaptive ferritin release, further ameliorating the oxidative damage induced by ALS. Apoferritin may also be delivered in N-acetylglucosamine coated liposomes, which preferentially target mannose receptors on M2 polarized microglia. ICV administration of these nanoparticles may deliver apoferritin to M2 microglia while avoiding off-target effects of widespread IL-4-dependent immunomodulation. By contrast, liposomes coated with LPS, which are traditionally used to target macrophages and microglia, should be avoided in ALS. LPS stimulates Toll-like receptor (TLR) 4, which drives M1 macrophage polarization and may be detrimental in ALS. Other immunomodulators, such as pidotimod to drive M2 polarization, may be given concurrently, either ICV or systemically. This may allow more precise compartment-based immunomodulation in conjunction with apoferritin delivery.
6.5 Statins Adversely Impact ALS: A Possible Mechanism via Impaired Microglia and Macrophage Chemotaxis

Our results suggest statins adversely impact SOD1 mice, consistent with clinical reports linking statin therapy to accelerated disease progression in patients with ALS (11, 12). SOD1 mice administered statins have accelerated disease progression and decreased survival. These effects were most pronounced in double transgenic mice harboring H67D HFE, highlighting the important of genotype in the response to pharmacotherapies. However, mitochondrial dysfunction did not contribute to the statin-induced effects. Mitochondrial enzymes were not perturbed by statin administration at 120 days, when differences in grip strength were greatest between groups, or at endstage. This suggests statins induce adverse effects in ALS independently of mitochondrial dysfunction.

The cholesterol-independent, pleiotropic effects of statins may underlie their adverse impact on ALS. Statins inhibit isoprenoid synthesis. Isoprenoids serve as lipid anchors for various signaling molecules, including the Rho, Ras, and Rac GTPases. Statins disrupt signaling pathways mediated by these enzymes, with the net effect of improving endothelial function, stabilizing atherosclerotic plaques, inhibiting thrombogenesis, and most importantly for ALS, decreasing inflammation (43). Because of these pleiotropic effects, statins have been evaluated in a number of autoimmune and thrombogenic disorders, with limited success (44). Given the evidence presented in this dissertation, the statin effect that appears most relevant to ALS pathophysiology is anti-inflammatory modulation mediated via macrophages. Evidence suggests statins also drive M2 macrophage polarization (45, 46). This conflicts with our animal data, which suggests a detrimental effect of statins on ALS disease progression and survival, as well as our proposed model, which argues for a beneficial role of M2 polarization. However, detailed
investigation of the precise, pleiotropic effects of statins suggests a mechanism underlying their adverse impact in ALS that is mediated by effects on M2 polarized microglia and macrophages.

New research suggests statins potently inhibit monocyte and macrophage chemotaxis, a mechanism that may partly explain their adverse effects in ALS despite anti-inflammatory M2 modulation. Statins inhibit Rho GTPases, which are essential for actin filament remodeling, and severely disrupt the microfilament network dynamics required for chemotaxis in monocytes and macrophages. Statins also inhibit the chemoattractant molecule MCP-1. The net effect is disruption of the ability of monocytes and macrophages to migrate to inflammatory foci (47). Thus, whereas statins drive M2 macrophage polarization, they may render monocytes and macrophages incapable of reaching areas of tissue damage to exert neuroprotective and anti-inflammatory effects. In effect, the inhibitory effects of statins on naive microglia and macrophage chemotaxis outweights their downstream effects on M2 polarization. This is consistent with our predictive biomarkers data suggesting adequate chemotactic signals, in the form of MCP-1 in CSF and IP-10 in plasma, prolong disease duration (10). Importantly, statin-induced inhibition of macrophage chemotaxis and MCP-1 secretion is most pronounced in females (47). This is consistent with clinical reports suggesting females on statins experience the greatest detriment to disease progression (12).

