Contribution of \textit{HFE} Polymorphisms to Pathogenetic
Mechanisms of Amyotrophic Lateral Sclerosis

A Dissertation in
Cell and Molecular Biology

by
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Relatively selective destruction of upper motor neurons in the motor cortex and lower motor neurons in the brainstem and spinal cord is the defining feature of amyotrophic lateral sclerosis (ALS). Motor neuron degeneration relentlessly progresses, with death occurring an average of three to five years after onset due to respiratory failure. Despite extensive research, definite causes of the disease remain unknown for the vast majority of patients. Risk factors for the disease also remain mostly elusive; however, recently studies have identified the \textit{H63D HFE} genetic variant, but not the \textit{C282Y HFE} polymorphism, as a possible risk factor. I begin by extending the genetic association of the \textit{H63D HFE} allele with sporadic ALS.

Genotyping of a large number of ALS patients and control subjects from across the United States, and combining the results with those from populations around the world revealed a dose-dependent association of the \textit{H63D HFE} allele with sporadic ALS. The association of this allele with other risk-associated alleles was explored, and suggests \textit{H63D HFE} is unlikely to be merely tagging another allele. Previous functional analyses of HFE also suggest a potential contributory role of HFE variants to ALS pathogenesis. Wildtype HFE is critically positioned as a regulator of cellular iron acquisition. The potential cellular milieu resulting from dysfunctional HFE stands to contribute to pathogenesis of ALS by a variety of mechanisms.

ALS pathogenesis is thought to involve a number of converging and cascading pathways including neuroinflammation, oxidative stress, glutamate excitotoxicity, and
growth factor deficiency. Cellular iron dysregulation may potentiate each of these mechanisms either through generation of reactive oxygen species leading to oxidative stress or through other specific means. I assessed biomarkers associated with neuroinflammation and trophic factor signaling for the ability to distinguish ALS patients from control subjects and elucidate mechanisms of disease pathogenesis, and determine the impact of HFE polymorphisms on biomarker expression. In cerebrospinal fluid, expression levels of cytokines suggested an inflammatory profile associated with ALS, and demonstrated utility in classifying subjects by disease status. HFE polymorphisms were also associated with altered expression of several markers. In plasma samples, expression of the iron homeostasis proteins L-ferritin and transferrin was altered by disease status and presence of the H63D HFE allele. These two markers also suggested efficacy in classifying subjects by disease status. Additionally, the H63D HFE allele was associated with changes in inflammatory cytokines and trophic factors suggesting both potentially beneficial and detrimental effects in ALS pathogenesis.

At the cellular level, our results suggest a contribution of the H63D HFE allele to glutamate excitotoxicity and inflammatory signaling. H63D HFE was associated with increased neuronal glutamate secretion as well as deficient cellular glutamate uptake, in contrast to C282Y HFE, which was associated with the opposite effects. Expression of H63D HFE, but not C282Y HFE, also resulted in increased cellular secretion of monocyte chemoattractant protein-1 (MCP-1), a potent recruiter and activator of microglia and macrophages. The effects of H63D HFE on glutamate regulation and secretion of MCP-1 could generally be mimicked by modulating cellular iron levels.
Minocycline, a multifaceted antibiotic, has been explored for use in treating ALS patients due to its myriad effects on multiple pathways associated with disease pathogenesis. Our results suggest that response to this agent may be affected by $HFE$ genotype.

Collectively, our results establish the $H63D HFE$ polymorphism as a risk factor for ALS and demonstrate cellular mechanisms by which it may contribute to pathogenesis of the disease.
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<th>Full Form</th>
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<tbody>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>ALSFRS-R</td>
<td>ALS Functional Rating Scale Score-Revised</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>β2M</td>
<td>beta 2 microglobulin</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>DFO</td>
<td>desferroxamine</td>
</tr>
<tr>
<td>DHK</td>
<td>dihydrokainic acid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagles Medium</td>
</tr>
<tr>
<td>EAAT</td>
<td>excitatory amino acid transport</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FAC</td>
<td>ferric ammonium citrate</td>
</tr>
<tr>
<td>Fe</td>
<td>iron</td>
</tr>
<tr>
<td>FTD</td>
<td>Frontotemporal dementia</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>GSK</td>
<td>glycogen synthase kinase</td>
</tr>
<tr>
<td>HH</td>
<td>hereditary hemochromatosis</td>
</tr>
<tr>
<td>HIF</td>
<td>hypoxia inducible factor</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>Hsp</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IRE</td>
<td>iron responsive element</td>
</tr>
<tr>
<td>IRP</td>
<td>iron regulatory protein</td>
</tr>
<tr>
<td>LMN</td>
<td>lower motor neuron</td>
</tr>
<tr>
<td>L-SOS</td>
<td>L-serine-O-sulfate</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
</tr>
<tr>
<td>MMP</td>
<td>mitochondrial membrane potential</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-kappa B</td>
</tr>
<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative reverse-transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RANTES</td>
<td>regulated on activation normal T-cell expressed and presumably secreted</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarco/endoplasmic reticulum Ca^{2+} ATPase</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SOD1</td>
<td>superoxide dismutase-1</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline-Tween</td>
</tr>
<tr>
<td>Tf</td>
<td>transferrin</td>
</tr>
<tr>
<td>TfR</td>
<td>transferrin receptor</td>
</tr>
<tr>
<td>THA</td>
<td>L-(-)-threo-3-hydroxyaspartic acid</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>UMN</td>
<td>upper motor neuron</td>
</tr>
<tr>
<td>USFDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VGLUT</td>
<td>vesicular glutamate transporter</td>
</tr>
<tr>
<td>Wt</td>
<td>wildtype</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

This thesis is dedicated to my wife, Rebecca P. Mitchell. Thank you for all of your love and support, which mean more than I can describe. I also want to thank my parents, grandparents, and the rest of my family for their love and support.

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All patients and volunteers who donated samples used in this work deserve many thanks and I am profoundly grateful for their willing assistance.
Chapter 1

An Overview of Amyotrophic Lateral Sclerosis and Potential Role of Iron Misregulation in Disease Cascades

Introduction

Amyotrophic lateral sclerosis (ALS), also referred to as Lou Gehrig’s disease or motor neurone disease, is defined by the selective and progressive degeneration of both upper motor neurons in the motor cortex and lower motor neurons in the brainstem and spinal cord (Brooks BR 2000). Symptoms of ALS typically begin as minor weakness in limb muscles or muscles controlling speech and swallowing. Approximately 75% of patients first experience symptoms in the limbs (limb onset), while 25% first experience deficits in speech and swallowing (bulbar onset) (Nalini, Thennarasu et al. 2008). The disease typically progresses to affect both limb and bulbar muscles and average survival time is 3-5 years following the onset of symptoms, with death usually due to respiratory insufficiency. Disease course is highly variable in terms of presenting symptoms and length of survival. By definition, ALS involves both upper motor neuron (UMN) and lower motor neuron (LMN) dysfunction, but patients early in the course of the disease may present with only one of the two. Reasons for the preferential destruction of motor neurons compared to other neurons are not known. The recent identification of mutations in genes, particularly SOD1, associated with inherited cases has led to the development of rodent models that have accelerated research into the underlying pathogenesis of the disease and enabled testing of therapeutic targets. Despite extensive research and
valuable discoveries, underlying causes and effective treatments have mostly remained elusive.

Current treatment of ALS mainly involves management of symptoms. A number of therapeutic measures have been evaluated in disease models and patients, but only one drug has shown consistent benefits in humans. Riluzole is the only drug with proven benefit in prolonging life of human ALS patients and was approved by the US Food and Drug Administration in 1995 for treatment of ALS patients. Riluzole is thought to prolong survival of ALS patients by several months on average (Lacomblez, Bensimon et al. 1996), but even with treatment, ALS is uniformly fatal.

Because ALS is a disease defined by neuronal death, the disease classification may encompass multiple etiologies that happen to have the same clinical manifestation. Research into the underlying cause and the pathogenesis of ALS may be hampered by this broadly based classification because different mechanisms of pathogenesis may have variable influence depending on genetic and environmental factors. Additionally, therapies may have different efficacy depending on etiology. The goals of past and current research include the identification of disease risk factors, earlier and more accurate diagnosis, patient stratification by etiology and pathogenesis, identification of therapeutic targets, and more accurate evaluation of response to therapies.

Despite extensive research knowledge of risk factors for sporadic ALS, mechanisms of disease pathogenesis, and effective treatments for ALS patients is currently insufficient.
The identification of risk factors for sporadic ALS would likely benefit patients by elucidating mechanisms of pathogenesis and allowing stratification of patients for individual therapy. A long-term goal of identifying patients at risk before disease onset would be a possibility once more risk factors are identified and preventive measures may be available.

**Epidemiology**

The overall incidence of ALS is 1-3/100,000 person-years (Van Damme, Dewil et al. 2005; Beghi, Logroscino et al. 2006; Cronin, Hardiman et al. 2007; van Es, van Vught et al. 2008). Estimations of the lifetime risk of developing ALS range from 1 in 600 to 1 in 2000 (Dunckley, Huentelman et al. 2007; Blauw, Veldink et al. 2008). The median age of onset of symptoms is approximately 56 years (Valdmanis and Rouleau 2008), though some researchers have suggested that age-adjusted incidence of ALS continuously increases as persons age (Beghi, Millul et al. 2007). Additionally, some familial forms of ALS are characterized by earlier onset. The incidence of ALS also varies by sex, with a male to female ratio of 1.3-1.6:1 (Valdmanis and Rouleau 2008). Frontotemporal dementia (FTD) may coexist with ALS, with clinically-evident dementia found in 10-15% of ALS patients, although extensive cognitive testing identifies frontotemporal deficits in up to 50% of ALS patients (Strong, Lomen-Hoerth et al. 2003; Ringholz, Appel et al. 2005).
ALS is typically classified as either familial (fALS) or sporadic (sALS). Familial ALS has been suggested to account for up to 10% of all ALS cases (Pasinelli and Brown 2006), though other estimates suggest less than 5% of cases are familial (Schymick, Talbot et al. 2007). Clinical presentation of fALS matches sALS except for earlier age of onset of fALS (46 vs. 56 years old) (Valdmanis and Rouleau 2008), and faster disease progression for fALS patients with the A4V SOD1 mutation (Ratovitski, Corson et al. 1999). Genes associated with familial ALS include \textit{SOD1}, \textit{alsin}, \textit{VAPB}, \textit{senataxin}, \textit{dynactin}, \textit{ALS3}, \textit{ALS6}, and \textit{ALS8}, and inheritance is autosomal dominant in most cases (Valdmanis and Rouleau 2008). The most widely known genetic mutations associated with familial ALS are those in the \textit{SOD1} gene, accounting for approximately 20% of familial cases (Rosen, Siddique et al. 1993). SOD1 is a ubiquitously-expressed enzyme which converts the highly toxic superoxide radical to hydrogen peroxide. More than 110 mutations affecting greater than 50% of the 153 amino acids in SOD1 have been identified in ALS patients, and most are inherited in an autosomal dominant pattern (Valdmanis and Rouleau 2008).

**Mutant SOD1 (mSOD1) Models**

The identification of mSOD1 as a cause of familial ALS led to development of transgenic rodent models overexpressing various human SOD1 mutations. Deletion of mouse \textit{SOD1} genes or overexpression of wildtype human SOD1 do not cause a motor neuron disease phenotype, also suggesting a toxic gain of function associated with SOD1 mutations (Reaume, Elliott et al. 1996; Shibata 2001). These models show similar adult-onset of
motor neuron degeneration with timing of onset generally proportional to copy number of transgenes (Shibata 2001). Due to various inherent deficiencies in animal models, cell culture, zebrafish and drosophila models expressing various mutant forms of SOD1 have also been created and studied (Lemmens, Van Hoecke et al. 2007; Watson, Lagow et al. 2008).

While these models may only be directly representative of genetic alterations in 1-2% of human patients, their actual relevance to human patients may be more extensive. fALS patients are clinically indistinguishable from sALS patients, reflecting similar disease pathogenesis. Basic science studies have established similar mechanisms of pathogenesis in animal models and human patients, and there has been recent speculation that oxidation and dysfunction of SOD1 may be involved in all cases of ALS (Kabashi, Valdmanis et al. 2007). Nevertheless, the vast majority of treatments showing benefits in mutant SOD1 rodents have failed to show benefits in human ALS patients. Some of these failures may reflect the fact that most treatments are initiated in the rodent models prior to disease onset, which is unlikely to be relevant to treatment of humans. Other failures may represent differences in pharmacology between rodents and humans, while other failures may be due to the heterogeneous nature of the disease. Further details of these rodent models will be explored below in terms of mechanisms of pathogenesis.
Genetic Risk Factors for Sporadic ALS

Sporadic cases of ALS account for 90-95% of ALS cases (Schymick, Talbot et al. 2007). The lack of known genetic causes of sporadic ALS has led to the search for genetic risk factors increasing the risk of ALS in persons exposed to certain environmental triggers. Different incidence rates of ALS between persons of different ethnicities, and variable incidence rates of ALS among persons of the same ethnicity in different geographic locations (Cronin, Hardiman et al. 2007) are further evidence of gene-environment interactions leading to the development of ALS. A number of genetic risk factors have been studied, but the best established risk factor for sporadic ALS is increased age (Brooks 1996).

While sALS is not directly inherited, a British twin study suggested that sporadic cases involve a significant genetic component, with heritability estimated between 0.38 and 0.85 (Graham, Macdonald et al. 1997). A number of other studies have sought to identify the genetic risk factors of ALS using candidate gene approaches to examine genes seemingly involved in ALS pathogenesis. Rare allelic variants in a number of genes including *apurinic endonuclease (APEX1)*, *angiogenin*, *chromatin modifying protein 2b (CHMP2B)*, *dynactin (DCTN1)*, neurofilaments, *paraoxonase, peripherin, progranulin, SOD1, survival motor neuron 1 and 2*, and *vascular endothelial growth factor (VEGF)* have been associated with a small number of ALS cases, and usually only in a single population or study (reviewed in (Schymick, Talbot et al. 2007)). The *H63D* and *C282Y* variants of the *HFE* gene has been examined in multiple populations (Wang, Lee et al.
2004; Yen, Simpson et al. 2004; Goodall, Greenway et al. 2005; Restagno, Lombardo et al. 2007; Sutedja, Sinke et al. 2007). Overall, these studies demonstrate an increased risk of developing ALS associated with the \( H63D \) allele.

Recently, genome-wide association studies of single nucleotide polymorphisms (SNPs) associated with sporadic ALS have allowed unbiased screening of the genome. Single stage genome-wide association studies have individually failed to identify genes or alleles associated with sporadic ALS, most likely due to small sample sizes and potentially overly-conservative multiple comparisons corrections. However, the sharing of genotyping data and multistage SNP genotyping have enabled the identification of several disease susceptibility genes. Collection of DNA samples from multiple populations is often necessary to obtain sample sizes large enough to detect risk alleles, but genetic heterogeneity among populations may confound these efforts. Some disease-associated alleles may only be risk factors in select populations depending on allele frequency and environmental factors.

Genome-wide association studies have identified \( ITRP2 \) as a susceptibility gene for sporadic ALS in the Netherlands, Belgium, and Sweden (van Es, Van Vught et al. 2007). One SNP in this gene, \( rs2306677 \) (odds ratio (OR) 1.58), was significantly associated with ALS after correcting for multiple comparisons. A SNP near the \( FLJ10986 \) gene was significantly associated with sALS (OR 1.35) in a multistage analysis (Dunckley, Huentelman et al. 2007) that also included pooling data from a previously published study (Schymick, Scholz et al. 2007). A common SNP in \( DPP6 \) has been associated with
a significantly increased risk of developing ALS in multiple populations, with an odds ratio of 1.30 for the minor allele (van Es, van Vught et al. 2008). The risk allele showed a dose-dependent effect, with an odds ratio of 1.20 for heterozygotes and an odds ratio of 1.60 for homozygotes (van Es, van Vught et al. 2008). Additionally, this risk allele has been confirmed in other populations (Cronin, Berger et al. 2007). The contribution of each of these genetic variants to disease pathogenesis remains unknown as they await functional assessment.

In addition to genetic polymorphisms which may impair function of proteins or alter expression levels of proteins, variations in gene copy numbers (copy number variants) may also affect protein expression levels to modify risk of disease. Copy number variations represent a large source of genetic diversity. High throughput screening techniques have recently allowed genome-wide copy number variant screening in ALS patients. A recent study identified several copy number variants unique to ALS patients as well as a smaller number unique to controls subjects. Each of these alleles was found in a very small number of subjects and none of the copy-number variants were significantly different between the groups (Blauw, Veldink et al. 2008). However, the group of ALS patients did have a significantly greater total number of heterozygous deletions.

Several important points have been elucidated by the groundbreaking genome-wide studies mentioned above. The first genome-wide association study in ALS patients failed to identify any significantly associated genetic variants; however, the sharing of data
allowed subsequent studies to pool genotypes for greater power. The public sharing of
data has resulted in the efficient identification of genetic variants that each have a small
collection to ALS pathogenesis (OR < 2.0). The small contributions of many genes
may combine to increase risk of developing ALS. One must be careful to note that
genome-wide association studies are generally designed to identify tagging SNPs and the
SNPs and genes identified in these studies may be in linkage disequilibrium with true
disease-susceptibility alleles. Functional assessment of identified genes is necessary to
determine the roles of these identified genes in ALS pathogenesis.

Many studies have been conducted to search for and explore genes and allelic variants
associated with sporadic ALS. Currently, no single genetic variant has been determined
to account for a large number of cases. The identification of a large number of variants,
each present in a small number of subjects suggests the presence of a large number of
genetic risk factors for sporadic ALS. Many of the genome-wide association studies have
been underpowered to detect rare variants associated with sporadic ALS, highlighting the
importance of publicly sharing DNA samples and genotyping data (e.g. Coriell Institute
for Medical Research) to permit meta-analyses (Cronin, Berger et al. 2007; Schymick,
Scholz et al. 2007; van Es, van Vught et al. 2008). Additionally, additive effects of
multiple disease-associated alleles within individual subjects likely contribute to disease
risk, but are difficult to assess.

Studies examining the incidence of ALS in different geographic regions and populations
may provide clues into the pathogenesis of the disease. A recent review compared the
incidence of ALS between ethnicities (Cronin, Hardiman et al. 2007). However, incidence rates are difficult to compare because of a lack of studies in non-Caucasian populations, differences in reporting of age and sex of subjects, different study types (prospective and retrospective), and differences in disease definition. Future studies with standardized data collection should provide more reliable data. The available data suggests that the incidence of ALS is lower in Asian populations compared to Caucasian populations (Moriwaka, Okumura et al. 1993; Fong, Yu et al. 1996; Cronin, Hardiman et al. 2007). Several mortality studies and migration studies conducted with multiple ethnic subgroups in a single geographic region found higher incidences of ALS and ALS mortality rates in Caucasians than other ethnic groups. It should be noted that different incidence rates between different ethnic groups suggest genetic components to disease pathogenesis. Genetic variants which are more common in Caucasians may be factors in the apparent higher incidence of ALS in Caucasians. Consistent with this concept, the \textit{H63D HFE} polymorphism—a common genetic variant in Caucasians—has recently been associated with an increased risk of sporadic ALS.

**HFE and ALS**

In light of studies describing the potential role of \textit{HFE} variants in other neurodegenerative diseases (Buchanan, Silburn et al. 2002; Dekker, Giesbergen et al. 2003; Connor and Lee 2006), studies were initiated to determine the association with \textit{HFE} gene variants with ALS. Each of these studies is a retrospective case-control study that compared rates of two \textit{HFE} polymorphisms, termed \textit{H63D} and \textit{C282Y}, between ALS
patients and controls. The control groups used for each study consisted of either neurological disease controls (patients with neurological symptoms who were ultimately found not to have ALS) (Wang, Lee et al. 2004), or neurologically normal controls with no symptoms of ALS (Yen, Simpson et al. 2004; Goodall, Greenway et al. 2005; Restagno, Lombardo et al. 2007; Sutedja, Sinke et al. 2007). In addition to comparing allele frequencies and carrier rates between ALS patients and controls, most of the studies also sought to determine the association of \textit{HFE} polymorphisms with region of onset (limb vs. bulbar), disease progression, and age at onset. The vast majority of patients and controls included in these studies consisted of non-Hispanic Caucasians, a population considered to have the highest frequencies of \textit{HFE} polymorphisms (Merryweather-Clarke, Pointon et al. 1997), and possibly the highest rate of ALS diagnosis (Cronin, Hardiman et al. 2007). One study in particular included black, Asian, and Hispanic ALS patients who are known to have relatively low rates of \textit{HFE} polymorphisms (Yen, Simpson et al. 2004).

Because \textit{HFE} polymorphism rates vary significantly by population (Merryweather-Clarke, Pointon et al. 2000), it is important that each of these studies has examined subjects representing different populations and most accounted for ethnicity differences between patients and controls. The populations represented in these studies included Texas (Yen, Simpson et al. 2004), Central Pennsylvania (Wang, Lee et al. 2004), England and Ireland (Goodall, Greenway et al. 2005), the Netherlands (Sutedja, Sinke et al. 2007), and Italy (Restagno, Lombardo et al. 2007). Altogether these studies have examined \textit{HFE} polymorphisms in 989 ALS patients and 6634 controls. All but one study
individually showed an association between the H63D polymorphism and ALS (Wang, Lee et al. 2004; Goodall, Greenway et al. 2005; Restagno, Lombardo et al. 2007; Sutedja, Sinke et al. 2007). My evaluation of the role of the H63D variant in ALS began with a meta-analysis including all five studies published to date examining the association between ALS and HFE polymorphisms, which showed an odds ratio of 1.26 (95% CI 1.09-1.46) for all H63D carriers (Table 1.1).
Table 1-1: Association of the H63D and C282Y HFE polymorphisms with ALS.

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<tr>
<td>n</td>
<td>51 ALS 47 control</td>
<td>121 ALS 133 control</td>
<td>379 ALS 400 control</td>
<td>289 ALS 5886 control</td>
<td>149 ALS 168 control</td>
<td>989 ALS 6634 control</td>
</tr>
<tr>
<td>H63D Odds Ratio (95% CI)</td>
<td>1.12 (0.44-2.82)</td>
<td>2.54 (1.36-4.74)</td>
<td>1.85 (1.35-2.54)</td>
<td>1.06 (0.81-1.38)</td>
<td>2.32 (1.33-4.04)</td>
<td>1.26 (1.09-1.46)</td>
</tr>
<tr>
<td>C282Y Odds Ratio (95% CI)</td>
<td>0.92 (0.12-6.80)</td>
<td>3.32 (0.13-82.43)</td>
<td>0.95 (0.66-1.36)</td>
<td>0.67 (0.42-1.08)</td>
<td>1.91 (0.45-8.13)</td>
<td>1.01 (0.80-1.26)</td>
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One population (Dutch) demonstrated significantly higher age of onset for $H63D$ heterozygotes, but this was not found in other populations (Sutedja, Sinke et al. 2007). Another study showed a higher $H63D$ allele frequency in ALS patients > 50 years old, but this did not reach statistical significance (Wang, Lee et al. 2004). The $H63D$ polymorphism did not appear to have any effect on any other parameter included in the studies. Additionally, the rates of other $HFE$ polymorphisms ($S65C$, $C282Y$) did not differ between ALS patients and controls, and no effects of these other polymorphisms on disease parameters were seen. It must be noted that these other polymorphisms are much less frequent than $H63D$, and thus necessitate larger sample sizes for appropriate power.

Four studies from four different populations reported an association of the $H63D$ $HFE$ allele with ALS, all showing higher frequencies of the $H63D$ polymorphism in ALS patients. One study did not find an association, though this study had the smallest number of samples and the ALS patient group included ethnicities with lower $HFE$ minor allele frequencies (Yen, Simpson et al. 2004). None of these studies excluded the possibility that $H63D$ is in linkage disequilibrium with another potentially relevant SNP. For this reason, my dissertation begins with a more in-depth analysis of the genetic association of the $H63D$ $HFE$ variant with ALS (Chapter 2). The known functional significance of the $H63D$ polymorphism suggests that this SNP plays a significant role in ALS, and these functions will be further explored in subsequent chapters.
The Hemochromatosis (HFE) Gene

*HFE* was originally identified as *HLA-H* in association with the systemic iron overloading condition known as hereditary hemochromatosis (HH) (Feder, Gnirke et al. 1996). The *HFE* gene resides on chromosome 6p21.3 and includes six coding and one non-coding exons. The gene product, HFE, is a 343-residue major histocompatibility complex (MHC) class I-like, or class Ib molecule with similarity to MHC class I molecules. The 49 kDa protein has domains consisting of a signal sequence, α1 and α2 domains similar to the peptide binding region of MHC class I molecules, an immunoglobulin-like domain (α3), a transmembrane region and a cytoplasmic region (Feder, Gnirke et al. 1996; Feder, Tsuchihashi et al. 1997; Lebron, Bennett et al. 1998). Despite HFE’s similarity to MHC class I molecules which bind peptides and present them to T lymphocytes, HFE has a narrowed peptide binding groove and does not bind peptides (Lebron, Bennett et al. 1998).

Common single nucleotide polymorphisms (SNPs) in *HFE* gene are *rs1799945* in exon 2 and *rs1800562* in exon 4. The ancestral allele at *rs1799945* is a C resulting in a histidine at the 63rd residue, while the minor allele is a G, resulting in an aspartic acid residue (H63D) at this position in the α1 domain. A histidine at the 63rd position normally forms a salt bridge with an aspartate residue at the 95th position. Substitution of an aspartate at position 63 would likely disrupt this salt-bridge, leading to a local change in conformation (Lebron, Bennett et al. 1998). The ancestral allele at *rs1800562* encodes a G resulting in a cysteine at the 282nd residue while the minor allele is an A resulting in a
tyrosine at this position (C282Y), located in the α3 domain. This substitution disrupts a disulfide bond critical for tertiary structure of the protein (Feder, Gnirke et al. 1996; Lebron, Bennett et al. 1998).

Not only are the H63D and C282Y HFE polymorphisms known to have functional deficits, they are relatively common in the general population. Accordingly, the effects of these polymorphisms stand to impact a large number of people whose disease pathogenesis and treatment are related to iron regulation.

Data from the International HapMap Project (www.hapmap.org) show the H63D and C282Y allele frequencies vary significantly by population. The highest minor allele frequency for H63D allele was found in the Utah residents with ancestry from northern and western Europe (0.129), followed by the Han Chinese (0.023), and the Japanese (0.022) populations. No H63D alleles were detected in the Yoruba population. The C282Y allele was only detected in the Utah residents, with an allele frequency of 0.042. A worldwide effort to determine the frequencies of the H63D and C282Y alleles showed similar results to the HapMap project (Merryweather-Clarke, Pointon et al. 1997). Overall, the C282Y HFE allele frequency was 1.9%, and highest in northern Europeans, particularly in the Irish population. The H63D HFE allele frequency overall was 8.1%, and was higher throughout European populations than American, Asia, or African populations. The H63D allele frequency was highest in the Spanish Basque population, and more widespread than the C282Y variant, which was mostly limited to persons from northern Europe and their descendants.
Function of HFE Protein

HFE was originally identified by its association with the iron overload disease, hereditary hemochromatosis (Feder, Gmirke et al. 1996), suggesting a role in iron regulation. HFE forms a heterodimer with beta2-microglobulin (β2M) in the endoplasmic reticulum before being transported to the plasma membrane (Waheed, Parkkila et al. 1997). The α3 domain is involved in this non-covalent interaction with β2M. H63D and Wt HFE show no difference in cellular processing and localization, while the C282Y HFE variant fails to form a heterodimer with β2-microglobulin and is retained in the ER and Golgi (Feder, Tsuchihashi et al. 1997; Waheed, Parkkila et al. 1997).

Transferrin-mediated cellular iron acquisition is the major route by which cells may obtain iron. After iron-loaded transferrin binds to the transferrin receptor, the complex is internalized, followed by acidification of the endosome by a H^+-ATPase, which reduces the affinity of transferrin (Tf) for iron and iron is released. This iron is reduced and transported into the cytosol via DMT1 where it enters either the labile iron pool, is stored in ferritin, or is incorporated into one of many proteins utilizing iron. HFE is thought to directly affect transferrin-dependent cellular iron acquisition. Co-immunoprecipitation studies have shown that wildtype (Wt) and H63D HFE form a complex with the transferrin receptor (TfR) (Feder, Penny et al. 1998). At the plasma membrane, Wt HFE is proposed to decrease the affinity of transferrin receptor for diferric transferrin, leading to decreased uptake of iron into cells (Feder, Penny et al. 1998). Both the H63D and
C282Y forms of HFE are proposed to be deficient in this role as H63D HFE does not appear to decrease the affinity of TfR for Tf, and little C282Y HFE is present at the plasma membrane (Feder, Tsuchihashi et al. 1997; Feder, Penny et al. 1998).

Despite evidence of the H63D and C282Y HFE variants having the same effects on cellular iron affinity in vitro (Feder, Penny et al. 1998), the contribution of H63D HFE to hemochromatosis has been the subject of debate. While the H63D variant is more common than C282Y, it is involved in far fewer cases of hereditary hemochromatosis, suggesting alternative cellular functions. It has even been suggested that H63D doesn’t affect iron parameters (Carella, D'Ambrosio et al. 1997), though others have suggested merely a lower penetrance of the H63D allele. The creation of mice expressing the mouse orthologs of H63D and C282Y HFE (H67D and C294Y, respectively) shed some light on this discrepancy (Tomatsu, Orii et al. 2003). Mice expressing one H67D allele had higher levels of hepatic iron compared to mice expressing one Wt allele, but lower levels compared to mice lacking HFE, suggesting a deficient but not absent function for H63D HFE. Additionally, H67D homozygous mice had higher levels of hepatic iron compared to homozygous Wt mice (though only significantly higher in females), but lower levels than C294Y/H67D compound heterozygotes or C294Y homozygotes, showing functionality of H67D HFE intermediate between that of Wt HFE and C294Y HFE.

An important function of HFE seems to be the regulation of hepcidin production. Hepcidin is a small protein that binds to the iron export protein, ferroportin, causing its internalization and degradation (Nemeth, Tuttle et al. 2004). This inhibits iron efflux
from monocytic lineage cells and enterocytes, leading to decreased iron release from the gut epithelial cells into the plasma pool, and increased iron retention in reticulendothelial cells. Hepcidin production is normally stimulated by inflammatory cytokines and by iron loading (Pigeon, Ilyin et al. 2001; Lee, Peng et al. 2005; Wrighting and Andrews 2006). By regulating gut iron absorption and cellular iron distribution, hepcidin may be a central mediator in iron homeostasis. Inappropriately low levels of hepcidin have been observed in hemochromatosis patients possessing HFE polymorphisms and in mouse models lacking HFE expression (Bridle, Frazer et al. 2003), suggesting a mechanism by which HFE variants affect systemic iron regulation. Wildtype HFE may serve as a sensor of extracellular iron and a mediator of pathways leading to hepcidin production (Schmidt, Toran et al. 2008). The sequestration of C282Y HFE in the ER would likely disrupt this normal pathway, but the degree of impairment of H63D HFE is uncertain. It is possible that the C282Y and H63D forms of HFE have differing effects on the regulation of hepcidin. Decreased hepcidin levels associated with the C282Y HFE are proposed to cause increased bodily iron levels resulting in hemochromatosis (Piperno, Girelli et al. 2007). It is possible that the apparent lesser impact of H63D HFE on bodily iron accumulation may result from a reduced impact of this variant on normal hepcidin regulation.

At the cellular level, expression patterns of HFE indicate that it is in position to influence iron homeostasis in every tissue. At the time the HFE gene (then called HLA-H) was identified, mRNA expression was confirmed by Northern blot in all tissues except the brain (Feder, Gnirke et al. 1996). Subsequently, more sensitive techniques have identified
HFE mRNA expression in the CNS (Connor, Milward et al. 2001). Within the CNS, expression of the protein has been detected in the brain capillary endothelial cells, ependymal cells, choroid plexus epithelium, neurons associated with senile plaques, and astrocytes associated with the blood brain barrier (Connor, Milward et al. 2001). Additionally, HFE expression has been identified in anterior horn neurons (unpublished data). Early on, the brain was considered immune from iron overload associated with hereditary hemochromatosis because the mechanisms of brain iron acquisition were not well described, and brain iron acquisition was thought to be constant over a wide range of systemic iron measures (Hallgren and Sourander 1958). Newer evidence suggest this is not so, and iron accumulation and/or imaging abnormalities have been demonstrated in the brains of HH patients (Berg, Hoggenmuller et al. 2000; Nielsen, Vainer et al. 2000).

**Iron, Age, and Disease**

Hallgren et al. (Hallgren and Sourander 1958) were the first to describe an increase in brain non-heme iron with increasing age. Additionally, they found the highest cortical iron levels in the motor cortex, although overall, the highest iron levels were found in the extrapyramidal system. Bartozkis et al. (Bartzokis, Mintz et al. 1994) and Loeffler et al. (Loeffler, Connor et al. 1995) have also described age-dependent increases in brain iron. Bartozkis et al. (Bartzokis, Tishler et al. 2007) additionally described age-related increases in brain ferritin iron levels which were higher in men than women, leading the authors to speculate that these differences may explain the relationship of several neurodegenerative diseases with age and sex. The increases in brain iron with increasing
age may be one factor in age-related neurodegenerative diseases, such as Alzheimer’s disease, Parkinson’s disease, and ALS. Additionally, increases in iron have consistently been found in brains of Parkinson’s and Alzheimer’s patients (Connor, Menzies et al. 1992; Morris and Edwardson 1994; Morris, Kerwin et al. 1994).

Iron is the most abundant transition metal in the body and is a required component of Fe-S clusters and heme groups and a number of cellular enzymes. Iron also has the potential to promote formation of reactive oxygen species (ROS) via the Fenton reaction, whereby hydrogen peroxide in the presence of ferrous iron can form highly reactive hydroxyl radicals. Oxidative stress is a normal byproduct of cellular metabolism and is regulated by a variety of antioxidant mechanisms. However, iron must be strictly regulated to avoid excessive generation of ROS.

Iron management consists of a complex interaction between a large number of molecules. Non-heme iron in blood is tightly bound to (Tf) which solubilizes the ferric (Fe$^{3+}$) ion with an affinity constant on the order $10^{22}$ M$^{-1}$ (Aisen and Listowsky 1980). It is thought that most cell types possess transferrin receptor 1 (TfR1). Uptake of transferrin-bound iron mediated by TfR is generally considered the major route of cellular iron acquisition. The main storage molecule for iron within cells is ferritin, a conglomerate of 24 subunits of H- and L-ferritin polypeptides present in different proportions depending on cell type. The H-ferritin subunit possesses ferroxidase activity necessary for oxidizing Fe$^{2+}$ while the L-ferritin subunit participates in nucleation of iron within the core of the ferritin molecule (Levi, Yewdall et al. 1992). Additionally, the presence of a mitochondrial
ferritin has recently been identified. Cellular iron homeostasis is known to involve posttranscriptional regulation of transferrin receptor (TfR) and cytosolic ferritin expression through the interaction of their mRNAs with iron regulatory proteins, IRP1 and IRP2. IRP1 exists as cytosolic aconitase/IRP1 depending on the presence (aconitase) or absence (IRP1) of a Fe-S cluster. IRP2 does not have an Fe-S cluster, but is degraded in the presence of adequate iron (Kuhn 1998). These IRPs are able to respond to cellular iron levels by binding iron responsive elements (IREs) in conditions of iron starvation. Despite similar in vivo expression (ubiquitous) and in vitro activity, IRP2 is proposed to be the dominant IRP, particularly in the brain because IRP2 knockout mice develop neurodegenerative disease (LaVaute, Smith et al. 2001; Meyron-Holtz, Ghosh et al. 2004). TfR mRNA contains multiple IREs in its 3’ untranslated region (UTR). In conditions of low cellular iron, IRPs bind IREs, which stabilizes the mRNA leading to increased translation. Ferritin mRNA, both H- and L- chains, possesses an IRE in the 5’ UTR. When the 5’ IRE is bound by an IRP, translation is prevented leading to decreased ferritin levels (reviewed in (Pantopoulos 2004)).

Iron and ALS

A number of studies have examined the association of iron regulation with neurological conditions, including ALS. One small study of 8 Guamanian ALS patients found elevated levels of iron in frontal gray matter compared to 4 normal Guamanian control subjects (Yasui, Ota et al. 1993). Another small study found insignificantly increased mean levels of tissue iron deposition in the cytoplasm and nuclei of spinal motor neurons and cervical
spinal cord capillaries in ALS patients compared to normal control patients (Kasarskis, Tandon et al. 1995). Each of these studies was likely hampered by small sample sizes. It is also important to note that each of these studies utilized tissue samples obtained at autopsy and these findings may reflect either disease-specific processes or non-specific disease end-stage processes.

Spinal cord samples from mice overexpressing human SOD1\(^{G93A}\) compared to mice overexpressing human SOD1 show an increase in mRNA levels of H-ferritin, L-ferritin, and transferrin receptor at end-stage disease (Olsen, Roberds et al. 2001). While an increase in expression of H- and L-ferritin transcripts may reflect a protective response of cells to sequester excess iron released during neurodegeneration, the increase in transferrin receptor transcript would potentially increase uptake of iron into cells. These findings may seem to contradict each other, but it is important to note that transcript expression may not reflect protein expression, especially in the case of transcripts including iron responsive elements (IREs). Messenger RNAs that include IREs, such as transferrin receptor and ferritin, are regulated by the binding of iron regulatory proteins to the IRE more so than they are regulated by transcript number (Pantopoulos 2004). More recently, an abstract presented at the Society for Neuroscience 2007 Annual Meeting demonstrated increased levels of iron in ventral spinal cord motor neurons in mice overexpressing human SOD1\(^{G93A}\) as early as 4 weeks of age in mice that develop ALS-like symptoms at an average age of 90 days. Additionally, this iron deposition was associated with increased free radical production which was abrogated by administration of the iron chelator desferrioxamine (Lee, Shin et al. 2007).
Altered regulation of iron, consisting of excessive cellular iron loading or altered subcellular iron distribution, occurring at any point in ALS pathogenesis has the potential to impact the disease process. Excessive oxidative stress may impact a large number of disease-associated pathways, while iron may influence these pathways independent of oxidative stress as well. The mechanisms by which iron misregulation may impact the pathogenesis of ALS are explored below.

Mechanisms of Pathogenesis

A complete understanding of the pathogenesis of the disease will aid the identification of risk factors and facilitate the identification of therapeutic targets. Current theories about the pathways leading to selective motor neuron death, the pathological hallmark of ALS, suggest that a multitude of converging pathways contribute to disease pathogenesis. As ALS is defined by selective motor neuron death, early studies focused primarily on pathogenetic mechanisms in neurons. More recent evidence suggests other cells may be just as or more important in disease pathogenesis. This concept is perhaps best exemplified using transgenic mice expressing mutant human SOD1. Selectively decreasing mSOD1 expression in microglia or astrocytes both reduced late stage disease progression, while selective decrease of mSOD1 expression in motor neurons slowed disease onset and early progression (Boillee, Yamanaka et al. 2006; Yamanaka, Chun et al. 2008). At least for cases caused by mSOD1, this suggests that initial events in the pathogenetic cascade occur in neurons, which may then incite toxic responses in
surrounding glial cells. Whether this same sequence occurs in cases not involving mSOD1 is not known; however, a large number of pathogenetic similarities exist between mSOD1 models and sporadic cases.

Given the genetic and clinical variability of ALS and the selectivity of the disease to motor neurons, it is highly unlikely that any single pathway is responsible for disease pathogenesis. Implicated mechanisms center around the roles of neuroinflammation (Henkel, Engelhardt et al. 2004), oxidative stress (Barber, Mead et al. 2006), glutamate excitotoxicity (Van Damme, Dewil et al. 2005), mitochondrial dysfunction (Shaw, al-Chalabi et al. 2001), growth factor deficiency (Ekestern 2004), protein aggregation (Cluskey and Ramsden 2001), and cytoskeleton dysfunction (Julien and Beaulieu 2000). These pathways have the potential to influence each other and risk factors affecting multiple pathways are more likely to account for larger numbers of disease cases. As will be described, iron dyshomeostasis has the potential to impact many of these mechanisms of pathogenesis and may be a central mechanism.

Neuroinflammation

A wealth of information supports the role of excessive inflammation in amyotrophic lateral sclerosis. The evidence that mice lacking mSOD1 expression in microglia and astrocytes show slower late disease progression highlights the importance of these cells in ALS pathogenesis. At the cellular level, increased levels of T lymphocytes, reactive astrocytes, activated microglia and macrophages have been observed in patients and
disease models in regions of motor neuron loss (Kawamata, Akiyama et al. 1992). This nexus of cells may produce an overexuberant inflammatory response to damaged motor neurons. Inflammatory mediators released from activated inflammatory cells may then result in progressive destruction of neighboring motor neurons. As the resident immune cell in the CNS, microglia may play a central role in the inflammatory response which leads to progression of ALS.

The recruitment of glial cells to regions of motor neuron destruction and the proliferation of these cells in these regions have been well documented. Astrogliosis is a common finding in regions of motor neuron destruction and may influence later stage disease progression through activation of microglia (Yamanaka, Chun et al. 2008), among other mechanisms. Microgliosis been demonstrated in spinal cords of ALS patients and transgenic animal models (Kawamata, Akiyama et al. 1992; Hall, Oostveen et al. 1998). Microglia, derived from the monocytic lineage, represent the resident immune cells of the CNS. Under normal conditions they exist in a resting state, with the capacity to become activated in response to various stimuli, including direct injury and signaling molecules.

Microglia and macrophages are recruited to sites of motor neuron degeneration and become activated in response to cytokines and chemotactic factors. Damaged or stressed astrocytes and neurons are major sources of monocyte chemoattractant protein-1 (MCP-1) (Wilms, Sievers et al. 2003; Henkel, Engelhardt et al. 2004), a potent member of the CC chemokine family involved in recruitment of monocyte-lineage cells. Activation is induced by a variety of different factors resulting in different activation pathways.
Classical activation mostly results from IFN-γ stimulation of microglia, leading to a respiratory burst and secretion of mostly pro-inflammatory factors. Sources of IFN-γ include T lymphocytes (Gordon 2003) as well as microglia (Wang and Suzuki 2007), astrocytes (Abbas, Bednar et al. 2002), and neurons (Miyatake, Ikeda et al. 2006). Activated microglia are major sources of inflammatory mediators including IL-1, IL-6, and TNF-α. In addition to inflammatory mediators, microglia are the source of the respiratory or oxidative burst which releases neurotoxic reactive oxygen and reactive nitrogen species including nitric oxide, superoxide, hydrogen peroxide, hydroxyl radical, and peroxynitrite. Classically activated microglia secrete various chemokines such as MCP-1, and macrophage inflammatory protein-1 (MIP-1) which further recruit and activate microglia and macrophages (Wilms, Sievers et al. 2003). Microglia have the capacity to recruit lymphocytes and increase their proliferation through the secretion of factors such as IL-2, IL-8, IL-12, and IL-15 (reviewed in (Block and Hong 2005)).

Microglia are also activated by IL-4 or IL-13 resulting in alternative activation leading to a reduced respiratory burst and inhibited secretion of pro-inflammatory cytokines. However, alternative activation increases expression of MHC class II molecules and the mannose receptor, as well as chemokines including MCP-1 and CCL22 (Gordon 2003). Inhibition of microglial activation can occur through the binding of IL-10, TGF-β, IFN-α/β or glucocorticoids, which decrease expression of MHC class II molecules, proinflammatory cytokines and chemokines, and inhibit the respiratory burst and increase secretion of anti-inflammatory cytokines (Gordon 2003).
Numerous studies have described increased microglial recruitment and activation near regions of motor neuron death in ALS patients and animal models. Henkel et al. (Henkel, Engelhardt et al. 2004) demonstrated increased numbers of monocytic lineage marker transcripts in spinal cord tissue of both sporadic and familial ALS patients compared to non-neurological disease controls. Additionally, they found increased numbers of CD68+ microglia surrounding ventral horn neurons in ALS patients compared to controls. Transcript levels and immunostaining of the chemokine MCP-1 were also demonstrated to be significantly elevated in spinal cord tissue of sporadic ALS patients. This study determined CSF levels of MCP-1 were significantly higher in ALS patients compared to non-neurological disease controls. Increased numbers of activated microglia are present in the ventral horns and MCP-1 is elevated in the spinal cord of mutant SOD1 transgenic mice prior to the onset of disease (Alexianu, Kozovska et al. 2001; Henkel, Beers et al. 2006). MCP-1 is also elevated in CSF and serum of ALS patients (Baron, Bussini et al. 2005) (Wilms, Sievers et al. 2003; Tanaka, Kikuchi et al. 2006), suggesting the findings in the rodent models are relevant to patients. It is not known whether microglia accumulate prior to disease onset in humans.

As major sources of inflammatory cytokines and other potential mediators of ALS pathogenesis, activated microglia likely play a central and coordinating role in the disease process. Microglia are capable of producing factors that are directly toxic to motor neurons, as well as cytokines that induce surrounding astrocytes and T lymphocytes to produce inflammatory mediators.
A number of studies have shown T lymphocyte margination in blood vessels near degenerating motor neurons, as well as infiltration of T lymphocytes into the spinal cord parenchyma (Troost, van den Oord et al. 1989; Kawamata, Akiyama et al. 1992). CD4+ T lymphocytes are present in spinal cord parenchyma near corticospinal tracts, and CD8+ and CD4+ T lymphocytes are present in the ventral horns of ALS patients at autopsy. T lymphocytes have also been found in the spinal cords of the mutant SOD1 transgenic mice, although not until late in the disease course (Alexianu, Kozovska et al. 2001). ALS patients may also be characterized by greater proportions of both CD4+ and CD8+ T lymphocytes expressing intracellular IL-13 compared to controls (Shi, Kawano et al. 2007). The significance of this finding lies in the known functions of IL-13 in stimulating humoral immunity, increasing MCP-1 synthesis in cells of monocytic lineage, and upregulating vascular cell adhesion molecule-1 (VCAM-1) (Gordon 2003). Thus, accumulation of T lymphocytes can in turn lead to further recruitment and activation of microglia and cytotoxic mediators produced by these cells may be detrimental to motor neurons.

Molecular markers of neuroinflammation have been demonstrated in animal models and human ALS patients. Numerous biomarker studies have demonstrated elevated levels of the inflammatory cytokines IL-1β, IL-6, and TNF-α in tissue and fluid samples of ALS patients and in animal models (Sekizawa, Openshaw et al. 1998; Hensley, Fedynyshyn et al. 2003; Moreau, Devos et al. 2005). Additionally, gene expression studies and immunohistochemistry of spinal cords from human ALS patients and animal models have revealed elevated transcript levels and positive immunostaining for inflammatory
markers. Sasaki et al. (Sasaki, Shibata et al. 2000) demonstrated positive immunostaining of microglial inducible nitrous oxide synthase (iNOS) in spinal cords of ALS patients, particularly in regions of motor neuron destruction. Cycloxygenase-2 (COX-2), which catalyzes the production of the potent inflammatory mediator, prostaglandin E2 (PGE2), is induced in astrocytes, microglia, and neurons in the spinal cords of ALS patients (Almer, Guegan et al. 2001; Drachman, Frank et al. 2002). In a SOD1^{G93A} mouse model, which has increased levels of PGE2 compared to nontransgenic mice, treatment with a selective COX-2 inhibitor decreased production of prostaglandin E2, delayed disease onset, and prolonged survival (Drachman, Frank et al. 2002). Unfortunately, clinical trials of celecoxib (a COX-2 inhibitor) failed to show any benefit of the drug in ALS patients (Cudkowicz, Shefner et al. 2006). Microglia are also sources of anti-inflammatory mediators such as TGF-β and IL-10, and trophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF). Recent opinions increasingly support the notion of variable activation pathways of microglia (Gordon 2003). The potential for microglia to exert a protective effect in the CNS exists, but evidence suggests a net negative effect on motor neuron survival. Additionally, the influence of microglial activation and inflammation may change throughout the disease course. The tetracycline derivative, minocycline, which has a well-characterized inhibitory effect on microglial activation, delayed both disease onset and death in mSOD1 mice in several studies (Kriz, Nguyen et al. 2002; Van Den Bosch, Tilkin et al. 2002; Zhu, Stavrovskaya et al. 2002; Zhang, Narayanan et al. 2003). Unfortunately, minocycline failed to show clinical benefit in ALS patients in a recent
phase III trial (Gordon, Moore et al. 2007). The potential reasons for this lack of efficacy are numerous, including insufficient doses of drug, toxicity to cells other than microglia, or treatment too late in the disease process.

As discussed more in following sections, neuroinflammation detrimentally influences a number of other pathways implicated in ALS pathogenesis. Activation of microglia is associated with a respiratory burst characterized by extracellular release of reactive oxygen and nitrogen species. In this manner, inflammatory pathways leading to microgliosis and activation of microglia are likely to lead to localized increases in oxidative stress. Prostaglandin E2, mainly produced in microglia by cyclooxygenase, induces astrocytes to release glutamate (Drachman, Frank et al. 2002). Astrocytes are the cells primarily responsible for removing synaptic glutamate to prevent glutamate excitotoxicity. Microglial activation may thus enhance oxidative stress and glutamate excitotoxicity, which may explain some of the benefits of anti-inflammatory treatments.

Regulation of iron within the cells involved in the inflammatory pathway affects their function and thus impacts inflammatory conditions. Evidence suggests that cell-specific increases in iron may enhance inflammatory signaling. Cyclooxygenase (COX) is a heme protein which catalyzes the conversion of arachidonic acid to a variety of prostaglandins involved in inflammatory signaling, and activity of cyclooxygenase is sensitive to the availability of iron in the form of heme groups (Mancuso, Perluigi et al. 2006). Lymphocyte proliferation is dependent upon transferrin-mediated iron uptake (Seligman, Kovar et al. 1992), and T<sub>H1</sub> cells in particular are sensitive to inhibition of the transferrin
receptor (Weiss 2005). Iron loading in microglia enhances their response to LPS and iron chelation diminishes the response, influencing the secretion of IL-1β and TNF-α (Zhang, Surguladze et al. 2006). It is unclear whether activation of microglia by IFN-γ or other mediators is equally sensitive to iron, but these results suggest a mechanism by which iron regulation may impact inflammatory pathways.

In turn, inflammation affects iron regulation, both at the local and systemic levels. Transcriptional regulation of hepcidin is upregulated by IL-6 (Wrighting and Andrews 2006) and IL-1 (Lee, Peng et al. 2005). Increases in hepcidin levels are typically associated with decreased gut absorption of iron and increased stores of iron in the monocyte-lineage cells comprising the reticuloendothelial system (Deicher and Horl 2006). This cellular redistribution may actually limit the potential iron-mediated toxicity in some cells, but increase inflammatory signaling pathways in macrophages and microglia (Zhang, Surguladze et al. 2006). Iron-mediated oxidative stress induces nuclear localization of NF-κB which upregulates transcription of a large number of inflammatory genes (Simmonds and Foxwell 2008). In contrast, deficient production of hepcidin, which frequently accompanies expression of the H63D and C282Y HFE variants, leads to reduced accumulation of iron in macrophages, which diminishes their release of select inflammatory cytokines (Wang, Johnson et al. 2008). However, the pro-inflammatory cytokine, IL-1β, induces expression of the ferroxidase ceruloplasmin in astrocytes (Kuhlow, Krady et al. 2003). Ceruloplasmin within the CNS may increase the binding of ferric iron to transferrin, thereby facilitating transferrin-mediated iron uptake by neurons. Thus iron and neuroinflammation are intimately related with increases in iron potentially
stimulating inflammatory signaling which in turn may influence distribution of iron stores.

Based on studies suggesting elevated levels of inflammatory biomarkers associated with ALS, the second major focus of my thesis focused on identifying a panel of biomarkers able to distinguish ALS patients from control subjects. While the previous studies mentioned above identified inflammatory markers associated with disease, we hypothesized that a panel would be necessary for accurate classification of health status and would provide more useful information concerning disease pathogenesis. Additionally, we hypothesized that HFE variants would impact the expression of these biomarkers due to the influence of iron regulation on inflammatory pathways.

**Oxidative Stress**

Oxidative stress generally can be defined as the overwhelming of antioxidant defenses by oxygen radicals, resulting in molecular modification and damage. Oxidative stress normally occurs during oxidative phosphorylation, and thus, mitochondria are a major source of oxygen radicals. One proposal for the selective vulnerability of motor neurons in ALS includes the large size and metabolic requirements of motor neurons, necessitating numerous mitochondria. As discussed above, activated microglia are sources of nitric oxide and superoxide as part of the respiratory burst. Most evidence of iron loading causing toxicity centers on the formation of reactive oxygen species by the Fenton reaction. These different mechanisms of generating oxidative stress are all implicated in ALS pathogenesis with widespread effects on other pathways.
Reactive oxygen species (ROS) including superoxide and hydroxyl radical, among others, cause cellular damage through several mechanisms (Scandalios 2005). ROS can react with lipids, particularly in membranes, to cause lipid peroxidation and membrane leakage. Commonly-measured biomarkers of oxidative stress include lipid peroxidation byproducts such as isoprostanes and malondialdehyde. ROS cause significant protein damage through amino acid modifications, including formation of carbonyl groups, and fragmentation of peptide chains. Carbonyl groups are formed specifically on lysine, arginine, proline, and threonine (Scandalios 2005), and are also frequently measured as biomarkers of oxidative stress. Alteration of proteins leads to aggregation, cross-linking, and increased susceptibility to proteolysis. Oxidative damage to DNA includes cross-linkage of DNA and proteins, and formation of DNA adducts leading to DNA deletions, mutations, and translocations. A frequent result of oxidative damage to DNA is the easily measured 8-hydroxydeoxyguanosine (8-oxo-dG). No cancers have been directly linked to 8-oxo-dG formation, but it is known to cause GC→TA transversions (Aust and Eveleigh 1999). Another frequent indicator of oxidative stress is the induction of antioxidant proteins. The production of reactive oxygen species is balanced by antioxidant defenses including glutathione, glutathione peroxidase, glutathione transferases, catalase, superoxide dismutases, heme oxygenase and other proteins.