If statins negatively impact ALS via inhibition of monocyte and macrophage chemotaxis, then the primary effect should be on disease progression and survival, rather than disease risk. Within this framework, neuroinflammation is not necessarily the primary driver of ALS pathogenesis. However, once the initiating pathogenic processes cause sufficient damage to induce symptoms, neuroinflammation arises as an important consequence. Statins may adversely impact ALS by blunting adaptive responses to this neuroinflammation, without modifying the
initiating event. This insight agrees with clinical reports showing inconsistent statin effects on ALS disease risk (48), and more robust effects on disease progression (11, 12), as well as our own results showing detrimental effects on disease acceleration and survival in animal models.

These results unify the range of intrinsic to extrinsic factors modifying ALS disease progression, analyzed in this thesis, into a framework consistent with our proposed model. Immunomodulatory M2 microglia and macrophage responses promote neuroprotection and limit neurotoxicity in ALS, as reflected by protein biomarker panels predicting longer survival. H63D HFE modifies these effects, by directly driving M2 polarization, preconditioning microglia and macrophages towards M2-based responses, and augmenting ferritin-based adaptive responses in activated M2 microglia. Statins adversely impact ALS by interfering with the region-specific coordination of immunomodulation necessary for neuroprotection, via inhibition of microglia and macrophage chemotaxis to areas of active inflammation. This hampers the ability of macrophages to aid in immunomodulation and tissue repair at sites of neuromuscular degeneration in periphery, and microglia to limit excessive neuroinflammation in CNS.

The statin effects on macrophage chemotaxis proposed in our model is testable using animal models. If the hypothesis is correct, then simvastatin and atorvastatin, which are lipophilic and readily cross the blood brain barrier (BBB), should have similar detrimental effects on SOD1 mice as pravastatin, which is hydrophilic and does not readily cross the BBB. Although the negative effects of pravastatin may be less than simvastatin or atorvastatin, nonetheless peripheral inhibition of monocyte and macrophage chemotaxis should still have a measureable negative impact on inflammatory modulation. However, if the hypothesis is incorrect, and statins exert a more direct effect on cells of the CNS, then only statins which cross the BBB should harm ALS mice, whereas those that do not should have no detrimental effect.
6.6 Conclusions

Evidence presented in this chapter connects the range of intrinsic to extrinsic factors modifying ALS disease progression together into a unified pathway mediated by M2 microglia and macrophages. Our proposed model is presented in Figure 6-1. Briefly, M1 activity promotes neuroinflammation, exacerbating ALS and causing a feedback loop that further drives M1-based responses. In contrast, M2 activity limits neurotoxicity and promotes neuroprotection in ALS. H63D HFE influences these effects, by directly driving M2 polarization, preconditioning microglia and macrophages towards M2-based responses, and augmenting adaptive, M2-based ferritin secretion. Statins act upstream of these mechanisms, by inhibiting microglia and macrophage chemotaxis to sites of injury.

Our model suggests treatment strategies that may benefit patients with ALS. Immunomodulation, specifically upregulation of M2 polarization and function, may limit the neuroinflammation characterizing the disease. This strategy may be enhanced by compartment-specific delivery of apoferritin into the CNS, or even targeted, nanomedicine based methods targeting apoferritin directly to M2 microglia. The goal is to achieve time- and region-specific immunoregulation that limits neuroinflammation.

Our studies did not address whether neuroinflammation is a primary or secondary insult in the pathogenesis of ALS. The answer to this larger question determines the degree to which immunomodulation can improve ALS phenotype. As highlighted by analysis of the genetic basis of ALS in the introduction to this dissertation, the disease is highly heterogeneous and involves multiple, interacting disease pathways. The genetic, biomarker-based, and pharmacotherapeutic factors analyzed in this dissertation suggest neuroinflammation is a converging pathway influencing disease progression. In this chapter, we conclude that M2 microglia and
macrophages play a critical role in the neuroinflammatory processes characterizing ALS. M2 dependent biological activity influences disease trajectory and quality of life in patients with ALS, and represents a pathway amenable to therapeutic modulation. Determining the place of neuroinflammation within the larger context of mechanisms contributing to ALS pathophysiology will be critical to understanding the disease, and developing novel therapies.
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Selected Publications
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