Whether oxidative stress occurs early in the disease process, or whether it occurs later as a result of other dysfunctional pathways is not known. Regardless, the effects of oxidative stress on cellular function likely have damaging consequences on the disease
process. Either through the respiratory burst of microglia, transition metal dysregulation, or inefficient oxygen utilization by damaged mitochondria, excessive oxidative stress is proposed to play a role in the pathogenesis of ALS.

Numerous studies have demonstrated evidence of increased oxidative stress at multiple levels in ALS patients compared to controls (reviewed in (Barber, Mead et al. 2006)). Shaw et al. (Shaw, Ince et al. 1995) found elevated levels of protein carbonyls in lumbar spinal cord samples of ALS patients compared to both normal controls and a neurological disease control group. Although the neurological disease control group included many subjects with neurodegenerative diseases, the involvement of the lumbar spinal cord in other conditions may be substantially less than in ALS. Ferrante et al. (Ferrante, Browne et al. 1997) described an increase in protein carbonyls and 8-oxo-dG in the primary motor cortex, particularly in sporadic ALS patients. Similar markers were not found in the parietal cortex or cerebellum, suggesting changes related to the ALS disease process. However, like other studies using post-mortem specimens, the timing of oxidative stress in disease pathogenesis was not clarified by either of these studies and may only reflect late stage developments. In contrast, biomarker studies using fluids collected at time of disease onset or at time of diagnosis might show more specific earlier changes associated with disease. Using CSF obtained from ALS patients an average of less than 9 months after disease onset, Siciliano et al. (Siciliano, Piazza et al. 2007) demonstrated elevated levels of oxidized proteins and reduced anti-oxidant capacity in ALS patients compared to normal controls. Such early changes in biomarkers may provide information more specific to ALS and may also aid in diagnosis.
Although these studies strongly suggest oxidative stress associated with ALS pathogenesis, this phenomenon is clearly not specific to ALS. Oxidative modification has been demonstrated with aging processes as well as numerous neurological conditions (reviewed in (Beal 2002)). Use of modified molecules as biomarkers to aid in diagnosis or to provide more specific clues into pathogenesis may require identification of specific cells or specific molecules affected by oxidative stress.

The most widely studied protein associated with ALS, SOD1, normally has a prominent role in the protection against oxidative stress through its conversion of superoxide to hydrogen peroxide. Initial hypotheses that deficient dismutase activity resulting from the various mutations was the cause of ALS proved untrue, as many SOD1 mutations are not thought to disrupt the dismutase activity of this enzyme (Valdmanis and Rouleau 2008). Both mutations disrupting the copper and zinc binding regions and those not affecting these regions produce similar phenotypes, suggesting copper and zinc-mediated activities are not involved in disease pathogenesis (Shaw and Valentine 2007). Aggregation of mutant SOD1 promoted by malformation or oxidative modification has thus been proposed as a common mechanism associated with all mutant forms of SOD1 (Shaw and Valentine 2007). SOD1 has been shown to be a major target of oxidative stress (Andrus, Fleck et al. 1998), and oxidized Wt SOD1 is thought to acquire many of the same properties as mutant SOD1. SOD1 has been recently proposed to play a role in all cases of ALS (Kabashi, Valdmanis et al. 2007), and as such, factors that increase oxidation of
SOD1 would increase the risk of developing ALS. This theory, if true, might implicate oxidized SOD1 as a specific biomarker of ALS.

Oxidative stress likely does not function as an independent mechanism of ALS pathogenesis. Oxidatively modified proteins and lipids have altered functionality, leading to decreased activity of enzymes, and structural defects in cellular membranes including the inner mitochondrial membrane. Proper function of the inner mitochondrial membrane is crucial for the generation of the proton gradient needed for ATP synthesis, and thus, oxidative stress ultimately may impair cellular energy production and pathways dependent on proper mitochondrial function. Excessive cytosolic calcium is buffered in mitochondria and motor neurons depend on this method for calcium buffering to a larger extent than many other neurons (Barber, Mead et al. 2006). Detrimental effects of excessive cytosolic calcium are discussed later.

Transition metals, particularly iron, can catalyze the formation of reactive oxygen species resulting in oxidative stress. Hydrogen peroxide in the presence of ferrous iron can form highly reactive hydroxyl radicals, forming ferric iron in the process. This reaction is commonly known as the Fenton reaction and is proposed to impact a large number of conditions associated with iron loading. Oxidative stress may also create a vicious cycle by increasing TfR expression and cellular iron acquisition via increased IRP1 activity. Aberrantly increased activity of IRP1 in the presence of iron loading has been proposed to result from oxidative modification of protein, inhibiting the functional Fe-S cluster needed for aconitase activity (Nunez-Millacura, Tapia et al. 2002). In this manner,
oxidative stress unrelated to local iron accumulation may in turn result in iron
misregulation and further oxidative stress.

*Mitochondrial Dysfunction*

Mitochondria have been proposed as both a source and a target of the ALS-associated
pathogenetic mechanisms. The majority of cellular oxygen and iron are utilized in
mitochondria, which also serve a major role in regulating apoptosis, ATP synthesis, and
calcium buffering. Dysfunction in mitochondria has the potential to impact many of the
pathways implicated in ALS pathogenesis.

Mitochondria are subjected to high levels of oxidative stress due to their roles in oxygen
and iron utilization. A gradual buildup of mitochondrial and cellular damage caused by
oxidative stress that slightly out-competes antioxidant defenses is a popular theory for the
aging process (Terman and Brunk 2006). Mitochondrial DNA, which comprises 37 genes
coding for proteins involved in the electron transport chain as well as rRNA and tRNA, is
particularly susceptible to oxidative damage (Chen, Hales et al. 2007). Mitochondria are
relatively lacking in DNA repair mechanisms, exposing the electron transport chain to the
mutagenic effects of oxidative DNA damage. Mitochondrial dysfunction may thus play a
role in diseases associated with advanced age, including ALS.

Mitochondria serve as a buffer for excessive cytosolic calcium, which, if not properly
regulated, is toxic to cells by a number of mechanisms. Elevated calcium levels may
activate phosphatases and degradative enzymes including proteases, lipases, and endonucleases, as well as alter exocytosis. Calcium loading in mitochondria may in turn also induce mitochondrial toxicity via generation of reactive oxygen species in mitochondria and opening of the permeability transition pore (Hansson, Mansson et al. 2008). Permeability transition ultimately results in collapse of the mitochondrial membrane potential, impaired ATP synthesis and release of cytochrome c.

A variety of studies support a role for mitochondrial dysfunction in the pathogenesis of ALS. Morphologically, conglomerates of mitochondria in skeletal muscle and anterior horn neurons of ALS patients and vacuolization of mitochondria in transgenic mouse models suggest mitochondrial abnormalities (Dupuis, Gonzalez de Aguilar et al. 2004). Functionally, decreased complex I activity, ATP synthesis, and mitochondrial superoxide dismutase-2 activity in ALS patients and disease models likewise suggest mitochondrial dysfunction involved in ALS pathogenesis (Dupuis, Gonzalez de Aguilar et al. 2004).

Mice overexpressing human SOD1$^{G93A}$ show impaired mitochondrial calcium buffering specific to the CNS prior to onset of symptoms, similar to mice overexpressing human SOD1$^{G85R}$ at symptom onset (Damiano, Starkov et al. 2006). However, the SOD1$^{G93A}$ but not the SOD1$^{G85R}$ mice are characterized by deficient ATP synthesis and oxygen consumption (Damiano, Starkov et al. 2006). These findings demonstrate mitochondrial dysfunction in two ALS mouse models, but suggest different contributions to overall pathogenesis. Additionally, wildtype SOD1 and several mutant forms of SOD1 have been
shown to localize to mitochondria, with increased generation of superoxide by the mutant forms (Zimmerman, Oberley et al. 2007).

Although most evidence of mitochondrial involvement in ALS has been provided by mouse models expressing mutant human SOD1, there is evidence of mitochondrial dysfunction in sporadic ALS patients. Mitochondria in spinal cords of sALS patients are characterized by morphologic abnormalities largely absent from control spinal cord sections (Sasaki and Iwata 2007). Decreased activity of cytochrome c oxidase has been demonstrated in sALS spinal cords (Fujita, Yamauchi et al. 1996; Borthwick, Johnson et al. 1999). Because several subunits of the cytochrome c oxidase complex are encoded in the mitochondrial genome, it was suggested this was the result of mutated mitochondrial DNA (Borthwick, Johnson et al. 1999). Alternatively, oxidative modifications of the protein subunits may impair enzymatic function. In contrast to these studies using post-mortem tissue, Murata et al. (Murata, Ohtsuka et al. 2008) demonstrated elevated levels of oxidized coenzyme Q10 in the CSF of sALS patients. Oxidized CoQ10 was proposed as a sensitive measure of mitochondrial oxidative stress, particularly compared to other antioxidants (Murata, Ohtsuka et al. 2008). Since the CSF was obtained from the majority of subjects less than two years after disease onset, and from many subjects less than one year after onset, this finding may indicate relatively early mitochondrial oxidative stress in sALS.

The fact that many of the mitochondrial abnormalities in ALS patients and mutant SOD1 transgenic mouse models are not limited to motor neurons or even the nervous system
does not contradict the involvement of mitochondria in ALS pathogenesis. It does suggest mitochondrial dysfunction is not the sole pathway contributing to disease pathogenesis. Mitochondria are intricately involved in other proposed pathogenic mechanisms and may be a central mediator of disease pathogenesis.

Mitochondria are the major site of iron utilization as the synthesis of Fe-S clusters and the incorporation of iron into heme groups occurs in mitochondria. Thus mitochondria are subjected to the potential toxicity of iron-mediated generation of reactive oxygen species. It has been proposed that mitochondria regulate cellular iron distribution through uptake and utilization of iron for functional groups and the export of these groups from the mitochondria (Rouault and Tong 2005). Some mitochondria contain mitochondrial ferritin, a molecule with high degree of similarity to H-ferritin, whose regulation is mostly unknown (Drysdale, Arosio et al. 2002). Mitochondrial ferritin may play an important role in sequestering iron away from reactive oxygen species produced by electron transport chain complexes. Upregulation of mitochondrial ferritin may also serve to limit labile iron available in the cytosol (Nie, Sheftel et al. 2005). Whether mitochondrial ferritin may be manipulated to limit iron-mediated oxidative stress and mitochondrial damage remains to be determined, but it is an exciting possibility. The sensitivity of mitochondria to oxidative stress and the accumulation of bodily iron with increasing age may combine to result in a greater influence of mitochondrial dysfunction on disease pathogenesis with increasing age. Additionally, the central role of mitochondria in producing energy needed for other pathways involved in ALS
pathogenesis and buffering calcium to prevent glutamate excitotoxicity (discussed below) demonstrate the need for proper mitochondrial function.

Glutamate Excitotoxicity

Glutamate is tightly regulated within the CNS because it can function as an excitatory neurotransmitter, yet has the propensity to cause excitotoxic damage. Glutamate excitotoxicity results from neurons being exposed to elevated extracellular glutamate levels or especially susceptible neurons being exposed to normal glutamate concentrations. Stimulation of ionotropic glutamate receptors resulting in postsynaptic calcium influx is considered to underlie the toxic effects of extracellular glutamate. A strong line of evidence supporting the role of glutamate toxicity in the pathogenesis of ALS is that riluzole, the only United States Food and Drug Administration-approved drug to treat ALS, has anti-glutamatergic properties.

Extracellular glutamate levels in the CNS are regulated through both secretion and uptake of glutamate. The glutamate-glutamine cycle is one pathway through which appropriate handling of glutamate is achieved. In this model, neurons synthesize and secrete glutamate which is taken up by astrocytes and converted to glutamine. The non-excitotoxic glutamine is then transferred back to neurons before being reconverted to glutamate by glutaminase (Daikhin and Yudkoff 2000).
The vesicular secretion of glutamate from neurons is a regulated component of the glutamate-glutamine cycle. Expression of vesicular glutamate transporters (VGLUTs) in a neuron is sufficient to confer glutamate secretion properties to the cell (Takamori 2006) (Takamori, Rhee et al. 2000). VGLUTs transport glutamate into synaptic vesicles via a proton-dependent electrochemical gradient in preparation for calcium-dependent exocytosis. To date, three isoforms of VGLUTs have been identified, labeled as VGLUT1-3. VGLUT1 and VGLUT2 are predominantly expressed in the brain, mostly in complimentary regions, though occasionally cells have been shown to co-express both (Boulland, Qureshi et al. 2004; Wojcik, Rhee et al. 2004; Billups 2005). Wojcik et al. (Wojcik, Rhee et al. 2004) also demonstrated that the level of expression of VGLUTs can influence the amount of glutamate secreted from neurons by showing that overexpression resulted in larger vesicular quantal size, while decreased VGLUT expression results in smaller vesicular quantal sizes (Moechars, Weston et al. 2006).

Following presynaptic release of glutamate, excitatory amino acid transporters (EAATs), primarily located on surrounding astrocytic as well as neuronal membranes, are responsible for clearing glutamate and preventing toxic concentrations of extracellular glutamate (Rothstein, Jin et al. 1993). Five EAATs, named EAAT1-5, have been identified. EAAT1 and EAAT2 are considered to be expressed exclusively on glial cell membranes throughout the CNS, while EAAT3 is widely expressed on neurons (Shigeri, Seal et al. 2004). EAAT4 expression is generally limited to the cerebellum and Purkinje cells in particular, but expression may be induced in astrocytes in select circumstances (Yi, Herrero et al. 2007). EAAT5 expression may be limited to the retina (Arriza, Eliasof
et al. 1997). Alternative expression of these transporters has been suggested; however, these trends at least largely appear true. Uptake of glutamate via EAATs is ultimately energy dependent since glutamate is co-transported with Na\(^+\) along its electrochemical gradient established by the Na\(^+\), K\(^+\)-ATPase. Astrocytes are responsible for clearing the majority of glutamate from the synaptic cleft, primarily via the EAAT2 transporter (Van Den Bosch, Van Damme et al. 2006).

Riluzole is the only USFDA-approved treatment for ALS, but the exact mechanism by which riluzole acts is not known. Riluzole has been proposed to inhibit both spontaneous and evoked synaptic release of glutamate, possibly by blockade of sodium channels (Cheramy, Barbeito et al. 1992; Martin, Thompson et al. 1993), calcium channels (Wang, Wang et al. 2004), or potassium channels (Zona, Siniscalchi et al. 1998). Riluzole has also been shown to increase the affinity of EAATs for glutamate and increase glutamate uptake (Fumagalli, Funicello et al. 2008). Additionally, inhibition of postsynaptic glutamatergic signaling by riluzole has been suggested (Debono, Le Guern et al. 1993).

The efficacy of riluzole in treating ALS was demonstrated in both mouse models and humans (Bensimon, Lacomblez et al. 1994; Gurney, Cutting et al. 1996; Lacomblez, Bensimon et al. 1996), suggesting a significant contribution of glutamate excitotoxicity to ALS pathogenesis. Both human ALS patients and mutant SOD1 transgenic rodent models have decreased expression of EAAT2 resulting in deficient glutamate uptake (Rothstein, Van Kammen et al. 1995; Bendotti, Tortarolo et al. 2001). A beta-lactam antibiotic, ceftriaxone, induces the expression of EAAT2 and has proven beneficial in delaying
onset of symptoms and prolonging survival in an ALS rodent model (Rothstein, Patel et al. 2005). CSF levels of glutamate have been examined in ALS patients, with variable results. While some studies have found no elevation of plasma or CSF glutamate associated with ALS (Perry, Krieger et al. 1990; Bendotti, Tortarolo et al. 2001), other have suggested elevated glutamate concentrations may reflect a disease subtype, particularly associated with limb onset (Camu, Billiard et al. 1993; Spreux-Varoquaux, Bensimon et al. 2002).

Excitotoxicity associated with extracellular glutamate is likely mediated through the ionotropic AMPA and NMDA glutamate receptors. Motor neurons, compared to other neurons, have been shown to be especially susceptible to AMPA-mediated glutamate excitotoxicity (Van Damme, Dewil et al. 2005). AMPA receptors are variably permeable to calcium depending on the presence of the GluR2 subunit, which reduces calcium permeability compared to other AMPA subunits (Hollmann, Hartley et al. 1991). A relative lack of the GluR2 AMPA receptor subunit in motor neurons is proposed to underlie the selective vulnerability of motor neurons in ALS (Van Damme, Dewil et al. 2005).

Many of the effects of glutamate excitotoxicity are mediated through increases in intracellular calcium levels. Motor neurons may be especially susceptible to glutamate excitotoxicity because of their particular sensitivity to fluctuations in cellular calcium levels. Buffering of cytosolic calcium is normally accomplished by sequestration in endoplasmic reticulum and mitochondria, and binding to cytosolic calcium binding
proteins, including parvalbumin and calbindin D-28K. In mice expressing mutant human SOD1<sup>G93A</sup>, expression of parvalbumin in affected neurons decreases in early stages of disease, while expression of calbindin D-28K is largely absent from affected spinal cord neurons in transgenic and control mice (Sasaki, Warita et al. 2006). Autopsy specimens from control subjects showed a lack of calbindin D-28K and parvalbumin expression in spinal ventral horn motor neurons and cranial nerve motor neurons typically affected by ALS (Alexianu, Ho et al. 1994). However, motor neurons relatively unaffected by ALS and other neurons generally showed expression of at least one of these two calcium binding proteins. Thus, the motor neurons involved in ALS pathogenesis appear predisposed to glutamate excitotoxicity.

Several studies have suggested the possibility that iron impacts cellular glutamate regulation. A recent metabolic analysis of mice deficient in H-ferritin, the predominant iron storage protein in neurons, revealed higher levels of glutamate in several brain regions (Ill, Mitchell et al. 2006), although exact location of the glutamate could not be determined. McGahan et al. (McGahan, Harned et al. 2005) demonstrated that iron-loading increased glutamate secretion by stimulating glutamate production through modulation of cytosolic aconitase activity in both neurons and retinal pigment epithelial cells. Iron-induced oxidative stress is also associated with increased release of glutamate from retinal cells (Rego, Santos et al. 1996). Glutamate clearance may also be impaired by oxidative stress which has the potential to increase glutamate excitotoxicity by modifying EAATs resulting in inactive transporters (Miralles, Martinez-Lopez et al. 2001). Thus there is compelling evidence that alterations in cellular iron status can impact
glutamate regulation and thus, potentially increase glutamate toxicity. Altered cellular iron status may also play a role in another recent topic of ALS research, that being the role of growth factor deficiency in ALS pathogenesis, and the potential use of growth factors for treatment.

**Growth Factor Deficiency**

A deficiency of neurotrophic factors or signaling pathways involving these factors could logically contribute to vulnerability of motor neurons to other insults. Vascular endothelial growth factor (VEGF) was first implicated in ALS when mice with reduced expression of VEGF developed adult-onset motor deficits and neurodegeneration (Oosthuyse, Moons et al. 2001). Subsequent studies have implicated growth factor deficiency as a contributing mechanism in ALS pathogenesis and suggested neurotrophic factors as potential therapeutic agents.

In addition to promoting angiogenesis, VEGF is also a neurotrophic factor which promotes neuronal growth during development and protects neurons from a variety of insults including hypoxia, glucose and serum deprivation, and glutamate excitotoxicity (reviewed in (Brockington, Lewis et al. 2004)). Deletion of the hypoxia response element in the promoter of the *VEGF* gene in mice resulted in decreased protein expression and adult-onset motor dysfunction (Oosthuyse, Moons et al. 2001), leading to studies of the role of VEGF in ALS. Initial genetic association studies have identified risk-associated haplotypes in the *VEGF* gene (Lambrechts, Storkebaum et al. 2003), although later
studies failed to confirm these results (Chen, Saeed et al. 2006; Del Bo, Scarlato et al. 2008). Angiogenin is an angiogenic factor with similar actions and regulation to VEGF. Genetic association studies have identified a risk-associated SNP in the *angiogenin* gene in some populations (Greenway, Alexander et al. 2004) (Greenway, Andersen et al. 2006), although studies in other populations also failed to verify this finding (Corrado, Battistini et al. 2007; Del Bo, Scarlato et al. 2008). Like *VEGF* haplotypes, *angiogenin* haplotypes may be associated with a small number of cases of ALS in select populations.

A number of studies have evaluated growth factors as biomarkers in ALS with mixed results. VEGF levels have been found to be both elevated in ALS patients with longer duration of disease (Ilzecka 2004) and reduced in the CSF of ALS patients early in the course of the disease (Devos, Moreau et al. 2004), suggesting VEGF may be a biomarker of disease progression. Longitudinal studies involving serial sampling of CSF from ALS patients throughout the course of the disease might further elucidate the pattern of VEGF expression with disease progression. Cronin et al. (Cronin, Greenway et al. 2006) found no difference in serum VEGF levels between ALS patients and healthy controls, but they found a decrease in serum VEGF levels in ALS patients over a 12 month period. In this same study, they found higher serum levels of angiogenin in ALS patients than healthy controls, but these levels did not change over the 12 month period.

Both *VEGF* and *angiogenin* genes have hypoxia response elements (HRE) in their promoters and are inducible by hypoxia. Respiratory insufficiency associated with ALS leading to hypoxia may be expected to increase the transcription of these hypoxia-
inducible genes. ALS patients may have abnormal regulation of VEGF in particular. In contrast to controls, hypoxia in ALS patients has been associated with lower CSF levels of VEGF and a negative correlation between arterial oxygen partial pressure and CSF VEGF (Moreau, Devos et al. 2006).

Treatment of ALS patients with neurotrophic factors is a logical therapeutic approach, even in patients without growth factor deficiencies. Breeding VEGF-deficient mice with transgenic mice expressing the mutant human SOD1 resulted in shortened survival (Lambrechts, Storkebaum et al. 2003), while exogenous delivery of VEGF prolongs survival of mutant SOD1 transgenic rodents (Azzouz, Ralph et al. 2004; Storkebaum, Lambrechts et al. 2005). Another neurotrophic factor, insulin-like growth factor-I (IGF-I) also delays disease endpoint in these models; however, it has not shown a consistent clinical benefit in humans (Mitchell, Wokke et al. 2002).

Expression of hypoxia-inducible genes is activated when hypoxia inducible factor (HIF) binds to the hypoxia response element (HRE) in the genes’ promoters. HIF subunits are constitutively expressed and HIF-1α is usually quickly degraded following hydroxylation of proline residues by the iron-containing enzymes HIF-prolyl 4-hydroxylases (PHDs). These enzymes have become pharmacologic targets, and studies have shown their activity to be inhibited by iron chelation, and thus lower iron levels may result in upregulation of hypoxia responsive genes, and vice versa (Callapina, Zhou et al. 2005; Siddiq, Aminova et al. 2007). Neuronal toxicity of oxygen and glucose deprivation can be reduced by pretreatment with the iron chelator, desferroxamine, mediated by HIF-1α.
(Hamrick, McQuillen et al. 2005). Cellular increases in iron may similarly prevent normal induction of hypoxia-inducible genes. In this manner, genetic or environmental factors causing iron loading may inhibit the protective upregulation of VEGF, angiogenin and other hypoxia-inducible genes. Cellular iron loading may also be a factor in the induction of protein aggregation, which has been proposed as a factor in the majority of ALS cases (Shaw and Valentine 2007), as discussed below.

*Protein Aggregation*

Like several other neurodegenerative disease, there is extensive evidence suggesting protein aggregation contributes to ALS pathogenesis. Similar to other pathways implicated in pathogenesis of the disease, it is unknown whether protein aggregation is an early process that precipitates other mechanisms of pathogenesis, or whether aggregation of aberrant proteins results from upstream inciting events. Also similar to other proposed mechanisms of pathogenesis, it is unclear why toxicity associated with protein aggregation would selectively affect motor neurons, although selective vulnerability has been suggested for a variety of reasons. Protein aggregation also suggests a link to the frontotemporal lobar degeneration that sometimes accompanies ALS.

One unique histopathological feature of ALS is the presence of neuronal Bunina bodies, lysosomal inclusion bodies immunopositive for cystatin c and transferrin (Mizuno, Amari et al. 2006; Okamoto, Mizuno et al. 2008). Interestingly, cystatin c has been identified as a potential biomarker for ALS as it is reportedly decreased in the CSF of ALS patients.
(Ranganathan, Williams et al. 2005; Pasinetti, Ungar et al. 2006). Another feature found in both ALS patients and rodent models is the presence of ubiquitinated inclusions, shown to stain positive for neurofilaments, protein chaperones, proteosomes, TDP-43, and SOD1 (Shibayama, Strong et al. 2005). The identification of SOD1 in aggregates suggests one mechanism by which mutant SOD1 may confer toxicity to motor neurons. Toxicity of mSOD1 may result from increased oxidative activity, or from misfolding and aggregation. The fact that over 100 different mutations in SOD1 all produce similar motor neuron degeneration has puzzled researchers, but may also provide important clues. A common pathway may involve the aggregation of mutant SOD1 resulting from oxidative modification, disrupted metal coordination, or altered surface charge of the protein (Shaw and Valentine 2007). Oxidative modification of wildtype SOD1 has been shown to alter the surface charge of SOD1 and decrease Cu\(^{2+}\) coordination, which may increase its tendency to aggregate (Fujiwara, Nakano et al. 2007). Some disease-associated SOD1 mutants have also been shown to decrease surface charge (Shaw and Valentine 2007). Other cellular processes leading to modification of SOD1 and causing aggregation would have the same result. There has been recent speculation that oxidation and dysfunction of SOD1 may be involved in all cases of ALS, not just those associated with SOD1 mutations (Kabashi, Valdmanis et al. 2007). Factors affecting aggregation of SOD1 may be one mechanism by which this could occur. However, one study did not find any SOD1-positive inclusions in sporadic ALS patients (Watanabe, Dykes-Hoberg et al. 2001), which would argue against involvement of SOD1 in these sporadic cases.
Frontotemporal lobar degeneration (FTLD) with ubiquitin positive tau-negative neuronal cytoplasmic inclusions may coexist with ALS symptoms. TDP-43 is commonly found in these ubiquitinated inclusions in spinal cord motor neurons and cortical neurons (Arai, Hasegawa et al. 2006). A recent study identified TDP-43 mutations in a family afflicted by fALS that segregated with the disease (Sreedharan, Blair et al. 2008). Additionally, this study screened 526 sALS patients and 1262 controls, revealing three mutants in the case group and none in the controls. Other studies have not found TDP-43 mutations in ALS patients, suggesting it is at most rarely associated with ALS (Gijselinck, Sleegers et al. 2007; Guerreiro, Schymick et al. 2008). Tau-positive frontotemporal dementia is commonly characterized by intracellular and extracellular tau-positive aggregates, which are also a hallmark of the ALS/parkinsonism-dementia complex seen in the Western Pacific (Shankar, Yanagihara et al. 1989). ALS may be a disease of proteinopathy with clinical variance depending on the proteins involved.

Misfolded and aggregated proteins may exhaust cellular responses that are necessary to protect against cellular damage. Aggregated proteins recruit and sequester protein chaperones (e.g. heat shock proteins), and are targeted for proteosomal degradation by ubiquitination. Indeed, heat shock proteins have been identified in cytoplasmic inclusions of mouse models and human fALS and sALS patients (Watanabe, Dykes-Hoberg et al. 2001). This may increase apoptosis of affected cells because several heat shock proteins function to inhibit apoptosis by sequestering Apaf-1 or cytochrome c or preventing Apaf-1 oligomerization (Shibayama, Strong et al. 2005). Increasing heat shock protein expression may prove beneficial in ALS cases involving protein aggregation. Injection of
heat shock protein 70 (Hsp70) delayed onset of symptoms and death in mutant SOD1 transgenic mice when started before disease onset, but not when started at onset (Gifondorwa, Robinson et al. 2007). Arimoclomol, a heat shock protein coinducer, benefited mutant SOD1 transgenic mice by delaying disease onset and death when initiated before disease onset and delayed death in mice treated at symptom onset (Kieran, Kalmar et al. 2004). While treating mice prior to onset or on the average day of onset may not apply to human patients, these results are promising and clinical trials of arimoclomol are underway. However, motor neurons may be relatively insensitive to induction of heat shock proteins (Batulan, Shinder et al. 2003), and thus induction of heat shock proteins by arimoclomol may be restricted to glial cells. Targeting protein aggregation in motor neurons may require exogenous administration of heat shock proteins or treatment by other mechanisms.

The evidence implicating excessive oxidative stress in ALS pathogenesis and the propensity of oxidatively modified SOD1 to form aggregates suggest one mechanism by which ALS occurs in the absence of SOD1 mutations. Any factors contributing to oxidative stress in motor neurons or surrounding glial cells may increase SOD1 modification and thus, aggregation. Wang et al. (Wang, Lee et al. 2004) reported decreased expression of SOD1 in neuroblastoma cells expressing the H63D HFE variant, which also showed higher intracellular labile iron and markers of oxidative stress (Lee, Patton et al. 2006). Using muscle biopsies from ALS patients, we subsequently found reduced expression of SOD1 in patients carrying at least one H63D HFE polymorphism compared to subjects homozygous for the wildtype HFE allele (unpublished data). The
cause of these findings is not known. However, both pieces of data may reflect either
decreased total SOD1 expression, or aggregation of SOD1 in SDS-insoluble fractions.
Iron-mediated protein oxidation and aggregation has been suggested to contribute to
other diseases, including aggregation of α-synuclein associated with Parkinson’s disease
(Hashimoto, Hsu et al. 1999). Induction of protein aggregation by local increases in labile
iron may be a common mechanism among diseases characterized by protein aggregates.
As cytoskeleton components have been identified in protein aggregates associated with
ALS, cytoskeletal dysfunction may also be a common theme among ALS patients.

Cytoskeletal Dysfunction

The cytoskeleton of neurons consists broadly of microtubules, actin, and a variety of
intermediate filaments. Additionally, a number of associated proteins are involved in
stabilization of the cytoskeleton, including microtubule-associated protein tau. Transport
over long distances (e.g. along axons) occurs mainly along microtubules driven by
kinesin family members in the anterograde direction, and dynein in the retrograde
direction (Chevalier-Larsen and Holzbaur 2006). Anterograde transport is involved in
transport of synaptic vesicle precursors, membrane receptors, cytoskeletal components,
and distribution of mitochondria throughout axons. Retrograde transport is involved in
the trafficking of neurotrophic factors, protein aggregates, and defective mitochondria to
the cell body. Thus, like other pathways implicated in ALS disease pathogenesis,
deficient axonal transport is likely to accentuate other mechanisms of pathogenesis. Also
like other pathogenetic mechanisms, motor neurons may be particularly susceptible to interruptions in axonal transport due to several unique features.

Motor neurons are particularly large neurons with high metabolic demands. As discussed above, this necessitates a high number of mitochondria strategically distributed throughout the cell. Primary motor neurons obtained from several different mutant SOD1 transgenic mice show deficient anterograde axonal transport leading to reduced numbers of mitochondria in axons (De Vos, Chapman et al. 2007). Mutant SOD1 transgenic mice with late disease onset show decreased expression of kinesin and dynein well before onset of symptoms, suggesting early impaired anterograde and retrograde axonal transport (Warita, Itoyama et al. 1999). Anterograde transport of both tubulin and neurofilament-L is impaired in multiple mutant SOD1 mouse models before symptom onset, and this may contribute to neurofilament accumulation in the cell body (Williamson and Cleveland 1999). Small numbers of ALS patients have been found to possess mutations in the tail region of the heavy neurofilament (NF-H) gene that may affect phosphorylation of the protein (Figlewicz, Krizus et al. 1994; Tomkins, Usher et al. 1998; Al-Chalabi, Andersen et al. 1999). Neurofilament accumulation is a common feature in many cases of both sporadic and SOD1-associated ALS (Munoz, Greene et al. 1988; Kokubo, Kuzuhara et al. 1999), which may reflect a convergence of pathogenetic factors that all lead to increased phosphorylation.

Factors affecting the dynein-dynactin complex would be expected to mostly affect retrograde transport. Several missense mutations in the p150 subunit of dynactin
(DCTN1) have been identified in a small number of familial and sporadic ALS patients (Puls, Jonnakuty et al. 2003; Munch, Sedlmeier et al. 2004). These mutations may have variable genetic association with ALS, but suggest a common deficit in axonal transport. Recently a mouse model has been developed which expresses mutant dynactin p150 (Laird, Farah et al. 2008). In addition to defects in vesicle trafficking, this mouse shows a phenotype similar to ALS in decreased number of motor neurons, gliosis, and ubiquitin-positive inclusions at the microscopic level, and weakness, muscle wasting, weight loss, and paralysis at the gross level.

The potential effects of iron regulation on cytoskeletal function are largely indirect. Tau normally functions in stabilization of microtubules and promotion of microtubule polymerization. Phosphorylation of tau tends to cause dissociation from microtubules and aggregation of tau with impaired function of microtubules (Hernandez and Avila 2007). A number of kinases are potentially involved in phosphorylation of tau, including glycogen synthase kinase-3β (GSK-3β), which may be influenced by cellular iron levels. Iron-mediated oxidative stress increases tau phosphorylation concomitant with an increase in GSK-3β activity (Lovell, Xiong et al. 2004). It most be noted, however, that tau has a number of specific phosphorylation sites, and the tendency of phosphorylated tau to form aggregates may not just depend on amount of phosphorylation, but also the specific sites of phosphorylation. Overall effects of iron regulation on tau aggregation and cytoskeletal function are likely to be complex and may involve other kinases in addition to GSK-3β.
Conclusion

While a substantial amount of evidence implicates each of the mechanisms of pathogenesis described above, the role of each pathway in the selective motor neuron death of ALS remains unclear. Much evidence supports the concept that multiple pathways act both simultaneously and in a cascading fashion to contribute to disease pathogenesis. The lack of identified genetic or environmental risk factors associated with a large number of cases may result from each individual risk factor acting in only pathway. Alternatively, any risk factor acting on a downstream or common pathway may only confer a small negative effect.

None of the pathogenetic mechanisms reviewed above are specific to ALS. Other neurodegenerative diseases, including Alzheimer’s disease and Parkinson’s disease, appear to share many of these processes. Why these mechanisms result in the selective motor neuron death characteristic of ALS is not known. ALS pathogenesis is likely to result from the cumulative effect of multiple risk factors and/or triggering events, including those yet to be identified. Because disruption in cellular iron homeostatic mechanisms can be associated with multiple pathways purportedly involved in ALS, the prevalence of the H63D genetic variant in ALS has become an attractive area for investigation.
The impetus for the work presented in the following chapters was the association of the $H63D$ $HFE$ polymorphism with sporadic ALS identified in previous studies. **The overall working hypothesis guiding the proposed experiments in this thesis is that the HFE variants are associated with increased risk of developing ALS through mechanisms at least partially involving cellular misregulation of iron.**

This work was begun with the hypothesis that the $H63D$ $HFE$ allele is genetically associated with an increased risk of ALS. However the $HFE$ gene lies in a large block of linkage disequilibrium (LD). Thus, it is not clear whether the $H63D$ variant is the genetic factor that alters an individual’s risk of developing ALS or whether some other variant that is in linkage disequilibrium with $H63D$ is a causative factor. It is possible that the $H63D$ allele is “tagging” some other variant or haplotype that increases risk of developing ALS. Even if this allele is tagging another risk-associated allele, the $H63D$ $HFE$ variant has known functional consequences and thus it is unlikely to merely be a tagging SNP. For this reason, functional assays were also performed to help clarify the role of $HFE$ variants in cellular processes contributing to ALS pathogenesis.

A second hypothesis was that functional effects of $HFE$ variants would contribute to mechanisms of pathogenesis associated with ALS, thus elucidating how the $H63D$ polymorphism may increase risk of disease. Using human CSF and plasma samples, we used multiplex biomarker analysis to efficiently evaluate a potential panel of cytokines, trophic factors, and proteins involved in iron homeostasis association with ALS. Our working hypothesis was that a panel of biomarkers would allow accurate distinction of
ALS patients versus controls, correlate with ALS disease characteristics including disease progression, and provide clues into the pathogenesis of the disease. A secondary hypothesis was that HFE variants, particularly the H63D variant, would impact expression of biomarkers in these panels, and begin to elucidate the role of this polymorphism in disease pathogenesis.

Further examination of the impacts of HFE variants on a cellular milieu contributing to an increased risk of developing ALS was performed in cell culture models. The working hypotheses for these experiments were that HFE variants detrimentally affect numerous pathways associated with ALS pathogenesis, including glutamate regulation and inflammatory signaling, in particular. Cellular effects of HFE variants were hypothesized to start with iron misregulation and resulting increases in oxidative stress. However, these effects were proposed to extend beyond iron regulation since the H63D and C282Y variants have previously been shown to have similar affects on cellular iron acquisition, but different end results and different association with diseases.

Understanding the mechanism of even one genetic risk factor may help to elucidate disease pathways and identify other genetic and environmental risk factors. Additionally, information obtained from these studies may lead to better understanding of other diseases associated with iron misregulation at the cellular level.
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neurologically normal controls: first stage analysis and public release of data."


Chapter 2

Association of H63D HFE with Amyotrophic Lateral Sclerosis

Abstract

Five independent groups have published the frequencies of HFE polymorphisms in ALS patients compared to various control groups. A meta-analysis of these studies shows that possession of at least one H63D HFE allele is associated with a 26% increased risk of developing ALS. The objective of this study was to expand upon these previous studies by genotyping a larger number of ALS patients and beginning to assess the linkage of the H63D HFE allele with other alleles. DNA samples were obtained from 115 ALS patients and 121 normal controls from a single outpatient Neurology clinic. DNA samples were also obtained for 557 ALS patients and 519 normal controls from the NINDS Neurogenetics Repository at the Coriell Institute for Medical Research. We genotyped for the H63D allele and surrounding SNPs on chromosome 6 between positions 25,783,000 and 26,414,000 spaced approximately every 10 kb by sequencing. In the current study, the H63D HFE allele was not significantly associated with ALS (heterozygotes: OR 0.90, 95% CI 0.70-1.17; homozygotes: OR 1.69, 95% CI 0.87-3.28). Combining our results with previous association studies shows a dose-dependent association of the H63D HFE allele with ALS (heterozygotes: OR 1.18, 95% CI 1.03-1.34; homozygotes: OR 2.04, 95% CI 1.48-2.80). The minimum common region containing the H63D allele spanned 44.3 kb including the genes HFE, HIST1H4C,
While the results of our current study show no significant association of the \( H63D \) \( HFE \) allele with sporadic ALS, our study may have been underpowered to detect a significant association, particularly for \( H63D \) homozygotes. The combined analysis of all available data shows a significant association of this allele with ALS in a dose-dependent fashion. The region containing the \( H63D \) \( HFE \) allele in our samples included several highly conserved genes and the \( H63D \) variant is known to have potentially broad-ranging effects resulting from iron dyshomeostasis. The high degree of linkage disequilibrium in the region containing the \( HFE \) gene does not exclude the contribution of other genetic variants to the risk of sporadic ALS, and the partial linkage disequilibrium between this region and other surrounding regions further extends the number of loci that may be associated with ALS. Functional analysis of genetic variants within the regions associated with ALS should contribute to our understanding of the roles of these variants to ALS pathogenesis.

**Introduction**

ALS is a progressive neurodegenerative disease that is universally fatal. Despite the identification of genetic loci associated with a small number of familial ALS cases, the causes of sporadic ALS, representing over 90% of cases, remain unknown. A number of studies have been undertaken to identify genetic risk factors associated with risk of developing sporadic ALS. Several polymorphisms have been individually studied, and recent advances in high throughput technology have enabled large genome-wide single
nucleotide polymorphism (SNP) association studies. Ultimately, most studies have either failed to identify genetic risk factor of sporadic ALS, or have identified risk factors accounting for only a small number of cases.

rs1799945, a SNP within the *hemochromatosis* (*HFE*) gene, located on chromosome 6p21.3, was initially reported to be associated with ALS by our group (Wang, Lee et al. 2004). Substitution of a G for the ancestral C at this position results in a histidine to aspartic acid change at the 63rd amino acid position of the HFE protein. Our group identified an increased frequency of the *H63D HFE* allele in ALS patients, which was subsequently replicated by several other studies across multiple populations (Goodall, Greenway et al. 2005; Restagno, Lombardo et al. 2007; Sutedja, Sinke et al. 2007), though one study failed to find an association between this allele and ALS (Yen, Simpson et al. 2004). However, this negative study had the smallest number of subjects and the ALS patient group included ethnicities with low frequencies of *HFE* variants. Several of these studies have reported approximately 30% of ALS patients carrying at least one copy of the *H63D HFE* allele, establishing this variant as a potential factor in a large number of patients. The wildtype HFE protein regulates cellular iron acquisition and likely mediates control of systemic iron levels and distribution (Feder, Penny et al. 1998; Bridle, Frazer et al. 2003). Regulation of iron homeostasis is also influenced by a number of environmental factors, which may contribute to the variable association of HFE variants with neurodegenerative diseases, including ALS.
In this study we set out to (a) genotype a large number of ALS patients from across the United States and pool our data with the other previously published data, and (b) assess the linkage of the $H63D$ $HFE$ allele with other potential risk factor alleles. Although variants of $HFE$ are consistently associated with altered functional consequences, the $HFE$ gene lies in a large block of linkage disequilibrium (LD), and thus, its association with diseases is not clear. The latter aim of this study addresses the haplotype associated with the $H63D$ $HFE$ allele between ALS patients and controls, as this allele may be part of a risk-associated haplotype, or it may be tagging a risk-associated allele.

**Methods**

**Patient Samples**

DNA samples were obtained from 115 mostly-Caucasian ALS patients referred to the Penn State University/Milton S. Hershey Medical Center Department of Neurology (PSU). DNA samples were also obtained from 121 mostly-Caucasian neurologically normal controls consisting of spouses and caregivers and other persons in central Pennsylvania. None of these samples were included in previously published studies. DNA samples were also obtained from the NINDS Neurogenetics Repository at the Coriell Institute for Medical Research (Camden, NJ). All patients and controls gave written informed consent to participate in the study. DNA samples from 557 unique and
unrelated, white, non-Hispanic individuals diagnosed with sporadic ALS were selected from precompiled panels (NDPT025, NDPT026, NDPT027, NDPT028, NDPT029, NDPT030). DNA samples from 519 unique and unrelated, white, non-Hispanic neurologically normal controls were selected from precompiled panels (NDPT019, NDPT020, NDPT021, NDPT022, NDPT023, NDPT024). The study was approved by the Penn State Hershey Medical Center and Penn State College of Medicine Institutional Review Board.

rs1799945 Genotyping

All subjects were genotyped for the H63D HFE allele (rs1799945). DNA samples obtained from PSU were genotyped by restriction fragment length polymorphism analysis. Briefly, DNA (50 ng) was amplified by PCR using the primers forward: 5’-TTG TTT GAA GCT TTG GGC TAC G-3’, and reverse: 5’-CAT ACC CTT GCT GTG GTT GTG A-3’. The PCR reaction was initiated at 95 °C for 15 min was followed by 40 cycles consisting of denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, and extension at 72 °C for 1 min 30 s. The 210-bp PCR product was digested with MboI, and those products carrying the 63D (G) allele gave restriction fragments of 147 and 63 bp, while fragments containing the H63 (C) allele resulted in fragments of 99, 63, and 48 bp. Samples were randomly selected and verified by sequencing, performed by the Penn State University College of Medicine Core Facility.
DNA samples obtained from the Coriell repository were genotyped the \textit{H63D} \textit{HFE} allele by sequencing. DNA (10 ng) was amplified by PCR using the primers forward: 5’-ACA TGG TTA AGG CCT GTT GC-3’, and reverse: 5’-CTT GCT GTG GTT GTG ATT TTC C-3’. The PCR reaction was initiated at 94 °C for 3 min was followed by 8 cycles consisting of denaturation at 94 °C for 20 s, annealing at 66 °C for 20 s, and extension at 72 °C for 30 s; followed by 16 cycles of denaturation at 94 °C for 20 s, annealing at 66 °C for 20 s decreasing by 1 °C each cycle, and extension at 72 °C for 30 s; followed by 16 cycles consisting of denaturation at 94 °C for 20 s, annealing at 54 °C for 20 s, and extension at 72 °C for 30 s. Each product was sequenced using the forward primer with Applied Biosystems BigDye terminator v3.1 sequencing chemistry as per the manufacturer's directions. The resulting reactions were run on an ABI3730 genetic analyzer and analyzed with Sequencher software (version 4.5, GeneCodes Corp., MI).

\textit{Pooled Analysis with Previously Published Data}

Five previously published studies have reported genotype frequencies in ALS patients compared to controls (Wang, Lee et al. 2004; Yen, Simpson et al. 2004; Goodall, Greenway et al. 2005; Restagno, Lombardo et al. 2007; Sutedja, Sinke et al. 2007). We found an abnormally large number of \textit{H63D} homozygotes compared to heterozygotes in both the ALS patient and neurological disease control groups. The other studies assessing the association of the \textit{H63D} allele with ALS patients noted much larger numbers of \textit{H63D} heterozygotes than homozygotes. The vast majority of DNA samples from our
previous study were not available for re-evaluation, and therefore we have conservatively
discarded these subjects from the following analysis. For the pooled analysis, we
combined genotyping data from the current study with the remaining four previous
studies.

*Minimum Common Haplotype Analysis*

Using default haplotype block definitions in Haploview 4.1 (Gabriel, Schaffner et
al. 2002), and data from the CEU Caucasian population included in the International
HapMap Project, rs1799945 is not included in any haplotype blocks. Eight ALS patients
and eight controls homozygous for the *H63D* allele were randomly selected to assess
linkage of this allele with other loci. rs1799945 resides on chromosome 6 at position
26,199,158 in a large region of linkage disequilibrium. Nearby SNPs were chosen based
on data from the CEU Caucasian population of northern and western European ancestry
in the International HapMap Project. SNPs on chromosome 6 between positions
25,783,000 and 26,414,000 were chosen spaced approximately every 10 kb with minor
allele frequencies greater than 0.25, where available. PCR primers were designed with
Primer3 software (v. 0.4.0) with reference sequences provided by Ensembl. All SNPs and
primers are given in Table 2-1. Each product was sequenced using either the forward or
reverse primer, as described above.

Table 2-1
Table 2-1: SNPs included in region assayed for the minimum common haplotype including the H63D HFE allele.

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*Statistical Analysis*

Statistical analyses were performed as indicated with GraphPad Prism 4.03 (GraphPad Software, Inc.; San Diego, CA). Allele and genotype frequencies were compared between the ALS and control groups by Chi-square test for the current data. Odds ratios were calculated to compare genotype frequencies for the current data and the pooled analysis. Differences were considered significant with \( p < 0.05 \).

**Results**

For the current study we obtained DNA samples from a total of 672 ALS patients and 640 neurologically normal controls (*Table 2-2*). Genotyping of SNP rs1799945 in these samples revealed no significant differences in genotype (Chi-square = 0.102, 1 df, \( p = 0.749 \)) or allele (Chi-square 3.293, 2 df, \( p = 0.193 \)) frequencies between the ALS and control groups (*Table 2-3*).
Table 2-2: Results of rs1799945 genotyping.

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Table 2-3: Association of the G allele with ALS.

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Odds  

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Ratio (95% CI)  

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<th>(0.70-1.17)</th>
<th>(ref)</th>
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A pooled analysis of all available data excluding the data reported by Wang et al. (Wang, Lee et al. 2004), on the association of the \textit{H63D HFE} allele with ALS shows persons heterozygous and homozygous for the \textit{H63D} allele are over-represented in the ALS population compared to the control populations. This analysis showed a dose-dependent increased risk of \textit{H63D} heterozygotes (OR = 1.18, 95% CI 1.03-1.34) and \textit{H63D} homozygotes (OR = 2.04, 95% CI 1.48-2.80) in ALS patients (Table 2-4).
Table 2-4: Pooled analysis of available H63D genotyping data.

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<th>the Netherlands</th>
<th>Italy</th>
<th>Coriell</th>
<th>PA</th>
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<td>149</td>
<td>557</td>
<td>115</td>
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<td>121</td>
<td>7141</td>
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To begin evaluation of linkage disequilibrium between \textit{H63D} and another potential disease-associated allele, we genotyped SNPs spread approximately every 10 kb in 8 ALS patients and 8 controls across a 631 kb region surrounding the \textit{HFE} gene. Each sample included in this analysis was homozygous for the \textit{H63D} allele to facilitate identification of the surrounding haplotype. The minimum common haplotype including \textit{H63D} was determined for each sample, as shown in Table 2-5. The boundaries of these blocks were chosen as the point farthest from \textit{rs1799945} at which the genotype at a locus was homozygous in an individual. The minimum common haplotype in each group spanned a 44.3 kb region from \textit{rs1799945} to \textit{rs2856645}, and includes the genes \textit{HFE}, \textit{HIST1H4C}, \textit{HIST1H1T}, \textit{HIST1H2BC}, \textit{HIST1H2AC}. We did not find any difference in the minimum common haplotypes between the two groups.
Table 2-5

Table 2-5: Haplotypes including the H63D allele were determined for 8 ALS patients and 8 controls, shown above in gray.

*rs1799945* is highlighted in yellow.
**Discussion**

The current study found no significant differences in \textit{H63D} frequency between the ALS and control groups; however, combining our results with previously published studies demonstrates an increased risk of ALS associated with the \textit{H63D HFE} allele. This is in agreement with previous smaller combined analyses that also found an overall increased risk of ALS in H63D carriers (Schymick, Talbot et al. 2007; Sutedja, Sinke et al. 2007). In a subset of ALS and control \textit{H63D} homozygotes, the minimum common haplotype containing the H63D allele spanned a large region, but was the same for both groups.

Previous studies have largely associated the \textit{H63D HFE} allele with an increased risk of ALS, however, small sample sizes have mostly prevented a determination of a dose-dependent risk associated with this allele. The pooled analysis of all available data, excluding that reported by Wang et al., allowed the analysis of 1540 ALS patients and 7141 controls, and showed a dose-dependent risk associated with the \textit{H63D HFE} allele. The consistent trends of these studies, particularly that \textit{H63D} homozygotes are over-represented among ALS patients, increases the validity of this association.

The frequency of the \textit{H63D HFE} allele is highly variable between different populations (Merryweather-Clarke, Pointon et al. 1997; Merryweather-Clarke, Pointon et al. 2000). Even within Caucasians the allele frequency varies significantly by geographic
region. Each previously published study has drawn ALS patients and controls from the same limited geographic region. While this strategy is valid for the region in question, the results may not be applicable for other populations and regions. The Coriell repository consists of DNA samples obtained from across the U.S., thus offering the opportunity for more broadly applicable results.

To begin to assess the association of the $H63D$ $HFE$ allele with other SNPs that may be implicated as genetic risk factors for ALS, we determined the minimum common haplotype containing this allele. Between ALS patients and controls, we did not determine any differences within this region. Our data suggest the $HFE$ gene may exist within a common haplotype block that includes several histone genes. One of these ($HIST1H1T$) is only expressed in spermatocytes (Nayernia, Meinhardt et al. 2003), and others ($HIST1H4C, HIST1H2AC$) have only loosely been associated with any diseases (Bogni, Cheng et al. 2006; Parssinen, Alarmo et al. 2008). Thus, in this region, $HFE$ variants have been most widely associated with disease, and variants in other genes within this region should be assessed by functional analysis for potential roles in ALS pathogenesis.

Any haplotype including the $H63D$ $HFE$ allele could be in linkage disequilibrium with another haplotype that is associated with ALS. Although not conclusive, the relative strength of association of different haplotypes might suggest one is functionally relevant to disease pathogenesis and others are merely genetically linked. Thus, the subsequent follow-up study was devised.
The association of haplotypes on chromosome 6 within the region 25,783,000 and 26,414,000 are to be assessed. Tagging SNPs were selected using the CEU HapMap database to identify haplotype blocks associated with ALS. Tagging SNPs were selected using Tagger to identify all SNPs within the region with minor allele frequency ≥ 5% based on a pairwise $r^2 \geq 0.8$ with at least one tagging SNP. Ninety-two SNPs (Table 2-6) were chosen for a custom-designed VeraCode GoldenGate assay kit for the BeadXpress platform (Illumina; San Diego, CA). This will allow the genotyping of 480 ALS patients and 480 controls and calculation of the association of each of these haplotypes with ALS. At the time of this thesis, this work could not yet be completed due to delays in manufacturing of the custom-designed assay.
Table 2-6: SNPs to be assayed for peak haplotype association study.

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<th>Position</th>
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<td>26292020</td>
</tr>
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</tr>
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</tr>
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<td>rs9358913</td>
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<td>rs6910741</td>
<td>26003564</td>
<td>rs11759720</td>
<td>26358233</td>
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<tr>
<td>rs16891142</td>
<td>26007118</td>
<td>rs11753610</td>
<td>26359580</td>
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<td>rs12207059</td>
<td>26030384</td>
<td>rs809871</td>
<td>26364505</td>
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<tr>
<td>rs199741</td>
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<td>26041517</td>
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</tr>
<tr>
<td>rs9393676</td>
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<td>rs7773004</td>
<td>26375734</td>
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<td>rs7740793</td>
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<td>rs1124999</td>
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</tr>
<tr>
<td>rs199738</td>
<td>26071435</td>
<td>rs9379842</td>
<td>26397299</td>
</tr>
</tbody>
</table>
Acknowledgements

Many thanks to Beth Stephens for her help in collecting samples and Bryan Traynor, M.D. for his significant contributions to this work. This project was supported by the ALS Association.
References


Chapter 3

CSF Biomarkers Can Identify ALS Patients and Reveal Influences of HFE polymorphisms

Portions obtained from:

Abstract

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease with complicated pathogenesis that poses challenges with respect to diagnosis and monitoring of disease progression. The objectives of this study were (1) identify a biomarker panel that elucidates ALS disease pathogenesis, distinguishes ALS patients from neurological disease controls, and correlates with ALS disease characteristics; and (2) determine the effect of *HFE* gene variants, a potential risk factor for sporadic ALS, on the biomarker profile. We obtained cerebrospinal fluid samples by lumbar puncture from 41 ALS patients and 33 neurological disease controls. All patients were genotyped for *HFE* polymorphisms. We performed a multiplex cytokine and growth factor analysis and assays for iron-related analytes. Classification statistics were generated using a support vector machine algorithm. The groups of ALS patients and neurological disease controls were each associated with distinct profiles of biomarkers. Fourteen biomarkers differed
between ALS patients and the control group. The five proteins with the lowest p-values differentiated ALS patients from controls with 89.2% accuracy, 87.5% sensitivity, and 91.2% specificity. Expression of IL-8 correlated negatively with ALSFRS-R. Expression of β2-microglobulin was higher in subjects carrying an H63D HFE allele, while expression levels of PDGF bb, IL-12(p70), and IL-7 were higher in subjects carrying a C282Y HFE allele. Thus, a CSF inflammatory profile associated with ALS pathogenesis may distinguish ALS patients from neurological disease controls, and may serve as a biomarker panel to aid in the diagnosis of ALS after further validation. Identification of biomarkers associated with polymorphisms of the HFE gene may begin to clarify the association of HFE variants with ALS.

Introduction

Amyotrophic lateral sclerosis (ALS) is a complex, progressive neurodegenerative disease that is difficult to diagnose early in its course because initial symptoms and signs often are similar to those of more common conditions, and there is no specific diagnostic test for ALS. The pathogenesis of ALS is largely unknown, thus identification of biomarkers associated with ALS could eventually assist early diagnosis and aid understanding of the disease by providing insights into its pathogenesis. As defined by the NIH Biomarkers Definitions Working Group, a biomarker is ‘a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention’ (2001). Biomarkers may also be used as indicators of disease progression and as measures of
treatment effects. Finally, a comprehensive panel of biomarkers may help guide stratification of patients for different treatments based on etiopathogenesis.

A number of studies have been undertaken to identify biomarkers associated with ALS (Henkel, Engelhardt et al. 2004; Ranganathan, Williams et al. 2005; Cronin, Greenway et al. 2006; Tanaka, Kikuchi et al. 2006; Mitsumoto, Ulug et al. 2007; Nagata, Nagano et al. 2007; Ranganathan, Nicholl et al. 2007). While many of these studies have provided clues about pathogenetic mechanisms involved in the disease, most have examined only a small number of proteins. Several studies have examined proteomic profiles of ALS patients compared to various control groups (Ramstrom, Ivonin et al. 2004; Pasinetti, Ungar et al. 2006; Ranganathan, Nicholl et al. 2007). These proteomic studies have provided valuable examples of proteins which can distinguish ALS patients from controls groups, but most proteomic techniques are better suited for abundant proteins. We provide in this study an analysis of a panel of analytes that are associated with inflammation and trophic support that may more accurately reflect early stages of disease and cell response.

Our first hypothesis was that a panel of biomarkers could be identified in the cerebrospinal fluid (CSF) that will support the clinical diagnosis. Extensive evidence has suggested ALS pathogenesis involves excessive neuroinflammation (McGeer and McGeer 2002), aberrant growth factor regulation (Ekestern 2004), and iron dyshomeostasis (Kasarskis, Tandon et al. 1995), among other factors. Despite many attempts to identify causes of sporadic ALS, no genetic polymorphisms have been
identified to account for a large number of cases (Cronin, Berger et al. 2007; Schymick, Scholz et al. 2007; Schymick, Talbot et al. 2007). The $H63D$ polymorphism in the hemochromatosis gene ($HFE$), has been examined in multiple published studies (Wang, Lee et al. 2004; Sutedja, Sinke et al. 2007), with an overall odds ratio for ALS patients possessing at least one $H63D$ allele of 1.26 (95% CI 1.09-1.46) making this genetic variant the most frequently associated with ALS. HFE is involved in mediating iron homeostasis, inflammatory responses, and innate immunity (Roy, Custodio et al. 2004). Therefore, we also tested the hypothesis that $HFE$ gene variants will be associated with altered profiles in the panel of CSF biomarkers.

**Methods**

*Patients and Samples*

We obtained blood samples by venipuncture and CSF samples by lumbar puncture at the time of evaluation from patients who were undergoing CSF examination as part of their diagnostic evaluation in the outpatient Neurology clinic. These patients were grouped into those with ALS (clinically definite, probable, probable laboratory-supported, or possible ALS) (Brooks BR 2000), and those who presented with neurological symptoms but ultimately were found not to have ALS (neurological disease controls). Demographic and selected medical data were obtained from the patients’ medical records. The ALS Functional Rating Scale Score-Revised (Cedarbaum, Stambler et al. 1999) was completed for each ALS patient at an outpatient visit within two months.
of the lumbar puncture. All patients provided informed consent. This study was approved by the Institutional Review Board of the Penn State Milton S. Hershey Medical Center and Penn State College of Medicine. CSF samples were obtained between eight AM and twelve PM, to avoid changes related to a circadian rhythm, from a mostly Caucasian population. Samples were frozen immediately after collection and were later thawed on ice and centrifuged to remove any particulate matter. Protease inhibitor cocktail (Sigma-Aldrich; St. Louis, MO) was then added 1:100, and samples were refrozen at -80°C in 200 μL aliquots.

**HFE Genotyping**

DNA was purified from white blood cells using the QIAamp DNA Mini kit (Qiagen; Valencia, CA). All patients were genotyped for the H63D and C282Y HFE polymorphisms by restriction fragment length analysis as previously reported (Wang, Lee et al. 2004).

**Multiplex Cytokine Bead Assay**

We performed multiplex analysis on undiluted CSF supernatants using the Bio-Plex Human 27-plex panel of cytokines and growth factors (Bio-Rad; Hercules, CA). The proteins in this panel included eotaxin, fibroblast growth factor basic (FGF basic), granulocyte colony stimulating factor (G-CSF), granulocyte-monocyte colony stimulating factor (GM-CSF), interferon-gamma (IFN-γ), IFN-γ induced protein-10 (IP-10),
interleukin-1beta (IL-1β), IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-8, IL-10, IL-12(p70), IL-13, IL-15, IL-17, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1β (MIP-1β), platelet derived growth factor (PDGF) bb, regulated on activation normal T-cell expressed and presumably secreted (RANTES), tumor necrosis factor-alpha (TNF-α), and vascular endothelial growth factor (VEGF). Briefly, 1% bovine serum albumin (BSA) (Sigma-Aldrich; St. Louis, MO) was added to 200 μL of each CSF sample and standards were reconstituted in PBS with 1% BSA. Fifty μL of each sample or standard was added in duplicate to a 96-well filter plate and mixed with 50 μL of antibody-conjugated beads for one hour at room temperature. After one hour, wells were washed and 25 μL of detection antibody was added to each well. After a 30 minute incubation, wells were washed and 50 μL of streptavidin-PE was added to each well and incubated for 10 minutes. A final wash cycle was then completed and 125 μl of assay buffer was added to each well. The plate was then analyzed using a Bio-Plex 200 workstation (Bio-Rad). Analyte concentration was calculated based on the respective standard curve for each cytokine.

**Immunoassays**

CSF levels of β2-microglobulin (US Biological; Swampscott, MA) and transferrin (Bethyl Laboratories; Montgomery, TX) were assayed by ELISA, each according to the manufacturer's instructions.
**Atomic Absorption Spectroscopy**

The amount of iron in the CSF was determined by digesting the CSF in ultrapure Nitric Acid (JT Baker, 9598-00; Phillipsburg, NJ), 1:4 v/v, and samples were heated to 60°C for 24 hours. The digested samples were diluted 1:100 in ddH2O, and then analyzed on a Perkin Elmer Atomic Absorption Spectrometer 600 series (Waltham, MA).

**Statistical Analysis**

Multifactorial analysis of analyte expression was performed using GeneSpring GX version 7.3.1 (Agilent Technologies; Santa Clara, CA). Biomarkers were compared between groups via t-test and differences were considered significant if p < 0.05. Normal distribution of analyte expression was assessed with the Kolmogorov-Smirnov test using SigmaStat 2.03 (SPSS, Inc.; Chicago, IL). Correlations between markers and ALSFRS-R, duration of symptoms, and age were assessed by the Spearman’s or Pearson correlation coefficient, as appropriate, with p < 0.05 considered significant. Classification statistics were generated using a support vector machine algorithm with radial basis and polynomial dot product kernel functions and a diagonal scaling factor ranging from zero to five. Classification of each sample by disease status was determined by crossvalidation in a ‘leave one out’ strategy in which each sample was sequentially blinded. Each sample is individually removed from the sample set and the algorithm seeks to classify the sample compared to the others.
**Results**

CSF was obtained from 39 sporadic ALS patients and two familial ALS patients for a total of 41 ALS patients (nine with bulbar onset, 32 with limb onset). CSF was also obtained from 33 neurological disease controls. The characteristics of the two groups are given in Table 3-1, and the diagnoses of neurological control patients are listed in Table 3-2.
Table 3-1: Comparison of CSF samples from ALS patients and neurological controls (NDC). Values are given as mean (SEM).

<table>
<thead>
<tr>
<th></th>
<th>ALS</th>
<th>NDC</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>41</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>age (y)</td>
<td>58.8 (±1.8)</td>
<td>43.9 (±2.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>male:female</td>
<td>27:14</td>
<td>11:22</td>
<td>0.005</td>
</tr>
<tr>
<td>H63D carriers (n)</td>
<td>14</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>C282Y carriers (n)</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>clinically definite (n)</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>clinically probable (n)</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>clinically probable-laboratory supported (n)</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>clinically possible (n)</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>onset (limb:bulbar)</td>
<td>32:9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time from onset (mo)</td>
<td>16.6 (±2.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALSFRS-R</td>
<td>39.5 (±1.0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3-2: Diagnoses of neurological disease control patients.

<table>
<thead>
<tr>
<th>Neurological Disease Controls</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>undetermined</td>
<td>14</td>
</tr>
<tr>
<td>multiple sclerosis</td>
<td>1</td>
</tr>
<tr>
<td>chronic fatigue</td>
<td>1</td>
</tr>
<tr>
<td>cervical and thoracic myelopathy</td>
<td>1</td>
</tr>
<tr>
<td>progressive memory loss and depression</td>
<td>1</td>
</tr>
<tr>
<td>chronic headache and peripheral neuropathy</td>
<td>1</td>
</tr>
<tr>
<td>monoclonal gammopathy of undetermined significance</td>
<td>1</td>
</tr>
<tr>
<td>chronic headache</td>
<td>1</td>
</tr>
<tr>
<td>benign primary headache disorder</td>
<td>1</td>
</tr>
<tr>
<td>possible multiple sclerosis</td>
<td>1</td>
</tr>
<tr>
<td>small vessel ischemic changes</td>
<td>1</td>
</tr>
<tr>
<td>movement disorder</td>
<td>1</td>
</tr>
<tr>
<td>spinal cerebellar ataxia</td>
<td>1</td>
</tr>
<tr>
<td>cognitive change</td>
<td>1</td>
</tr>
<tr>
<td>mild optic disc edema</td>
<td>1</td>
</tr>
<tr>
<td>lower back pain</td>
<td>1</td>
</tr>
<tr>
<td>systemic lupus</td>
<td>1</td>
</tr>
<tr>
<td>fibromyalgia and psychiatric/psychological illness undetermined</td>
<td>1</td>
</tr>
<tr>
<td>pain, undetermined</td>
<td>1</td>
</tr>
<tr>
<td>peripheral polyneuropathy</td>
<td>1</td>
</tr>
</tbody>
</table>
All biomarkers on the panel were detectable in the CSF of the majority of the samples, with the exception of IL-1β, which was not detected in any sample. The analysis revealed that expression of several proteins correlated significantly with age (Table 3-3), and thus the values for these proteins were age-adjusted by linear regression. The expression of three biomarkers differed by sex (Table 3-3).
Table 3-3: Correlation of biomarkers with age and differences by sex. All values given as pg/mL, except transferrin (μg/mL). Values are given as mean (SEM).

**Age**

<table>
<thead>
<tr>
<th>Marker</th>
<th>r</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-17</td>
<td>0.389</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.389</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-13</td>
<td>0.351</td>
<td>0.002</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>0.353</td>
<td>0.002</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.345</td>
<td>0.002</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.324</td>
<td>0.004</td>
</tr>
<tr>
<td>PDGF bb</td>
<td></td>
<td>0.310</td>
</tr>
</tbody>
</table>

**Sex**

<table>
<thead>
<tr>
<th>Marker</th>
<th>males</th>
<th>females</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>12.76</td>
<td>16.98</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(0.68)</td>
<td>(0.88)</td>
<td></td>
</tr>
<tr>
<td>transferrin</td>
<td>27.35</td>
<td>19.64</td>
<td>0.032</td>
</tr>
<tr>
<td></td>
<td>(2.96)</td>
<td>(3.43)</td>
<td></td>
</tr>
</tbody>
</table>
Between ALS patients and neurological controls, the expression of 12 biomarkers was significantly higher in the CSF of ALS patients, while the expression of two biomarkers was significantly higher in controls, as shown in Table 3-4. A support vector machines algorithm was used to classify each sample as ALS or control in a ‘leave one out’ crossvalidation strategy with an accuracy of 86.5% using all assayed markers. The best accuracy was obtained using the five markers with the most significant p-values (IL-10, IL-6, GM-CSF, IL-2, and IL-15) with a first order polynomial dot product kernel function and a diagonal scaling factor of one. Limiting the analysis to the five most significant biomarkers enabled us to distinguish ALS patients from controls with 89.2% accuracy, 87.5% sensitivity, and 91.2% specificity.
Table 3-4: Biomarkers significantly different between ALS patients and neurological controls. All values given as mean (SEM), in pg/mL.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control</th>
<th>ALS</th>
<th>p</th>
<th>Fold Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>23.74 (0.96)</td>
<td>29.74 (0.98)</td>
<td>0.000001</td>
<td>1.25</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>47.59 (2.38)</td>
<td>66.66 (3.29)</td>
<td>0.000021</td>
<td>1.40</td>
</tr>
<tr>
<td>IL-2</td>
<td>45.72 (1.59)</td>
<td>54.35 (1.73)</td>
<td>0.000428</td>
<td>1.19</td>
</tr>
<tr>
<td>IL-15</td>
<td>19.64 (1.13)</td>
<td>25.31 (1.33)</td>
<td>0.001511</td>
<td>1.29</td>
</tr>
<tr>
<td>IL-17</td>
<td>59.65 (2.74)</td>
<td>77.52 (3.73)</td>
<td>0.002101</td>
<td>1.30</td>
</tr>
<tr>
<td>MIP-1b</td>
<td>14.99 (0.57)</td>
<td>17.58 (0.47)</td>
<td>0.002165</td>
<td>1.17</td>
</tr>
<tr>
<td>FGF basic</td>
<td>46.81 (8.91)</td>
<td>78.06 (6.54)</td>
<td>0.005317</td>
<td>1.67</td>
</tr>
<tr>
<td>G-CSF</td>
<td>4.26 (0.51)</td>
<td>6.42 (0.52)</td>
<td>0.007361</td>
<td>1.51</td>
</tr>
<tr>
<td>VEGF</td>
<td>18.74 (0.92)</td>
<td>24.40 (1.17)</td>
<td>0.00742</td>
<td>1.30</td>
</tr>
<tr>
<td>MIP-1a</td>
<td>8.12 (0.20)</td>
<td>8.74 (0.23)</td>
<td>0.042665</td>
<td>1.08</td>
</tr>
<tr>
<td>MCP-1</td>
<td>114.54 (5.58)</td>
<td>143.02 (7.65)</td>
<td>0.047217</td>
<td>1.25</td>
</tr>
<tr>
<td>IL-10</td>
<td>19.03 (0.81)</td>
<td>11.46 (0.44)</td>
<td>&lt;0.000001</td>
<td>1.66</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>55.05 (3.19)</td>
<td>43.63 (2.14)</td>
<td>0.032836</td>
<td>1.26</td>
</tr>
</tbody>
</table>
No markers differed significantly between ALS patients with limb onset versus bulbar onset or significantly correlated with duration of symptoms, although the expression of IL-8 correlated negatively with ALSFRS-R (Table 3-5).
Table 3-5

Table 3-5: Biomarkers associated with characteristics of ALS.

<table>
<thead>
<tr>
<th>ALS Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker</td>
</tr>
<tr>
<td>ALSFRS-R</td>
</tr>
<tr>
<td>IL-8</td>
</tr>
<tr>
<td>FGF basic</td>
</tr>
</tbody>
</table>

Duration of Symptoms

none

Limb vs. Bulbar Onset

none
Fourteen ALS patients carried an \textit{H63D HFE} variant (three homozygotes, 11 heterozygotes), and seven control patients (one homozygote, six heterozygotes) carried an \textit{H63D HFE} variant. Both the ALS patient group and the neurological control patient group each had five subjects carrying \textit{C282Y HFE} variants (one control homozygote, all others heterozygous). Heterozygotes and homozygotes were grouped together because of the relatively small number of homozygotes. Additionally, the two disease groups were considered together to determine the association between biomarker expression and HFE genotypes. Biomarkers differing by \textit{HFE} genotype are given in Table 3-6. There were no significant differences of iron concentration in the CSF for any of the comparison groups.
Table 3-6: Biomarkers stratified by HFE genotype at the 63^{rd} amino acid position and the 282^{nd} amino acid position. Values given in pg/mL, except β2M (ng/mL). Values are given as mean (SEM).

<table>
<thead>
<tr>
<th>Impact of HFE Variants</th>
<th>Wt/Wt</th>
<th>H63D/</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2M</td>
<td>689.34 (23.11)</td>
<td>799.37 (30.10)</td>
<td>0.002</td>
</tr>
<tr>
<td>IL-8</td>
<td>4.11 (0.20)</td>
<td>4.49 (0.24)</td>
<td>0.070</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Wt/Wt</th>
<th>C282Y/</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF</td>
<td>42.84 (3.90)</td>
<td>68.02 (10.48)</td>
<td>0.009</td>
</tr>
<tr>
<td>IL-12</td>
<td>28.62 (1.11)</td>
<td>37.34 (3.33)</td>
<td>0.026</td>
</tr>
<tr>
<td>IL-7</td>
<td>59.96 (1.79)</td>
<td>68.95 (3.35)</td>
<td>0.047</td>
</tr>
</tbody>
</table>
Discussion

The results of this study support our hypotheses that the profile of inflammatory and anti-inflammatory cytokines, growth factors, and analytes reflective of iron homeostasis differs between ALS patients and a group of neurological control patients. Of the 32 analytes that comprised the biomarker panel examined in this analysis, 14 were significantly different between the neurological disease control and ALS groups.

Because this study used CSF taken from ALS patients an average of less than 17 months after the onset of symptoms, it can be argued that this biomarker panel reflects relatively early changes in the CNS. There were no differences between limb and bulbar onset in the biomarker panel suggesting that activation and early progression of the disease could be the same regardless of site of disease onset. Each of the five most significantly different cytokines between ALS patients and neurological controls can be linked to disease pathogenesis. IL-10 is an anti-inflammatory cytokine known to inhibit microglial activation (Lee, Nagai et al. 2002). IL-6 is a cytokine generally considered to be inflammatory, but is known to also have anti-inflammatory and neurotrophic properties (Sekizawa, Openshaw et al. 1998). GM-CSF is usually regarded as a proliferator and differentiator of neutrophilic, eosinophilic and monocytic cellular lineages (Lee, Liu et al. 1994; Franzen, Bouhy et al. 2004). IL-2 is produced mainly in T lymphocytes and acts to stimulate growth and cytotoxicity of activated T lymphocytes (Grande, Firvida et al. 2006). IL-15 is a T lymphocyte chemoattractant also shown to
activate microglia (Huang, Ha et al. 2007) and upregulate the production of other chemoattractants including IL-8 and MCP-1 (Badolato, Ponzi et al. 1997). Indeed, expression MCP-1 as well as MIP-1\(_\alpha\), and MIP-1\(_\beta\) were all significantly increased in ALS patients compared to controls. Additionally we found a trend toward higher levels of IL-8 in carriers of the \(H63D\) \(HFE\) variant.

While this study provides initial development for a panel of CSF biomarkers at one point during the ALS disease process, it does not clarify whether inflammatory processes precede disease onset or result from it. Prognostic biomarkers which predict future development of ALS in asymptomatic patients would be of great use, but were not part of the study design and will be challenging to develop. Nonetheless, this study is clearly consistent with the theories that suggest ALS pathogenesis involves inflammatory activation (Boillee, Yamanaka et al. 2006). The biomarker panel also is consistent with the overall concept that microglial recruitment and activation is a key component of the disease pathogenesis (Kawamata, Akiyama et al. 1992; Engelhardt, Tajti et al. 1993; Graves, Fiala et al. 2004; Tanaka, Kikuchi et al. 2006). Activated microglia are a source of the leukocyte chemoattractant IL-8 (Ehrlich, Hu et al. 1998) and this was the only protein that had a direct correlation between expression and ALSFRS-R. Fittingly, this relationship showed an increase in IL-8 in the CSF with decreasing performance on the ALSFRS-R, which is consistent with evidence suggesting that T lymphocytes accumulate in regions of motor neuron loss and contribute to the disease (Engelhardt, Tajti et al. 1993). Accumulation of T lymphocytes can in turn lead to further recruitment and
activation of microglia, and cytotoxic mediators produced by these cells may be
detrimental to motor neurons.

Several studies have reported an increased risk of ALS in carriers of the \textit{H63D HFE} polymorphism. The \textit{C282Y} variant of the \textit{HFE} gene has not been reported to
increase in ALS although this rarer allelic variant may simply be underrepresented in the
studies to date. Both of the common \textit{HFE} gene variants have been proposed to have a
similar effect on cellular iron accumulation (Feder, Gnirke et al. 1996; Wang, Lee et al.
2004; Sutedja, Sinke et al. 2007), but the phenotype of cells carrying the different alleles
is different (Lee, Patton et al. 2006). While the nature of the \textit{HFE} association is still
under investigation, \textit{in vivo} studies in humans and animals reveal that \textit{HFE} variants are
associated with loss of iron homeostasis, exacerbated inflammatory responses, and
alterations in innate immunity (Roy, Custodio et al. 2004). In a recent series of cell
culture studies, human neuroblastoma cells carrying the H63D allele have alterations in
 glutamate metabolism that could suggest vulnerability to glutamate toxicity (Chapter 5).
All of these physiological changes are considered part of the pathogenic process in ALS,
and our panel of biomarkers showed differential expression of several analytes based on
\textit{HFE} genotypes. Beta-2 microglobulin (\textit{β2M}) and the chemokine IL-8 were increased or
trended toward an increase in the presence of an \textit{H63D HFE} variant, while PDGF bb, IL-
12, and IL-7 were increased in the presence of a \textit{C282Y HFE} variant. The increase in
\textit{β2M} may reflect increased turnover of the HFE-\textit{β2M} complex or may be a marker of
more general membrane protein turnover, particularly from immune cells (Nielsen,
Vainer et al. 2000). A study in an Irish population of hemochromatosis patients
demonstrated that patients carrying the *H63D HFE* allele had higher plasma levels of the chemokine MCP-1 than patients homozygous for the *C282Y* variant, or wildtype controls (Lawless, White et al. 2007). Consistent with previous studies (Nagata, Nagano et al. 2007), in our biomarker panel MCP-1 was elevated in ALS patients compared to controls. MCP-1 expression did not differ by *HFE* genotype, but IL-8 trended toward an increase in those individuals carrying the *H63D* allele, which may increase leukocyte recruitment and microglial activation in the *H63D* carriers. These data support the concept that the *H63D HFE* gene variant is associated with exacerbated inflammatory responses and perhaps an overexuberant microglial response that promotes an enabling environment for the triggering events associated with ALS. In the current study, we see a mixed profile of inflammatory (IL-12 and IL-7) and trophic (IL-7 and PDGF bb) profiles associated with the C282Y variant. Inflammatory influences which may increase risk of ALS in persons carrying the C282Y variant may be countered by the trophic influences so as not to increase the overall risk of developing ALS. Future studies incorporating larger numbers of subjects with *HFE* variants as well as subjects homozygous for the H63D and C282Y alleles will be necessary to further define the biomarker profiles associated with these variants.

It has been proposed that ALS results from triggering events causing a cascade leading toward selective motor neuron death in genetically susceptible individuals (Majoor-Krakauer, Willems et al. 2003). Our data clearly indicate a substantial pro-inflammatory environment in ALS. No markers differed between ALS patients with limb onset versus patients with bulbar onset suggesting similar pathologic processes
irrespective of the site of disease onset. We did not find any biomarkers in our panel that significantly correlated with duration of symptoms prior to lumbar puncture, but our patients were selected early in the disease process (average < 17 months). Serial sampling of CSF in patients throughout the course of the disease and correlation between clinical measures and biomarker expression may provide more accurate assessment of disease progression.

The biomarker panel accurately distinguished ALS cases from neurological disease controls, consistent with other proteomic studies (Ranganathan, Williams et al. 2005; Ranganathan, Nicholl et al. 2007). Thus our data and others show promise that a biomarker panel could be developed for aiding in the diagnosis of ALS. Future validation studies should incorporate samples from multiple clinics and from patients with syndromes which mimic ALS such as progressive muscular atrophy, spinal and bulbar muscular atrophy, and multifocal motor neuropathy.

Acknowledgements

Many thanks to all of the patients who participated in this study. I express my profound gratitude to Willard M. Freeman, Ph.D. for guidance in experimental design and data analysis. I also thank Beth Stephens and Ally Brothers for collecting samples, Scot Kimball, Ph.D. and Lydia Kutzler for their technical expertise, and Dave Mauger, Ph.D. for statistical assistance. This work was supported by funds from The Judith and Jean Pape Adams Charitable Foundation, The Paul and Harriett Campbell Fund for ALS
References


neurologically normal controls: first stage analysis and public release of data.


Chapter 4

Plasma Biomarkers Associated with ALS and the H63D HFE Polymorphism

Abstract

The objectives of this study were to determine if there are biomarkers in plasma that may characterize individuals with amyotrophic lateral sclerosis (ALS) and determine if that profile of biomarkers is affected by the H63D HFE variant. The H63D HFE polymorphism has been associated with ALS in multiple studies, and can be expected to affect indices of oxidative stress and inflammation. Plasma samples were obtained from 29 ALS patients (18 Wt/Wt, 11 H63D/Wt) and 36 healthy controls (19 Wt/Wt, 17 H63D/Wt), approximately matched for age and sex. A multiplex immunoassay system was used to screen a number of cytokines and growth factors, as well as β2-microglobulin, pro-hepcidin, transferrin (Tf), H-ferritin, and L-ferritin, and total plasma iron. ALS patients had significantly higher levels of L-ferritin and lower concentrations of Tf in the plasma samples when compared to healthy controls. These two biomarkers were used to classify a test group of subjects with 82% accuracy. Expression of MCP-1 was positively correlated, and GM-CSF was negatively correlated, with duration of symptoms in the ALS patient group. Comparing Wt/Wt subjects versus H63D/Wt subjects irrespective of disease status, the H63D allele was associated with higher plasma levels of L-ferritin and Tf, and lower plasma levels of IFN-γ, RANTES, and G-CSF. These data suggest that iron homeostasis may be disrupted in ALS patients and that the presence of
the $H63D$ $HFE$ allelic variant alters cytokine expression and the relationship between Tf and ferritin in plasma. G-CSF has been proposed as a potential treatment for ALS, and our data suggest patients carrying the $H63D$ allele may be less responsive to G-CSF treatments because of their lower baseline levels. Given the prevalence of $H63D$ $HFE$ gene variants in ALS, the data suggest that biomarker studies in this disease and perhaps other neurodegenerative diseases should consider HFE polymorphisms in evaluating their data.

**Introduction**

Amyotrophic lateral sclerosis (ALS) is characterized by degeneration of both upper and lower motor neurons (Brooks BR 2000). Its heterogeneity with regard to region of onset and rate of progression has likely impeded research into mechanisms and treatment, and may impose barriers to prompt and accurate diagnosis. A number of genetic and environmental risk factors have been proposed although each is likely to account for only a small proportion of disease cases.

One proposed genetic risk factor for ALS, the $H63D$ $HFE$ variant, has been associated with the disease in four published studies (Wang, Lee et al. 2004; Goodall, Greenway et al. 2005; Restagno, Lombardo et al. 2007; Sutedja, Sinke et al. 2007), although one study found no association (Yen, Simpson et al. 2004). This genetic polymorphism impairs the normal function of HFE to limit cellular iron uptake resulting in cellular iron accumulation (Feder, Penny et al. 1998). Because iron is part of functional
groups in many proteins involved in cellular energy metabolism, neurotransmitter synthesis, and regulation of transcription factors, the effects of iron accumulation are potentially widespread. Iron misregulation may contribute to many of the proposed mechanisms of ALS pathogenesis, which include oxidative stress (Barber, Mead et al. 2006), neuroinflammation (Henkel, Engelhardt et al. 2004), growth factor dysregulation (Ekestern 2004), glutamate excitotoxicity (Van Damme, Dewil et al. 2005), mitochondrial dysfunction (Shaw, al-Chalabi et al. 2001), protein aggregation (Cluskey and Ramsden 2001), and cytoskeleton dysfunction (Julien and Beaulieu 2000).

Previous attempts to identify biomarkers characteristic of ALS patients and disease processes have highlighted the lack of information provided by single biomarkers due to overlap in expression levels between groups (Henkel, Engelhardt et al. 2004; Ranganathan, Williams et al. 2005; Cronin, Greenway et al. 2006; Tanaka, Kikuchi et al. 2006; Mitumoto, Ulug et al. 2007; Nagata, Nagano et al. 2007; Ranganathan, Nicholl et al. 2007). Several studies have examined proteomic profiles of ALS patients compared to various control groups and suggested panels of biomarkers which may aid classification of subjects (Ramstrom, Ivonin et al. 2004; Pasinetti, Ungar et al. 2006; Ranganathan, Nicholl et al. 2007). However, most proteomic techniques are labor-intensive and are better suited for abundant proteins. Unlike several studies using proteomic technologies that are ultimately biased toward more abundant proteins, we have chosen to focus on a panel of low-abundance cytokines and trophic factors with an antibody-based approach.
Using the multiplex immunoassay, we previously identified a profile of biomarkers in cerebrospinal fluid (CFS) altered by ALS disease and by HFE genotypes (Mitchell, Freeman et al.). The availability of a biomarker assay for blood rather than CSF would greatly facilitate the uses noted above. Therefore, the current study was designed in a similar manner to our CSF-based biomarker analysis. The first question addressed in the current study was whether a panel of biomarkers could be identified in the plasma that will support the clinical diagnosis and clarify mechanisms of disease pathogenesis. We also tested the hypothesis that HFE gene variants will be associated with altered profiles in the panel of biomarkers.

Methods

Patients and Samples

We obtained blood samples by venipuncture from ALS patients attending a single ALS clinic and from normal controls between eight AM and twelve noon. All patients met revised El-Escorial criteria for clinically definite, probable, probable laboratory-supported, or possible ALS (Brooks BR 2000). The normal controls consisted of spouses and nonrelated caregivers of ALS patients, and of volunteers without neurological disease from the community. Although individual ethnicity data was not available for the study subjects, the majority of subjects were Caucasian. Subjects were approximately matched for age, sex, and HFE genotype at the 63rd amino acid position (dbSNP rs1799945). Subjects carrying C282Y HFE polymorphisms were excluded from the analysis, due to
small numbers of subjects. All subjects provided informed consent. This study was approved by the Institutional Review Board of the Penn State Milton S. Hershey Medical Center and Penn State College of Medicine. Samples from both ALS and control subjects were handled identically. When the samples were obtained they were centrifuged immediately and plasma was separated and placed into a -80°C freezer.

Samples were later thawed on ice and centrifuged to remove any particulate matter. A protease inhibitor cocktail (Sigma-Aldrich; St. Louis, MO) was then added at a dilution of 1:100, and samples were refrozen at -80°C in 200 μL aliquots until use.

**HFE Genotyping**

DNA was purified from leukocytes using the QIAamp DNA Mini kit (Qiagen; Valencia, CA). All patients were genotyped for the *H63D* and *C282Y HFE* polymorphisms by restriction fragment length analysis as previously reported.(Wang, Lee et al. 2004).

**Multiplex Cytokine Bead Assay**

We performed a multiplex analysis on plasma samples diluted 1:3 using the Bio-Plex Human 27-plex panel of cytokines and growth factors (Bio-Rad; Hercules, CA). The proteins measured in this panel include eotaxin, fibroblast growth factor basic (FGF basic), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), interferon-gamma (IFN-γ), IFN-γ induced protein-10 (IP-
10), interleukin-1beta (IL-1β), IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-8, IL-10, IL-12(p70), IL-13, IL-15, IL-17, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1β (MIP-1β), platelet derived growth factor (PDGF) bb, regulated on activation normal T-cell expressed and presumably secreted (RANTES), tumor necrosis factor-alpha (TNF-α), and vascular endothelial growth factor (VEGF). Briefly, 50 μL of each diluted sample or standard was added in duplicate to a 96-well filter plate and mixed with 50 μL of antibody-conjugated beads for one hour at room temperature. After one hour, wells were washed and 25 μL of detection antibody was added to each well. After a 30 minute incubation, wells were washed and 50 μL of streptavidin-PE was added to each well and incubated for 10 minutes. A final wash cycle was then completed and 125 μL of assay buffer was added to each well. The plate was then analyzed using a Bio-Plex 200 workstation (Bio-Rad). Analyte concentration was calculated based on the respective standard curve for each cytokine.

**Immunoassays**

MCP-1 was not detected by the multiplex assay in any sample and was thus analyzed separately by anti-human MCP-1 ELISA (GE Healthcare; Piscataway, NJ). Plasma levels of β2-microglobulin (US Biological; Swampscott, MA), Tf (Bethyl Laboratories; Montgomery, TX), c-reactive protein (R&D Systems; Minneapolis, MN), and pro-hepcidin (DRG International; Moutainside, NJ) were assayed by ELISA according to each manufacturer’s protocol. CRP data were only available for 54 subjects.
Levels of H-ferritin were assayed by ELISA using rabbit anti recombinant H-ferritin antiserum as we have previously reported (Cheepsunthorn, Palmer et al. 1998) and goat anti-rabbit secondary antibody. L-ferritin was measured by immunoradiometric assay (Siemens Medical Solutions; Malvern, PA) according to the manufacturer's instructions. The antibody used in this assay targets human spleen ferritin which is largely composed of the L-ferritin subunit, and thus is referred to as “L-ferritin” herein.

Iron Measurement and Calculation of Tf Saturation

The total amount of iron in the plasma was determined by digesting samples in ultrapure Nitric Acid (JT Baker, 9598-00; Phillipsburg, NJ), 1:4 v/v, and samples were heated to 60°C for 24 hours. The digested samples were diluted 1:100 in ddH₂O, and then analyzed on a Perkin Elmer Atomic Absorption Spectrometer 600 series (Waltham, MA). Tf saturation was calculated as Tf saturation (%) = plasma iron (mol/L) / (2 x Tf (mol/L)) x 100.

Statistical Analysis

Normal distribution of analyte expression was assessed with the Kolmogorov-Smirnov test. Biomarker concentrations were compared between groups (ALS vs. control
and wt/wt vs. H63D/wt) via t-test or Mann-Whitney U test, as appropriate. Correlations between markers and ALSFRS-R, duration of symptoms, and age were assessed by the Spearman’s or Pearson correlation coefficient, as appropriate. Chi-square test was used to assess frequency differences between groups. Data for individual markers were adjusted for age differences by linear regression. Samples were separated into two sets matched for disease and HFE genotype for disease status classification using logistic regression. Roughly two-thirds of the samples comprised the training set which was used to determine a logistic regression function characteristic of disease status (dichotomous dependent variable). Independent variables were assessed by forward stepwise regression automatically including sex and HFE genotype. The p-value to enter each marker into the logit function (“p to enter”) was then assessed for each variable. Each variable was then forced into the logit function in increasing order of p-value. Classification of the test set of samples was performed incorporating expression of L-ferritin and Tf into the logit function. Statitical analyses were performed using SigmaStat 2.03 (SPSS, Inc.; Chicago, IL) and GraphPad Prism 4.03 (GraphPad Software, Inc.; San Diego, CA). Differences and correlations were considered significant with p < 0.05.

Results

Patient demographics are described in Table 4-1. No significant differences were found between control subjects and ALS patients for age or sex. No significant differences were found between ALS patients for region of onset, duration of symptoms, or El Escorial classification based on HFE genotype.
Table 4-1: Subject demographics given as median (interquartile range). P-values indicate comparisons for all groups.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ALS</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1799945 genotype</td>
<td>wt/wt</td>
<td>H63D/wt</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>19</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>age (y)</td>
<td>54 (47-75)</td>
<td>58 (50-70)</td>
<td>61 (48-75)</td>
</tr>
<tr>
<td>sex (male:female)</td>
<td>12:7</td>
<td>10:7</td>
<td>11:7</td>
</tr>
<tr>
<td>onset (limb:bulbar)</td>
<td>12:6</td>
<td>6:5</td>
<td></td>
</tr>
<tr>
<td>duration of disease (months)</td>
<td>19 (12-28)</td>
<td>11 (9-19)</td>
<td></td>
</tr>
<tr>
<td>clinically definite (n)</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>clinically probable-lab.</td>
<td>6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>clinically possible (n)</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>
Correlation of analyte expression with age was determined independent of health status. A number of inflammatory cytokines increased with increasing age as did B2M and the trophic factor VEGF. Only pro-hepcidin decreased with advancing age. Age-adjusted values of each of these markers were used for subsequent analyses. All age-associated markers are given in Table 4-2.
Table 4-2

Table 4-2: Correlation of plasma biomarkers with age.

<table>
<thead>
<tr>
<th>Marker</th>
<th>r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2M</td>
<td>0.645</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.437</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>0.377</td>
<td>0.002</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.373</td>
<td>0.002</td>
</tr>
<tr>
<td>IL-7</td>
<td>0.348</td>
<td>0.005</td>
</tr>
<tr>
<td>MIP-1b</td>
<td>0.346</td>
<td>0.005</td>
</tr>
<tr>
<td>IP-10</td>
<td>0.342</td>
<td>0.006</td>
</tr>
<tr>
<td>IL-12(p70)</td>
<td>0.341</td>
<td>0.006</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.340</td>
<td>0.006</td>
</tr>
<tr>
<td>IFN-gamma</td>
<td>0.329</td>
<td>0.008</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>0.324</td>
<td>0.009</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.290</td>
<td>0.020</td>
</tr>
<tr>
<td>pro-hepcidin</td>
<td>-0.281</td>
<td>0.026</td>
</tr>
<tr>
<td>IL-13</td>
<td>0.251</td>
<td>0.046</td>
</tr>
<tr>
<td>PDGF</td>
<td>0.248</td>
<td>0.048</td>
</tr>
</tbody>
</table>
To examine our primary hypothesis, we first determined the biomarker profile associated with a diagnosis of ALS, region of onset, and disease progression independent of $HFE$ genotype. These results are shown in Table 4-3. L-ferritin and Tf levels were both associated with the diagnosis of ALS, while plasma pro-hepcidin expression was associated only with region of onset. Two proteins MCP-1 and GM-CSF were associated with duration of symptoms; MCP-1 levels increased whereas GM-CSF expression decreased.
Table 4-3

Table 4-3: Biomarkers affected by disease status. Values given as median (interquartile range).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ALS</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-ferritin (ng/mL)</td>
<td>25.78 (14.64-49.84)</td>
<td>66.17 (26.10-91.23)</td>
<td>0.002</td>
</tr>
<tr>
<td>Tf (ug/mL)</td>
<td>3098 (2058-3945)</td>
<td>2262 (1754-3365)</td>
<td>0.052</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ALS Patients</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Limb</td>
<td>Bulbar</td>
<td>p</td>
</tr>
<tr>
<td>pro-hepcidin (ng/mL)</td>
<td>213.5 (43.5-400.0)</td>
<td>483.3 (330.9-745.2)</td>
<td>0.004</td>
</tr>
<tr>
<td>IP-10 (pg/mL)</td>
<td>545.8 (364.0-715.1)</td>
<td>338.8 (234.1-480.1)</td>
<td>0.060</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Duration of Symptoms</th>
<th>r</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1</td>
<td>0.500</td>
<td>0.006</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>-0.435</td>
<td>0.018</td>
</tr>
</tbody>
</table>
To determine the ability of the biomarker panel to classify ALS patients from control subjects, both groups were randomly separated into a training set (2/3 of samples) and a test set (1/3 of samples). Subjects were matched for disease status and \textit{HFE} genotype, according to the demographics in Table 4-4. No differences were found between the two groups based on age, sex, duration of symptoms, \textit{HFE} genotype, or El Escorial classification of the ALS patients, indicating similar characteristics between the groups.
Table 4-4: Demographics of training and test sets. Values given as median (interquartile range).

<table>
<thead>
<tr>
<th></th>
<th>Training Set</th>
<th>Test Set</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n)</td>
<td>24</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>ALS (n)</td>
<td>19</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>clinically definite</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>clinically probable-laboratory supported</td>
<td>5</td>
<td>5</td>
<td>0.39</td>
</tr>
<tr>
<td>clinically probable</td>
<td>6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>clinically possible</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>onset (limb:bulbar)</td>
<td>11:8</td>
<td>7:3</td>
<td>0.52</td>
</tr>
<tr>
<td>duration of disease (months)</td>
<td>18 (11-27)</td>
<td>12 (10-25)</td>
<td>0.60</td>
</tr>
<tr>
<td>Age (y)</td>
<td>58 (49-72)</td>
<td>59 (43-77)</td>
<td>0.94</td>
</tr>
<tr>
<td>Sex (male:female)</td>
<td>27:16</td>
<td>13:9</td>
<td>0.99</td>
</tr>
<tr>
<td>HFE (wt/wt:H63D/wt)</td>
<td>22:21</td>
<td>15:7</td>
<td>0.19</td>
</tr>
</tbody>
</table>
The training set was used to determine a logistic regression function characteristic of disease status (dichotomous dependent variable). Independent variables were assessed by forward stepwise regression automatically including sex and HFE genotype. The p-value to enter each marker into the logit function (“p to enter”) was then assessed for each variable. Only L-ferritin and Tf had a p-value < 0.1 (L-ferritin: p = 0.031; Tf: p = 0.063). These two markers were used to classify the test set, resulting in a maximum classification accuracy of 82%, as shown in Table 4-5.
Table 4-5: Classification of subjects by disease status using logistic regression.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Classification</th>
<th>Accuracy</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Training Set</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-ferritin</td>
<td>0.031</td>
<td>67.4%</td>
<td>68.2%</td>
<td>100.0%</td>
</tr>
<tr>
<td>Tf</td>
<td>0.063</td>
<td>66.7%</td>
<td>81.8%</td>
<td>80.0%</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.153</td>
<td>71.4%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSF</td>
<td>0.165</td>
<td>71.4%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-13</td>
<td>0.245</td>
<td>73.8%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.248</td>
<td>76.2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1ra</td>
<td>0.296</td>
<td>71.4%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>0.399</td>
<td>78.6%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.406</td>
<td>78.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eotaxin</td>
<td>0.461</td>
<td>78.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.471</td>
<td>75.6%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td>0.472</td>
<td>84.6%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tf saturation</td>
<td>0.480</td>
<td>84.6%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-9</td>
<td>0.485</td>
<td>84.6%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-12</td>
<td>0.485</td>
<td>82.1%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP-1β</td>
<td>0.510</td>
<td>94.9%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP-1α</td>
<td>0.541</td>
<td>100.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF</td>
<td>0.562</td>
<td>100.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-5</td>
<td>0.565</td>
<td>100.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>0.651</td>
<td>100.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IP-10</td>
<td>0.657</td>
<td>100.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>0.673</td>
<td>100.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-7</td>
<td>0.674</td>
<td>100.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANTES</td>
<td>0.774</td>
<td>100.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pro-hepcidin</td>
<td>0.777</td>
<td>100.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-CSF</td>
<td>0.779</td>
<td>100.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDGF</td>
<td>0.828</td>
<td>100.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β2M</td>
<td>0.973</td>
<td>100.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-ferritin</td>
<td>0.982</td>
<td>100.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>1.000</td>
<td>100.0%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The secondary aim for this study was to assess biomarkers associated with the 
\( H_{63D} HFE \) polymorphism and determine if these alter the ALS profile. All subjects were 
grouped according to \( rs1799945 \) genotype (\( Wt/Wt \) vs. \( H_{63D}/Wt \)) regardless of health 
status (Table 4-6). L-ferritin and Tf were both increased in the plasma of \( H_{63D} \) carriers, 
and IFN-\( \gamma \), G-CSF, and RANTES were decreased in the plasma of these subjects 
regardless of health status. The expression of these five proteins was then assessed 
considering ALS patients and controls separately (Table 4-6). Within the healthy control 
group, \( HFE \) genotype had no impact on the expression on any markers. Within the ALS 
patient group, those patients with an \( H_{63D} \) allele had higher plasma levels of L-ferritin, 
and lower levels of IFN-\( \gamma \), G-CSF, and RANTES, compared to the ALS \( Wt/Wt \) group. 
The presence of the \( H_{63D} \) allele negated the increase in TF associated with ALS, and 
thus Tf levels were not different between the ALS \( Wt/Wt \) and ALS \( H_{63D}/Wt \) groups.
Table 4-6: Biomarkers affected by *HFE* genotype. Values given as median (interquartile range).

<table>
<thead>
<tr>
<th></th>
<th>All Subjects</th>
<th>Control Subjects</th>
<th>ALS Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt/wt</td>
<td>H63D/wt</td>
<td>p</td>
</tr>
<tr>
<td>IFN-gamma (pg/mL)</td>
<td>132.8 (116.6-178.3)</td>
<td>121.3 (100.8-145.0)</td>
<td>0.022</td>
</tr>
<tr>
<td>G-CSF (pg/mL)</td>
<td>38.17 (32.41-45.28)</td>
<td>34.80 (31.85-38.54)</td>
<td>0.043</td>
</tr>
<tr>
<td>Tf (ug/mL)</td>
<td>2281 (1831-3234)</td>
<td>3098 (2251-4189)</td>
<td>0.045</td>
</tr>
<tr>
<td>RANTES (pg/mL)</td>
<td>7394 (4966-9752)</td>
<td>5951 (4675-7753)</td>
<td>0.049</td>
</tr>
<tr>
<td>L-ferritin (ng/mL)</td>
<td>27.88 (15.46-67.51)</td>
<td>40.15 (23.04-87.53)</td>
<td>0.050</td>
</tr>
<tr>
<td>IFN-gamma (pg/mL)</td>
<td>129.4 (117.6-175.8)</td>
<td>129.4 (108.6-152.4)</td>
<td>NS</td>
</tr>
<tr>
<td>G-CSF (pg/mL)</td>
<td>35.94 (31.98-43.97)</td>
<td>35.56 (31.85-39.74)</td>
<td>NS</td>
</tr>
<tr>
<td>Tf (ug/mL)</td>
<td>2487 (1952-3408)</td>
<td>3225 (2640-4198)</td>
<td>NS</td>
</tr>
<tr>
<td>RANTES (pg/mL)</td>
<td>7305 (5471-9934)</td>
<td>6540 (4446-8899)</td>
<td>NS</td>
</tr>
<tr>
<td>L-ferritin (ng/mL)</td>
<td>23.04 (14.60-49.17)</td>
<td>34.22 (15.18-59.82)</td>
<td>NS</td>
</tr>
<tr>
<td>IFN-gamma (pg/mL)</td>
<td>167.1 (112.7-221.5)</td>
<td>103.2 (93.83-130.0)</td>
<td>0.033</td>
</tr>
<tr>
<td>G-CSF (pg/mL)</td>
<td>39.74 (33.32-46.85)</td>
<td>34.62 (29.05-35.65)</td>
<td>0.028</td>
</tr>
<tr>
<td>Tf (ug/mL)</td>
<td>2214 (1433-3428)</td>
<td>2465 (2153-3496)</td>
<td>NS</td>
</tr>
<tr>
<td>RANTES (pg/mL)</td>
<td>6851 (4966-10590)</td>
<td>5536 (4964-6925)</td>
<td>0.044</td>
</tr>
<tr>
<td>L-ferritin (ng/mL)</td>
<td>41.17 (18.90-79.52)</td>
<td>82.92 (38.78-145.4)</td>
<td>0.029</td>
</tr>
</tbody>
</table>
Plasma ferritin and Tf levels are affected by inflammatory states as well as systemic iron levels (Gabay and Kushner 1999; Raja, Pountney et al. 1999; Torti and Torti 2002). Therefore plasma levels of c-reactive protein were measured as a general indicator of inflammation. No significant differences were found between any groups (Figure 4-1). No significant differences in total plasma iron were identified between any groups. Additionally, no correlation was found between L-ferritin, Tf, CRP, and iron levels.
Figure 4-1: Subgroup Analyses. Plasma levels of L-ferritin, Tf, Tf saturation, total iron, and c-reactive protein (CRP) were determined as described in Methods. L-ferritin was elevated in the plasma of ALS H63D/Wt group (median 82.92 ng/mL) versus the ALS Wt/Wt group (median 41.17 ng/mL, p = 0.029) and the control H63D/Wt group (median 34.22 ng/mL, p = 0.007). No other differences reached the level of significance (p<0.05). Each data point represents one sample and horizontal lines represent medians. NS = not significant.
A number of significant findings in the current study reflect potentially disrupted iron regulation in association with either the diagnosis of ALS or the H63D HFE allele. A key regulator of iron is hepcidin and this protein is also an acute phase reactant whose synthesis is increased in inflammatory states (Nemeth, Valore et al. 2003). For these reasons, we determined the correlation of pro-hepcidin, the precursor of hepcidin, with plasma iron and inflammatory markers (Table 4-7). Although previous studies have suggested IL-6 is a potent inducer of pro-hepcidin transcription (Wrighting and Andrews 2006), we found a negative correlation between IL-6 and pro-hepcidin in control subjects, but not ALS patients (Table 4-7 and Figure 4-2). In control subjects, pro-hepcidin was also negatively correlated with IL-1β and IL-10, whereas pro-hepcidin was positively correlated with IL-1β in ALS Wt/Wt subjects and ALS patients grouped together independent of HFE genotype.
### Table 4-7

**Table 4-7**: Correlations of markers with pro-hepcidin.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th></th>
<th></th>
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<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wt/Wt</td>
<td>H63D/Wt</td>
<td>All</td>
<td>Wt/Wt</td>
<td>H63D/Wt</td>
<td>All</td>
<td>Wt/Wt</td>
<td>H63D/Wt</td>
<td>All</td>
<td>Wt/Wt</td>
<td>H63D/Wt</td>
</tr>
<tr>
<td>Iron</td>
<td></td>
<td>-0.265</td>
<td>-0.412</td>
<td>-0.253</td>
<td>-0.358</td>
<td>-0.297</td>
<td>0.026</td>
<td>0.145</td>
<td>0.405</td>
<td>0.897</td>
<td>0.133</td>
<td>0.156</td>
</tr>
<tr>
<td>L-ferritin</td>
<td></td>
<td>-0.197</td>
<td>0.039</td>
<td>-0.182</td>
<td>-0.185</td>
<td>0.436</td>
<td>0.113</td>
<td>0.463</td>
<td>0.180</td>
<td>0.558</td>
<td>0.433</td>
<td>0.881</td>
</tr>
<tr>
<td>H-ferritin</td>
<td></td>
<td>0.156</td>
<td>-0.447</td>
<td>-0.191</td>
<td>0.158</td>
<td>0.612</td>
<td>0.319</td>
<td>0.531</td>
<td>0.060</td>
<td>0.098</td>
<td>0.564</td>
<td>0.082</td>
</tr>
<tr>
<td>Tf</td>
<td></td>
<td>-0.253</td>
<td>0.206</td>
<td>0.047</td>
<td>0.123</td>
<td>0.318</td>
<td>0.232</td>
<td>0.657</td>
<td>0.340</td>
<td>0.227</td>
<td>0.345</td>
<td>0.443</td>
</tr>
<tr>
<td>IL-1b</td>
<td></td>
<td>-0.335</td>
<td>-0.287</td>
<td>-0.462</td>
<td>0.579</td>
<td>0.236</td>
<td>0.413</td>
<td>0.012</td>
<td>0.484</td>
<td>0.026</td>
<td>0.172</td>
<td>0.264</td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td>-0.373</td>
<td>-0.360</td>
<td>-0.483</td>
<td>0.162</td>
<td>-0.073</td>
<td>0.019</td>
<td>0.521</td>
<td>0.832</td>
<td>0.921</td>
<td>0.128</td>
<td>0.155</td>
</tr>
<tr>
<td>IL-10</td>
<td></td>
<td>-0.300</td>
<td>-0.306</td>
<td>-0.368</td>
<td>-0.040</td>
<td>-0.045</td>
<td>-0.071</td>
<td>0.874</td>
<td>0.894</td>
<td>0.713</td>
<td>0.212</td>
<td>0.232</td>
</tr>
<tr>
<td>TNF-alpha</td>
<td></td>
<td>-0.240</td>
<td>-0.230</td>
<td>-0.175</td>
<td>0.340</td>
<td>-0.368</td>
<td>0.007</td>
<td>0.338</td>
<td>0.376</td>
<td>0.307</td>
<td>0.038</td>
<td>0.376</td>
</tr>
<tr>
<td>CRP</td>
<td></td>
<td>-0.056</td>
<td>-0.429</td>
<td>-0.256</td>
<td>-0.147</td>
<td>-0.433</td>
<td>-0.305</td>
<td>0.587</td>
<td>0.250</td>
<td>0.138</td>
<td>0.837</td>
<td>0.144</td>
</tr>
</tbody>
</table>

**Note**: Significant correlations are indicated with asterisks: **p < 0.01**, *p < 0.05*.
Figure 4-2: Correlation of plasma pro-hepcidin and IL-6 in disease and genotype subgroups. Pro-hepcidin and IL-6 levels were measured in each plasma sample, as described in Methods. The relationship between pro-hepcidin and IL-6 was determined within each group. These two proteins were correlated in control subjects grouped together, independent of HFE genotype ($r = -0.483$, $p = 0.003$), but not in ALS patients.
Discussion

The results of this study support our hypotheses that biomarkers can be identified in plasma that differ between ALS patients and normal control subjects. Within the ALS patient group the observation that plasma levels of MCP-1 are increased and GM-CSF levels decrease with duration of symptoms suggest these proteins could be biomarkers for disease progression. The secondary aim of this study was to determine if the H63D HFE variant, which has been associated with an increased risk of developing ALS, was associated with an altered expression of biomarkers and if the H63D-associated biomarker profile altered any of the ALS relevant biomarkers. The presence of H63D HFE was associated with five specific protein changes from the profile examined and two of these proteins were found in the ALS profile. Thus, the H63D allelic variant does have a biological impact and is a confounder in studies aimed at identifying biomarkers in ALS. This concept is consistent with our previous report on CSF biomarker panels for ALS (Mitchell, Freeman et al.).

Classification of ALS Patients

The ability of the biomarkers associated with the ALS population to accurately classify ALS patients versus healthy control subjects was tested using expression levels of L-ferritin and Tf. These two biomarkers were used in a logistic regression model that also accounted for sex and HFE genotype to classify presence or absence of ALS with 82% accuracy. This study demonstrates the potential utility of these two biomarkers in identifying ALS patients. A number of previous analyses in plasma or serum have
identified altered expression of individual biomarkers between ALS patients and various control groups; however, single biomarkers are unlikely to provide clinically useful information for distinguishing ALS patients versus controls. A recent study identified increased serum levels of ferritin in ALS patients but did not find any changes in transferrin levels and did not classify subjects by health status using biomarker expression (Goodall, Haque et al. 2008). Previous studies have variably found altered plasma or serum cytokine expression associated with ALS (Wilms, Sievers et al. 2003; Baron, Bussini et al. 2005; Moreau, Devos et al. 2005; Cereda, Baiocchi et al. 2008), although we found no changes in cytokine expression between ALS patients and controls. We were, however, able to demonstrate efficacy of biomarkers to classify subjects by health status using plasma samples which are more easily obtained than CSF samples used in other studies.

Most panel-based biomarker studies in ALS have focused on the CSF (Mitchell, Freeman et al.; Ranganathan, Williams et al. 2005; Pasinetti, Ungar et al. 2006; Ranganathan, Nicholl et al. 2007). We previously published a CSF biomarker analysis using a similar approach as in this study. Our previous study identified a number of biomarkers in CSF associated with ALS patients (Mitchell, Freeman et al.). These alterations suggested an inflammatory profile associated with ALS pathogenesis, and allowed accurate classification of ALS patients versus a group of neurological disease control subjects. In the current study, we did not identify altered plasma expression of cytokines associated with ALS patients versus normal controls; however, altered
expression of L-ferritin and Tf allowed classification of ALS subjects in more than 80% of the cases. In our study using CSF our analysis reached classification accuracy of 89%.

Despite the relative similarity in diagnostic accuracy, none of the proteins distinguishing ALS patients from controls in the current study using plasma were the same as found in the CSF analysis. The CSF markers most strongly associated with health status were inflammatory cytokines, which were elevated in ALS patients, and the anti-inflammatory marker, IL-10, which was decreased in ALS patients (Mitchell, Freeman et al.). None of these markers were altered in plasma, suggesting inflammation in ALS is not prevalent throughout the body but limited to the CNS.

A combination of CSF and plasma biomarkers may be the optimal approach to obtaining a panel that can be used to support clinical diagnosis and monitor disease. For example, in CSF we indentified an increase in IL-8 expression with declining ALS Functional Rating Scale scores, while expression of MCP-1 and GM-CSF in plasma was associated with duration of symptoms. Independently these biomarkers had only a weak to moderate association with disease progression ($r \leq 0.5$), but combining them together may provide a useful measure of disease progression. Considering all three together was not possible in the current study because we did not have CSF data and plasma together for many subjects.
Insights into Disease Pathogenesis

In addition to providing clinical support, biomarkers may provide insights into the biological basis of the disease. Elevated plasma L-ferritin levels were a common characteristic of both ALS patients and carriers of an $H63D HFE$ allele, and elevated serum ferritin in ALS patients was recently reported in a study on ALS patients in the UK (Goodall, Haque et al. 2008). Cellular synthesis and secretion of ferritin is upregulated by iron loading and by inflammatory cytokines (reviewed in (Torti and Torti 2002)). The source of L-ferritin in the blood is generally considered to originate from macrophages of the reticuloendothelial system (Crichton, Wilmet et al. 2002). Therefore the increased L-ferritin observed in plasma from ALS patients should reflect increased iron stores or inflammation in ALS patients. To address the possibility that the changes in L-ferritin and Tf simply reflected an inflammatory state, we examined the expression of c-reactive protein (CRP), a marker of both acute and chronic inflammation (Wilson, Ryan et al. 2006). There was no increase in CRP in the ALS patients, and there was no correlation of CRP levels with L-ferritin, total plasma iron, Tf or Tf saturation. Thus, we do not interpret the increase in plasma ferritin in ALS to reflect general inflammation. This interpretation is in agreement with that offered by the UK group.(Goodall, Haque et al. 2008) Another source of L-ferritin in the plasma is a result of release from macrophages of the reticuloendothelial system. Activated macrophages increase synthesis of ferritin (Kim and Ponka 2000). Activation of macrophages is usually associated with inflammation but as indicated there was no evidence of inflammation in our subjects.
As mentioned, increased plasma L-ferritin is thought to reflect body iron stores (Torti and Torti 2002), particularly in the liver. There is no direct evidence that ALS patients have higher iron stores, but these data suggest this concept should be explored. Increased plasma L-ferritin may also indicate increased release from parenchymal cells, particularly hepatocytes, which may be characterized by altered protein synthesis and secretion in ALS (Nakano, Hirayama et al. 1987). Increased plasma ferritin is associated with allelic variants of the \textit{HFE} gene (Njajou, Houwing-Duistermaat et al. 2003) which is consistent with our study and further supports the notion that the L-ferritin could originate from hepatocytes because the macrophages in this population have relatively less iron (Wang, Johnson et al. 2008).

The other biomarker associated with ALS was Tf, which was decreased in the plasma of ALS patients, although this decrease was not observed in the ALS patients carrying \textit{H63D HFE}. Iron in the blood is mostly bound to Tf for transport throughout the body and the majority of cells acquire Tf-bound iron via Tf-receptor mediated endocytosis. Plasma Tf mostly originates from the liver and typically decreases with elevated iron stores (Raja, Pountney et al. 1999) or inflammation (Gabay and Kushner 1999; Cunietti, Chiari et al. 2004). Thus a reduced level of Tf in plasma with ALS could potentially reflect an increase in iron stores or inflammation with disease. As with L-ferritin levels, Tf levels were not associated with CRP or total iron levels. Thus, consistent with the interpretation of the increase in ferritin, the decrease in Tf is not interpreted as reflective of iron stores or inflammation.
Similar to increased L-ferritin in plasma, there is a possibility that reduced Tf levels may indicate hepatic dysfunction. Hepatic morphological and functional abnormalities have been reported in a study of ALS patients (Nakano, Hirayama et al. 1987). These data suggest the relationship between liver iron stores and liver function and activation of macrophages is a potential area for investigation in ALS. The lack of a decrease in Tf in the ALS patients with \( H63D \) \( HFE \) could support delivery of more iron to tissues including the brain (Burdo and Connor 2003) and the additional iron could be a source of oxidative stress consistent with the conceptual framework we have presented (Connor and Lee 2006) that \( HFE \) gene variants promote a milieu of iron induced oxidative stress that could contribute to ALS.

*Association of Markers with Disease Progression*

Another potential clinical use of biomarkers is for determination of disease progression. MCP1 and GM-CSF were both associated with duration of symptoms. MCP-1, a potent chemoattractant for monocyte lineage cells, increased with longer duration of symptoms. Elevated levels of MCP-1 have been associated with ALS (Mitchell, Freeman et al.; Baron, Bussini et al. 2005), and may contribute to the microgliosis and neuroinflammation associated with later stage disease progression (Henkel, Engelhardt et al. 2004; Boillee, Yamanaka et al. 2006). The elevation in MCP-1 could also be consistent with the increase in L-ferritin since both may originate from macrophages and secretion of each increases with macrophage activation (Lambotte, Cacoub et al. 2003; Thorley, Ford et al. 2007). Additionally, in our previous study we
reported an association between IL-8 levels in the CSF and disease progression (Mitchell, Freeman et al.). IL-8 is a lymphocyte chemoattractant produced in microglia and macrophages (Badolato, Ponzi et al. 1997), and functions to recruit lymphocytes to regions of neuronal degeneration.

GM-CSF levels declined in the plasma with disease progression but we previously reported an increase in CSF levels of GM-CSF in ALS patients (Mitchell, Freeman et al.). GM-CSF is synthesized by lymphocytes, macrophages and microglia and is an activator and differentiator of granulocyte and monocyte lineages therefore it is considered a potent immune stimulator (Ponomarev, Shriver et al. 2007; Volmar, Ait-Ghezala et al. 2008). GM-CSF is able to cross the blood brain barrier (Schabitz, Kruger et al. 2008). Thus decreased GM-CSF in plasma could be associated with increased transport into the CSF from plasma, but CNS sources of GM-CSF also readily exist (Volmar, Ait-Ghezala et al. 2008). Therefore, we cannot unequivocally interpret the increased CSF levels as directly related to the decreasing plasma levels but it would be consistent with increased CNS inflammation with this disease. Recently, receptors for GM-CSF have been identified on neurons and exposure to GM-CSF preserved neurons in ischemic stroke models by upregulating the anti-apoptotic molecules Bcl-2 and Bcl-XL (Schabitz, Kruger et al. 2008). Therefore GM-CSF could have a protective role in the brain and increased movement of GM-CSF from blood to brain could have a beneficial role.
Despite the potential neuroprotective effects of GM-CSF, increased CNS levels of GM-CSF and increased MCP-1 with increasing duration of disease support activation of microglia and macrophages. Microgliosis with associated neuroinflammation is proposed to contribute to late-stage ALS progression (Boillee, Yamanaka et al. 2006). The release of inflammatory mediators, and reactive oxygen and nitrogen species from microglia may directly contribute to motor neuron toxicity, and may also influence surrounding astrocytes and microglia to contribute to disease pathogenesis. For example, inflammatory cytokines downregulate expression of glutamate transporters in astrocytes, thus promoting glutamate excitotoxicity, a pathway commonly implicated in ALS pathogenesis (Tilleux and Hermans 2007). Hence, activation of microglia and recruitment of macrophages to the CNS may strongly influence progression of ALS.

Region of Onset

An additional use of the biomarker analyses in this study was to test the hypothesis that there would be a different biomarker profile for limb and bulbar onset of ALS symptoms. Any differences may provide insight into sensitivities to pathogenetic mechanisms that may vary between the brainstem and the spinal cord, underlying the phenotypic heterogeneity. Biomarker and genetic association studies have mostly failed to detect differences associated with region of onset, although a recent study identified differences in serum angiogenin levels between patients with bulbar versus limb onset (Cronin, Greenway et al. 2006). In the current study, one biomarker, pro-hepcidin, was elevated in the plasma of ALS patients with bulbar onset compared to patients with limb
onset of symptoms. Pro-hepcidin was the only protein in the biomarker panel that
decreased with increasing age; however, the site of onset of the ALS was not related to
age of onset.

Pro-hepcidin expression is generally used as an indicator of hepcidin, a systemic
regulator of iron absorption and distribution. Hepcidin complexes with the iron export
protein, ferroportin on cell membranes and the complex is internalized and degraded,
resulting in decreased iron efflux from cells (Nemeth, Tuttle et al. 2004). Declining levels
of pro-hepcidin associated with advanced age may contribute to cellular iron
redistribution, and it is possible that cellular sensitivity to iron accumulation differs
between the brainstem and spinal cord regions. The synthesis of pro-hepcidin is increased
in response to increasing levels of IL-6 (Wrighting and Andrews 2006). In our study
there was a negative correlation between the levels of IL-6 and pro-hepcidin in control
subjects, but not ALS patients. Additionally, the inflammatory cytokine, IL-1β, and the
anti-inflammatory cytokine, IL-10, were both negatively correlated with pro-hepcidin
levels. In ALS patients, only IL-1β was associated with pro-hepcidin levels, and this
relationship had a positive correlation. These results demonstrate a potential influence of
ALS disease on the relationship between inflammation and iron regulation. It should be
noted, however, that baseline influences of inflammatory cytokines on pro-hepcidin have
not been well studied, and none of these markers were elevated in ALS patients.
Impact of H63D HFE on the Biomarker Profile

Patients with the H63D HFE allele because of the reports it is more common in individuals with ALS (Wang, Lee et al. 2004; Schymick, Talbot et al. 2007; Sutedja, Sinke et al. 2007). We proposed a second hypothesis that the biomarkers are influenced by HFE genotype because, in addition to its involvement in cellular iron regulation, the HFE protein has been proposed to influence immune and inflammatory responses (Bridle, Frazer et al. 2003; Roy, Custodio et al. 2004), which have been reported as contributing to ALS (Moisse and Strong 2006). Consistent with our secondary hypothesis, five biomarkers from the panel were altered by HFE genotype, two of which were also identified in the ALS population. Similar to the increase of L-ferritin in ALS patients, L-ferritin in subjects carrying an H63D HFE allele is also increased. As previously mentioned, elevated ferritin in plasma may reflect increased iron stores but can also increase due to increased synthesis and secretion from macrophages during inflammation. Tf was increased in carriers of the H63D allele when ALS patients and controls were grouped together, which may also indicate altered iron or inflammation, but Tf levels were not associated with HFE genotype when controls and ALS patients were analyzed separately.

Neither L-ferritin nor Tf was associated with total plasma iron or c-reactive protein levels suggesting the plasma levels reflect altered tissue iron stores rather than inflammation. This interpretation is consistent with reports that HFE variants disrupt normal iron homeostasis in hepatocytes, which are the major cell type involved in
regulating synthesis and secretion of Tf (Beutler, Gelbart et al. 2000). The increase in L-ferritin may suggest that there is more parenchymal iron storage in ALS patients carrying the $H63D$ allelic variant than those with the $Wt HFE$ allele and more parenchymal iron could be associated with increased potential for oxidative stress, one of the pathogenic mechanisms under consideration in ALS.

Three cytokines, G-CSF, RANTES and IFN-γ, that were reduced in the plasma of ALS patients carrying an $H63D$ allele but were not part of the profile seen in the ALS patients with $Wt HFE$. The decrease in these cytokines could have direct relevance to disease pathogenesis and treatment strategies. For example, G-CSF has been explored for use in ALS patients to induce mobilization of hematopoietic stem cells to cross the blood brain barrier (Cashman, Tan et al. 2008). This concept is that these cells will provide a neuroprotective function in regions of motor neuron death (Cashman, Tan et al. 2008). Hence, ALS patients carrying the $H63D$ allele may be less responsive to G-CSF treatments because of their lower initial baseline levels.

Both RANTES (CCL5) and IFN-γ were decreased in the plasma of ALS patients with the $H63D HFE$ allele compared to $Wt$ homozygous ALS patients. RANTES is a chemokine expressed in a number of immune cells (Krensky and Ahn 2007), and acts to recruit and activate cells expressing the CCR5 receptor including monocytic lineage cells and $T_{H1}$ lymphocytes (de Nadai, Chenivesse et al. 2006). IFN-γ is classically produced by $T_{H1}$ lymphocytes and activates macrophage-family cells inducing them to produce IL-1, IL-6, TNF-α and a number of other potentially toxic intermediates (Gordon 2003). The
The combined effects of RANTES and IFN-γ in recruitment and activation of microglia and macrophages in the CNS likely contributes to ALS pathogenesis (Boillee, Yamanaka et al. 2006). At a superficial level, it could be argued that the decrease in RANTES and IFN-γ in the H63D HFE patients with ALS may be associated with reduced activation of microglia and there is a suggestion in some of the population studies of a later onset of disease in H63D carriers (Wang, Lee et al. 2004; Sutedja, Sinke et al. 2007). However, the interaction between iron and cytokines is complex. For example, IFN-γ also induces apoptosis in T lymphocytes and iron is a limiting factor in lymphocyte proliferation (Simka and Rybak 2008). Thus, lower levels of IFN-γ and the normal levels of Tf in the H63D HFE group might contribute to increased populations of T cells.

**Conclusion**

Our results suggest utility of plasma expression of L-ferritin and Tf in aiding the classification of ALS patients versus normal subjects. The specificity of these changes for ALS versus other diseases, particularly those mimicking ALS, should be assessed in future studies. A combined biomarker panel assessing proteins in both CSF and plasma might provide the most clinically-useful information. Compared to blood samples, CSF is not as readily collected, but our studies suggest the best biomarker analyses may include both plasma and CSF. Proteins associated with health status differed between CSF and plasma and expression of biomarkers in ALS patients greatly overlapped expression in controls, thus demonstrating the necessity of larger panels of biomarkers.
A number of studies including the present one have begun to implicate mechanisms by which the H63D HFE variant may be associated with an increased risk of ALS and alter pathogenetic mechanisms of the disease. Assessment of the total impact of the H63D HFE allele might be bolstered by a forthcoming mouse model. Cellular iron distribution is likely to be altered in the presence of HFE variants, as suggested above, possibly reducing iron accumulation in macrophages and microglia and cell type-specific iron-mediated toxicity in parenchymal cells.

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Chapter 5

HFE Polymorphisms Affect Cellular Glutamate Regulation

Abstract

Polymorphisms in the HFE gene are common genetic variants in Caucasians. One polymorphism, the H63D HFE variant, is reportedly increased in individuals with amyotrophic lateral sclerosis (ALS) and may be associated with Alzheimer’s disease (AD) and ischemic stroke. We have developed a human neuroblastoma cell line that expresses the different HFE polymorphisms to begin to explore the mechanism by which the H63D allele could increase risk of late onset neurodegenerative disorders. Glutamate excitotoxicity is a leading theory behind the pathogenesis of ALS and AD, and is thought to be a factor in ischemic stroke. We therefore tested the hypothesis that cells with the H63D allelic variant have a phenotype that is consistent with a profile that would promote glutamate toxicity. Specifically, we tested the effects of Wt, H63D, and C282Y HFE on thapsigargin-induced glutamate secretion and uptake of radioactive glutamate. In support of this hypothesis, compared to Wt HFE, expression of H63D HFE is associated with a 57% increase at 3 hours and a 19% increase at 24 hours in neuronal glutamate secretion. Sodium-dependent glutamate transport in cells expressing H63D HFE was approximately 40% of that in cells expressing Wt HFE. The polymorphism-associated changes could be mimicked by treating vector-transfected cells with the iron chelator, desferroxamine, and ferric ammonium citrate. The ability of pharmacological agents
Trolox, the water-soluble form of vitamin E, and minocycline to increase sodium-dependent glutamate uptake differed by \( HFE \) genotype and implicate oxidative stress in the effect on glutamate transport. In addition, intracellular calcium status is also altered in a genotype-specific manner which could further impact glutamate secretion. This study supports the concept that the H63D HFE variant fundamentally alters the cell phenotype in mechanisms beyond iron regulation, and suggests that the \( H63D \) allele could be a risk factor for neurodegenerative diseases by increasing the potential for glutamate toxicity.

Introduction

Glutamate is tightly regulated within the CNS because it functions as an excitatory neurotransmitter, yet it can potentially cause excitotoxic damage. Glutamate excitotoxicity has been implicated in many neurological conditions including ischemic stroke and neurodegenerative diseases (Doble 1999; Kemp and McKernan 2002; Van Damme, Dewil et al. 2005; Van Den Bosch, Van Damme et al. 2006). Riluzole, an anti-glutamatergic agent, is approved by the U.S. Food and Drug Administration for treatment of amyotrophic lateral sclerosis (ALS). A class of NMDA glutamate receptor antagonists has been approved for the treatment of Alzheimer’s disease (AD). Several anti-glutamatergic compounds have shown efficacy in stroke models or stroke patients (Hazell 2007).

Because of the possible toxic effects of glutamate, cellular regulatory mechanisms exist to minimize exposure to glutamate. The glutamate-glutamine cycle is one pathway
through which appropriate handling of glutamate is achieved (Daikhin and Yudkoff 2000). In this model, neuronal glutamate is transported into synaptic vesicles via vesicular glutamate transporters (VGLUTs) (Moechars, Weston et al. 2006). Excitatory amino acid transporters (EAATs), located on surrounding astrocytic and neuronal membranes as well as endothelial cells, are responsible for reducing extracellular glutamate concentrations (Rothstein, Jin et al. 1993; O'Kane, Martinez-Lopez et al. 1999; Hawkins, O'Kane et al. 2006). Glutamate in astrocytes is then converted to glutamine which is transferred back to neurons and reconverted to glutamate by glutaminase (Daikhin and Yudkoff 2000).

Several studies have suggested that iron impacts cellular glutamate regulation. An analysis of mice deficient in H-ferritin, the predominant iron storage protein in neurons, revealed higher levels of glutamate in several brain regions (Ill, Mitchell et al. 2006). Iron-loading in neurons and retinal pigment epithelial cells has been shown to increase glutamate secretion (McGahan, Harned et al. 2005). Iron-induced oxidative stress is also associated with increased release of glutamate from retinal cells (Rego, Santos et al. 1996). Recently, treatment with the iron chelator desferroxamine has been shown to inhibit hippocampal increases in glutamate associated with hypoxia/ischemia (Papazisis, Pourzitaki et al. 2008) Therefore, it is logical to hypothesize that alterations in the function of the HFE protein, a protein that is associated with regulation of iron uptake by cells, would influence glutamate regulation.
The wild-type (Wt) HFE protein regulates intracellular iron by reducing the affinity of transferrin receptor for diferric transferrin (Feder, Penny et al. 1998). The proteins resulting from the \textit{H63D} and \textit{C282Y} allelic variants in the \textit{HFE} gene are deficient in this function (Feder, Penny et al. 1998). The \textit{H63D} variant reportedly occurs with increased frequency in ALS, AD, and ischemic stroke (Wang, Lee et al. 2004; Goodall, Greenway et al. 2005; Connor and Lee 2006; Ellervik, Tybjaerg-Hansen et al. 2007; Restagno, Lombardo et al. 2007; Sutedja, Sinke et al. 2007), although not all studies have found this association, particularly in AD (Connor and Lee 2006). Because HFE is intricately involved in iron regulation and innate immunity, and iron regulation also contributes to lymphocyte function, there is likely a strong environmental interaction between the presence of \textit{HFE} gene variants and their role as modifiers and risk factors of disease.

To gain insights in the relationship between the HFE gene variants and their potential contribution to neurodegenerative disease, we developed a stably-transfected human neuroblastoma cell model (Lee, Patton et al. 2006). In addition, we identified several commercially available human astrocytoma cell lines endogenously expressing different HFE variants for this analysis. The significance of this research is that the \textit{C282Y} and \textit{H63D} \textit{HFE} allelic variants are common in Caucasians, thus, the ability of these variants to impact cell function and possibly treatment outcome is relevant to a significant percentage of the overall population at risk for neurodegenerative disease.
Methods

Materials

SH-SY5Y human neuroblastoma cell lines and human astrocytoma cell lines U251, LN-18, U118 MG, U138 MG, T98G, CCF-STTG1 were all obtained from American Type Culture Collection (Manassas, VA). Cell culture reagents including Dulbecco’s Modified Eagle’s Medium (DMEM), DMEM/F12, penicillin/streptomycin, penicillin/streptomycin/glutamine and Geneticin were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Gemini Bio-Products (West Sacramento, CA). Polymerase chain reaction (PCR) primers for VGLUT1-3, and EAAT1-5 were purchased from SuperArray (Frederick, MD). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) reagent was purchased from Promega (Chatsworth, CA). Mouse anti-glutaminase and rabbit anti-excitatory amino acid transporter-2 (EAAT2) were purchased from ABR-Affinity Bioreagents (Golden, CO). Rabbit anti-vesicular glutamate transporter-1 (VGLUT1) was purchased from Abcam (Cambridge, MA). Rabbit anti-EAAT1 and rabbit anti-EAAT3 were purchased from Alpha Diagnostic International (San Antonio, TX). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from GE Healthcare (Princeton, NJ). The DNeasy Tissue kit and RNaseasy Mini kit were purchased from Qiagen (Valencia, CA). Dihydrokainic acid (DHK) was purchased from Tocris Bioscience (Ellisville, MO). Polyacrylamide gels (4-20% Criterion, and 5% Criterion TBE) and DC protein assay were obtained from Bio-Rad (Hercules, CA). L-[3,4-3H]-glutamate was purchased from Perkin Elmer (Boston, MA). Amplex Red
Glutamic Acid Assay Kit and Fura-2 acetoxymethyl (AM) ester were purchased from Invitrogen (Carlsbad, CA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

**Cell Culture**

As previously reported (Wang, Lee et al. 2004), we created stably-transfected human neuroblastoma SH-SY5Y cell lines expressing wildtype, H63D, and C282Y forms of HFE. This cell line was chosen because it lacks endogenous HFE expression (Wang, Lee et al. 2004; Lee, Patton et al. 2006). Cells transfected with vector only served as controls. Transfected cells were maintained in DMEM/F12 medium supplemented with 10% FBS, 1% penicillin/streptomycin/glutamine, 1x nonessential amino acids, 1.8 g/L sodium bicarbonate, and 500 μg/mL Geneticin. Cell culture medium contained 20 μM glutamine. Prior to use, cells were differentiated for four to six days in 10 μM all-trans retinoic acid and the genotype of each cell line was confirmed by sequencing. All astrocytoma cell lines were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were generally used after seven to ten (maximum) passages.
**HFE Genotyping**

Genomic DNA was extracted from astrocytoma cell lines using the DNeasy Tissue kit according to the manufacturer's instructions. The PCR-amplified DNA samples were digested by restriction enzymes to determine the HFE genotype (Merryweather-Clarke, Pointon et al. 1997). DNA (50 ng) was amplified by PCR using the primers forward: 5’-ACA TGG TTA AGG CCT GTT GC-3’, and reverse: 5’ CTT GCT GTG GTT GTG ATT TTC C-3’ for detection of the H63D allele, and primers forward: 5’ CAA GTG CCT CCT TTG GTG AAG GTG ACA CAT-3’, and reverse: 5’ CTC AGG CAC GTG CCT CCT TTG GTG AAG GTG ACA CAT-3’ for the C282Y allele. The PCR reaction was performed in a total volume of 50 μL containing 100 pmol of each primer in a PTC-200 Peltier Thermal Cycler (MJ Research, Woburn, MA). The PCR reaction was initiated at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 45 s, annealing at 58 °C for 45 s, and extension at 72 °C for 1 min 30 s. A 294 bp product was obtained for the H63D polymorphism. Following digestion with MboI, the 63D allele gave restriction fragments of 237 and 57 bp, while the H63 allele resulted in three fragments (138, 99 and 57 bp). For the C282Y polymorphism, the primers resulted in a 343 bp product. Following digestion with RsaI, the 282Y allele resulted in products of 203, 111 and 29 bp, whereas the C282 allele yielded restriction fragments of 203 and 140 bp. Polymorphisms were detected by restriction fragment length analysis in 5% TBE polyacrylamide gel.
RT-PCR

We performed RT-PCR for VGLUT1, VGLUT2, VGLUT3, EAAT1, EAAT2, EAAT3, EAAT4, and EAAT5 using primers commercially available from SuperArray (Frederick, MD). Total RNA was extracted from cells using the RNeasy Mini kit and 5 μg of total RNA was used in a 20 μL cDNA reaction mixture. PCR was performed on cDNA using primers commercially available. The PCR reaction was initiated at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The PCR product was then analyzed on a Criterion 5% TBE gel.

Western Blotting

Cells were grown in T-75 cell culture flasks and then lysed with a RIPA buffer supplemented with 1% Triton X-100 and protease inhibitor cocktail. Forty micrograms total protein was separated by electrophoresis in a 4-20% Criterion polyacrylamide gel. Protein was then transferred to a nitrocellulose membrane and blocked for 1 hr at room temperature in tris-buffered saline-tween (TBS-T) with 5% nonfat milk. Membranes were then probed with anti-VGLUT1, anti-glutaminase, anti-EAAT1, anti-EAAT2, and anti-EAAT3 primary antibodies in TBS-T with 5% nonfat milk overnight at 4°C. HRP-conjugated secondary antibodies were then added in 5% nonfat milk for 1 hr at room temperature. Signals were obtained by chemiluminescence and visualized by CCD camera. Protein expression was quantified by densitometry and normalized to the amount
of protein loaded per well. Each blot shows results obtained from three independent cell cultures and each blot was run at least three times.

*Intracellular Iron and Calcium Measurement*

Intracellular labile iron was determined in the SH-SY5Y cells using the cell-permeable dye calcein-AM. Cells were grown in 24-well plates to 80% confluency. Calcein-AM was then added in medium at a final concentration of 0.25 μM and cells were incubated at 37°C for 30 minutes. Cells were then washed three times in ice-cold Hanks’ balanced salt solution and lysed in 200 μL RIPA buffer plus 1% Triton X-100. The fluorescence of the lysates was measured using an excitation wavelength of 485 nm and an emission wavelength of 538 nm. The fluorescence was normalized to the protein content of the lysate, determined by BioRad DC protein assay, after subtracting a blank consisting of cells incubated with medium lacking the dye. Relative intracellular calcium levels were determined in SH-SY5Y cells using the cell-permeable calcium-sensitive fluorescent dye, fura-2AM. SH-SY5Y cells were grown in a black-walled, clear-bottom 96-well plate until confluent. The medium was rinsed and replaced with fresh medium with or without 500 nM thapsigargin, a selective inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) which causes elevation of intracellular calcium. After a 1.5 hour incubation at 37°C, the medium was again rinsed and replaced with fresh medium with or without 1 μM fura-2AM. After one hour at 37°C, the cells were washed three times in medium and incubated in fresh medium for 30 minutes. Cells were then washed three times in ice-cold Hanks’ balanced salt solution. Relative calcium levels in
the cells were determined by measuring fluorescence emission at 510 nm with alternate excitation wavelengths of 340 nm and 380 nm. Binding of calcium to fura-2 shifts the optimal excitation wavelength from 380 nm to 340 nm, and thus data are reported as the 340/380 ratio of fluorescence (RFU).

Cell Viability Assays

Differentiated SH-SY5Y cell lines were grown in 96-well plates until 70-80% confluent. Cells were treated with various concentrations of desferroxamine (DFO), ferric ammonium citrate (FAC), Trolox, or minocycline, with starting doses and lengths of treatment based on previous reports. Cell viability was assessed with the colorimetric agent MTS and compared to untreated cells.

Glutamate Secretion

Stably-transfected SH-SY5Y cells were plated in 24-well plates. When the cells reached 80% confluence, the medium was replaced with 500 μL medium containing either 500 μM L-(-)-threo-3-hydroxyaspartic acid (THA, an inhibitor of glutamate uptake), or 500 μM THA plus 500 nM thapsigargin. The THA was used in this experiment because glutamate secretion can be influenced by uptake. Glutamate concentration in the medium was determined over time by removing 5 ul aliquots after 15 minutes, 3 hours, and 24 hours of thapsigargin exposure. These timepoints were chosen
to ensure accurate measurement of secreted glutamate and stability of EAAT inhibition. The amount of glutamate was measured by the Amplex Red Glutamic Acid Assay Kit. This assay involves oxidation of glutamate to produce hydrogen peroxide which then converts 10-acetyl-3,7-dihydroxyphenoxazine to the fluorescent product resorufin in an amount proportional to the starting glutamate concentration. Following removal of the medium, cells were lysed in RIPA buffer and the protein from each well was measured by DC protein assay. Intracellular glutamate content was determined by measuring glutamate in the cell lysate. Results are reported as net glutamate secreted or total intracellular glutamate normalized to the protein content of each well. The experiment was performed three times and results are given as the average ± SEM.

**Sodium-Dependent Glutamate Uptake**

Sodium-dependent glutamate uptake was measured similar to that reported by Sala et al. (Sala, Beretta et al. 2005). SH-SY5Y cells were plated in 24-well plates in 500 μL of medium at a density of 5 x 10^5 cells/mL and grown until 80-90% confluent. Astrocytoma cells were plated in 24-well plates in 500 μL of medium at a density of 1 x 10^5 cells/mL and grown until 80-90% confluent. After removing the cell culture medium, cells were washed 3 times in a HEPES-choline buffer (10 mM HEPES, 135 mM choline chloride, 5 mM KCl, 0.6 mM MgSO₄, 2.5 mM CaCl₂, 6 mM D-glucose, pH 7.4), and then incubated for 20 min in 500 μL HEPES buffer with either sodium chloride or choline chloride. After 20 min, 25 uL of 30 μM L-[3,4-³H]-glutamate (45.4 Ci/mmol) (9 parts non-radioactive:1 part radioactive) was added and the plates were incubated for 30
min at 37°C. After 30 min, cells were washed 3 times with ice-cold 0.32 M sucrose solution. Cells were then lysed in 200 μL 0.25 M NaOH, and 150 μL was added to 5 mL scintillation fluid. Consistent protein concentrations between replicates were confirmed by DC protein assay and the level of radioactivity in each sample was measured with a β-counter (Beckman Coulter, Fullerton, CA). Disintegrations per minute were converted to picomoles of glutamate based on its specific activity. Glutamate uptake was normalized to protein content and expressed as picomoles/g of protein/30 min. Glutamate uptake in the presence of sodium represented total glutamate uptake, while glutamate uptake in the absence of sodium represented sodium-independent uptake. Sodium-dependent glutamate uptake was determined by subtracting sodium-independent uptake from total uptake.

Sodium-dependent glutamate uptake was also determined in the presence of 500 μM L-(-)-threo-3-hydroxyaspartic acid (THA), which inhibits EAAT1-5, 1 mM dihydrokainic acid (DHK), which selectively inhibits EAAT2, and 500 μM L-serine-O-sulfate (L-SOS), which selectively inhibits EAAT1/3. Additionally, glutamate uptake was determined in the presence of Trolox, a water-soluble vitamin E analog, at empirically determined doses and exposure times, and minocycline, an anti-inflammatory and antioxidant, at empirically determined exposure times. A dose of 25 μM was chosen for minocycline treatment based on previous literature reports (Fernandez-Gomez, Galindo et al. 2005; Kraus, Pasieczny et al. 2005).
Statistical Analysis

All data are presented as mean ± SEM. The data were analyzed by one-way ANOVA with Tukey’s post-hoc analysis or Student’s t test, as appropriate. Differences among means were considered statistically significant when the p-value was < 0.05.

Results

Neurons derive a large portion of glutamate by deamination of glutamine to glutamate, catalyzed by glutaminase. Therefore glutaminase expression was determined in the SHSY5Y neuroblastoma cells (Figure 5-1). Glutaminase expression was 13% higher in the cells expressing the H63D allele than the cells expressing Wt HFE (p < 0.05), and 71% higher in the H63D cells than in the cells expressing C282Y HFE (p < 0.001).

Because expression of the vesicular glutamate transporters (VGLUTs) allows vesicular secretion of glutamate by transporting glutamate from the cytosol to membrane bound vesicles (Takamori, Rhee et al. 2000), we determined expression of VGLUTs in the SH-SY5Y cells. Expression at the mRNA level was confirmed for VGLUT1, but not VGLUT2 or VGLUT3 (Figure 5-1). The expression of VGLUT1 was confirmed at the protein level and quantified by western blot analysis. Cells expressing C282Y HFE had on average a 9-fold higher expression of VGLUT1 than all other cell lines (p < 0.001 for
each comparison), but no differences were observed between vector-transfected, Wt-, and H63D-expressing cells (Figure 5-2).
Figure 5-1: **Expression of VGLUT mRNA.** Expression of GAPDH and VGLUT1 mRNA was confirmed by RT-PCR in stably-transfected SH-SY5Y cells (A). These cells did not express mRNA for either VGLUT2 or VGLUT3 (B).
Figure 5-2: Expression of VGLUT1 and glutaminase. Expression of glutaminase (GLS) and vesicular glutamate transporter 1 (VGLUT1) were measured in stably transfected SH-SY5Y cells by western blot analysis, followed by quantification by densitometry. GLS expression was 13% higher in the cells expressing the H63D allele than the cells expressing Wt HFE, and 71% higher in the H63D cells than in the cells expressing C282Y HFE. Expression of VGLUT1 was measured in our SH-SY5Y cell lines by western blotting. This figure shows that all cell lines expressed VGLUT1. Cells from three different cultures are shown and the experiment was performed at least three times. The differences in expression were determined by densitometric analysis of the western blot. Cells expressing C282Y HFE expressed significantly higher levels of VGLUT1 than all other cells (p <0.001). * and *** represent p<0.05 and p<0.001 compared to Wt, respectively.
Vesicular glutamate secretion is regulated by intracellular calcium; therefore relative cytosolic calcium levels were determined in stably-transfected SH-SY5Y cells (Figure 5-3). Intracellular calcium at baseline conditions and following exposure to thapsigargin was determined using the calcium-sensitive fluorescent dye, fura-2AM. Under baseline conditions, intracellular calcium levels in both C282Y HFE (p<0.05) and H63D HFE (p<0.001) cell lines were greater than in Wt HFE cells, in the order Wt<C282Y<H63D. Thapsigargin exposure increased mean cytosolic calcium levels in each cell line, though the increase did not reach significance in the C282Y HFE cell lines (p = 0.06). Intracellular calcium levels were greatest in the H63D HFE cells, revealing altered cellular calcium handling with respect to HFE variants.
Figure 5-3: **Intracellular calcium.** Relative intracellular calcium levels were determined in SH-SY5Y cells using the calcium-sensitive fluorescent dye, fura-2AM, with and without treatment with thapsigargin. Binding of calcium to fura-2 shifts the optimal excitation wavelength from 380 nm to 340 nm, and thus data are reported as the 340/380 ratio of fluorescence (RFU). We determined intracellular levels of calcium in these cells in the pattern of C282Y = H63D > Wt at baseline, and H63D > Wt = C282Y after addition of thapsigargin. n = 4 for each. *, **, and *** represent p<0.05, p<0.01, and p<0.001, respectively, compared to Wt or Wt + thapsigargin (B).
Glutamate secretion was induced in each of the SH-SY5Y cell lines with thapsigargin in the presence of THA, an inhibitor of EAAT1-5. The latter was present to prevent cellular glutamate uptake which would lead to underestimating the amount of glutamate released. Over a 24 hour time period, a net accumulation of glutamate in the medium was demonstrated for each cell line. Conditioned medium from cells expressing H63D HFE contained 57% more glutamate than medium from cells expressing Wt HFE at 3 hours (p<0.05) and 19% more at 24 hours (p<0.05). Medium from cells expressing C282Y HFE contained 44% less glutamate at 24 hours compared to medium from cells expressing Wt HFE (p<0.001). Cell lysates showed similar levels of intracellular glutamate in vector-transfected cells and cells expressing Wt HFE and H63D HFE, while the lysate from cells expressing C282Y HFE contained 70% less intracellular glutamate compared to Wt HFE cells (p<0.001) (Figure 5-4).
Figure 5-4: Glutamate secretion. Glutamate secretion was determined in stably-transfected SH-SY5Y cells in the presence of 500 μM THA (an EAAT1-5 inhibitor) and 500 nM thapsigargin. After 15 minutes, 3 hours, and 24 hours, 5 uL of medium (1% of total medium) was collected and glutamate concentration was measured after subtracting a medium blank. Data are presented as mean ± SEM of the medium glutamate concentration normalized to the protein content of the cell lysate, with n = 3. (A) Induced glutamate secretion at 15 minutes, 3 hours, and 24 hours. Cells expressing H63D HFE secreted 57% more glutamate than cells expressing Wt HFE at 3 hours and 19% more at 24 hours. Cells expressing C282Y HFE secreted 44% less glutamate at 24 hours compared to cells expressing Wt HFE. (B) Intracellular glutamate measured in the cell lysate compared to extracellular glutamate at the 24 hour timepoint. Intracellular glutamate levels were not different between vector, H63D HFE, and Wt HFE cells. Cells expressing C282Y HFE had 70% less intracellular glutamate than Wt HFE cells. * and *** represent p<0.05 and p<0.001 compared to Wt at the same timepoint, respectively.
To further explore potential mechanisms by which the HFE genotype could influence glutamate secretion, we tested the hypothesis that the differences may be related to the differences in cellular iron loading caused by the HFE variants. Measurement of intracellular labile iron confirmed our previous report (Lee, Patton et al. 2006) of iron levels in the pattern C282Y (p<0.001 vs. Wt) > H63D (p<0.001 vs. Wt) > Wt (Figure 5-5A). To directly test the hypothesis that cellular iron status affects glutamate secretion, cells transfected with vector only were exposed to the iron chelator desferroxamine (DFO) or ferric ammonium citrate (FAC) to decrease or increase intracellular iron, respectively. Intracellular labile iron was not directly measured with these treatments as previous studies have shown the ability of these agents to modulate cellular non-heme and total iron levels (Starreveld, Kroos et al. 1995; Zhang, Haaf et al. 2005; Lee, Patton et al. 2006; Messner and Kowdley 2008), and they are commonly used for this purpose. Toxicity of DFO and FAC were first assessed at varying doses in the vector-control cell line (Figure 5-5B). Exposure to DFO resulted in a 52% decrease (p<0.05) in glutamate secretion over a 24 hour period, while treatment with FAC was associated with a 51% increase (p<0.05) in glutamate secretion over this time period, compared to control conditions (Figure 5-5C). Thus the effects of HFE polymorphisms on glutamate secretion can be mimicked by changing cellular iron status in this cell line.
Figure 5-5: Impact of cellular iron on glutamate secretion. (A) Intracellular labile iron was determined in the differentiated SH-SY5Y cells using the cell-permeable dye calcein-AM, as described in Methods. Fluorescence of calcein is quenched in the presence of iron, thus fluorescence is inversely proportional to labile iron levels. Intracellular labile iron levels were in the order C282Y > H63D = Vector > Wt. (B) Cell viability of vector-transfected cells was assessed after treatment with various concentrations of DFO and FAC for 48 hours, as reported in methods. (C) SH-SY5Y cells transfected with the vector alone were treated with various concentrations of the iron chelator desferroxamine (DFO) or ferric ammonium citrate (FAC), as described in Methods. Glutamate secretion was determined in the presence of 500 μM THA and 500 nM thapsigargin. Data are presented as mean ± SEM of the ratio of glutamate concentration to the protein content of the cell lysate and normalized to the control condition. Cells treated with 10 μM DFO secreted 52% less glutamate and cells treated with 90 μM FAC secreted 51% more glutamate compared to control at 24 hours. n = 4 for each. *, **, and *** represent p<0.05, p<0.01, and p<0.001, respectively, compared to Wt (A) or control (B and C).
Following secretion, excitatory amino acid transporters (EAATs) are responsible for clearing the majority of extracellular glutamate via a sodium-dependent mechanism. Expression of EAAT1-5 was determined at the mRNA level by RT-PCR. Each of the SH-SY5Y cell lines was determined to express mRNA for EAAT1, EAAT2, and EAAT3, but not EAAT4 or EAAT5 (Figure 5-6A). Likewise, expression of EAAT1-3 was verified by western blot analysis (Figure 5-6B). Expression of EAAT1 did not differ significantly by HFE genotype, while expression levels of both EAAT2 and EAAT3 were significantly greater in cells expressing C282Y HFE compared to all other cells (p < 0.001 for each).

Figure 5-6A
Figure 5-6: Expression of EAATs in SH-SY5Y cell lines. (A) Expression of EAAT1, 2, and 3 mRNA was confirmed by RT-PCR in stably-transfected SH-SY5Y cells. These cells did not express mRNA for either EAAT4 or EAAT5. (B) We determined the expression of excitatory amino acid transporters (EAATs) in the SH-SY5Y cells by western blotting, and found these cells to express EAAT1, EAAT2, and EAAT3. Cells from three different cultures are shown. EAAT1 expression did not differ between any of the cell types, though expression of EAAT2 and EAAT3 was significantly higher in cells expressing C282Y HFE than other cells (p < 0.001 for each).
Figure 5-6B

B

EAAT1

EAAT2

EAAT3

Vec  Wt  H63D  C282Y

Vec  Wt  H63D  C282Y

Vec  Wt  H63D  C282Y

***

Relative OD (EAAT1)

Relative OD (EAAT2)

Relative OD (EAAT3)
The uptake of glutamate was measured in each of the SH-SY5Y cell lines over a 30 minute period (Figure 5-7A). Expression of Wt HFE in the SH-SY5Y cells was associated with glutamate uptake at a rate 2.5-fold higher than cells expressing H63D HFE (p < 0.05). Expression of C282Y HFE was associated with glutamate uptake at 5.5 times the rate of cells expressing H63D HFE (p < 0.001). Because we determined that expression levels of EAAT1, EAAT2, and EAAT3 did not differ between cells expressing Wt and H63D HFE, it appears that expression levels of the transporters are not solely responsible for the different rates of transport between these cells.

We used selective inhibitors of the EAATs to isolate the contribution of various transporters to cellular glutamate uptake (Figure 5-7B). The non-selective inhibitor of EAAT1-5, THA, completely blocked sodium-dependent glutamate uptake in each cell line at a concentration of 500 μM. Likewise, L-SOS, an EAAT1/3 inhibitor, completely inhibited sodium-dependent glutamate uptake in each cell line at 500 μM. Glutamate uptake in the presence of 1 mM DHK, a selective EAAT2 inhibitor, did not significantly differ from uptake in the absence of DHK in any of the cell lines. These data indicate that EAAT1 and/or EAAT3 is responsible for all sodium-dependent glutamate uptake in these cells, in agreement with a previous report (Sala, Beretta et al. 2005).

Figure 5-7
Figure 5-7: Glutamate uptake in SH-SY5Y cells. (A) The uptake of glutamate from medium by sodium-dependent transport via the EAATs was measured in each of the cell lines similar to that described by Sala et al. (Sala, Beretta et al. 2005). Expression of Wt HFE in the SH-SY5Y cells was associated with glutamate uptake at a rate 2.5-fold higher than cells expressing H63D HFE (p < 0.05). Expression of C282Y HFE was associated with glutamate uptake at 5.5 times the rate of cells expressing H63D HFE (p < 0.001).

(B) Glutamate uptake was determined in the presence and absence of EAAT inhibitors and reported as percentage of baseline glutamate uptake to determine the roles of individual glutamate transporters. The EAAT1-5 non-selective inhibitor THA completely inhibited all sodium-dependent glutamate uptake in each of the cell lines at 500 μM. The inhibitor L-SOS, which selectively inhibits EAAT1 and EAAT3, also completely inhibited sodium-dependent glutamate uptake at 500 μM. DHK, which selectively inhibits EAAT2, did not significantly reduce sodium-dependent glutamate uptake at 1 mM. n = 5.
In light of the different rates of glutamate uptake in the SH-SY5Y cells with different HFE variants, we sought to expand our findings to another cell type and to a non-transfected cell line. Several commercially-available human astrocytoma cell lines expressing HFE variants were obtained from ATTC. We identified two cell lines homozygous for the \textit{Wt HFE} allele (U251, LN-18), three heterozygous for \textit{H63D HFE} (U118, U138, T98G), and one heterozygous for \textit{C282Y HFE} (CCF-STTG1). Similar to the SH-SY5Y neuroblastoma cells, astrocytoma cells expressing only Wt HFE transported glutamate on average at a rate approximately 2.5 times the rate of astrocytoma cells expressing H63D HFE. Astrocytoma cells expressing C282Y HFE transported glutamate at approximately 2.8 times the rate of the astrocytoma cells expressing Wt HFE (\textbf{Figure 5-8}). All comparisons between cells of different genotypes were statistically significant at the p < 0.05 level except between LN-18 vs. U138 astrocytoma cells where the difference reached p < 0.1. All comparisons between cells of the same genotype were not statistically significant (p > 0.05). Given the heterogeneity of the genetic backgrounds of the human astrocytoma cells, the similarity of the findings of the astrocytoma cells and the neuroblastoma cells is a compelling argument for the role of HFE in our results.
Figure 5-8: Glutamate uptake in astrocytoma cell lines. We measured glutamate uptake in several astrocytoma cell lines expressing different HFE variants. Similar to the SH-SY5Y cells, we found glutamate uptake differed by HFE genotype. Astrocytoma cells expressing only Wt HFE transported glutamate on average at a rate approximately 2.5 times the rate of the astrocytoma cells expressing H63D HFE. Cells expressing C282Y HFE transported glutamate at approximately 2.8 times the rate of the astrocytes expressing only Wt HFE. n = 5.
A potential mechanism by which H63D may impact the EAAT-mediated glutamate transport is through increased oxidative stress. Previous studies have demonstrated the ability of oxidative stress to impair EAAT-mediated glutamate transport (Trotti, Rolfs et al. 1999; Sala, Beretta et al. 2005) and we previously showed H63D cell lines have elevated oxidative stress compared to the other SH-SY5Y cells used in this study (Lee, Patton et al. 2006). Toxicity of the water-soluble vitamin E analog, Trolox, and the multifaceted antibiotic, minocycline, were first assessed in transfected SH-SY5Y cell lines at varying doses and lengths of treatment (Figure 5-9). SH-SY5Y neuroblastoma cells were pre-treated with Trolox for 72 hours prior to measuring glutamate uptake to determine the impact of this antioxidant on glutamate uptake (Figure 5-10). Trolox pre-treatment significantly increased glutamate uptake in each cell line, except for cells expressing C282Y HFE, which had a significant decrease in glutamate uptake with Trolox treatment. This study shows the potential for antioxidant treatment to either improve or worsen glutamate uptake, depending on HFE genotype.

Minocycline, a pharmaceutical agent which has been explored for the treatment of neurodegenerative diseases, has been shown to have direct antioxidant properties (Kraus, Pasieczny et al. 2005) as well as anti-inflammatory (Yrjanheikki, Tikka et al. 1999) and mitochondria-protective properties (Mansson, Hansson et al. 2007). Prior to measuring glutamate uptake, we pretreated SH-SY5Y cells with minocycline for 48 hours at a concentration of 25 μM, based on previous literature reports (Figure 5-10). Minocycline treatment significantly increased glutamate uptake in vector cells by 69% (p < 0.05), and
significantly decreased glutamate uptake in Wt HFE cells by 77% (p < 0.001). The effects of minocycline on the cells expressing other forms of HFE were not statistically significant.
Figure 5-9: **Cell viability assays.** Cytotoxicity of various durations and doses of Trolox and minocycline treatments were determined in differentiated stably-trasnfected SH-SY5Y cells. (A) Cell lines were treated with various doses of Trolox for 48 hours (n = 3). (B) Vector-transfected cells were treated with 200 μM Trolox for various times (n = 3). (C) Cell lines were treated with 200 μM Trolox for 72 hours. (D) Cell lines were treated for various times with 25 μM minocycline (n = 4). *, **, and *** represent p < 0.05, 0.01, and 0.001, respectively, compared to control (A), 0 hr (B and D), or 100% (C).
Figure 5-10: Glutamate uptake in SH-SY5Y cells with Trolox and minocycline treatment. Glutamate uptake was determined in each of the cell lines after pretreating the cells for 72 hours with 200 μM Trolox or 48 hours with 25 μM minocycline. Uptake is reported as a percentage of untreated cells. Trolox treatment resulted in glutamate transport rate increases of 40%, 44%, and 56% in vector, Wt, and H63D cells, respectively, but a 29% decrease in glutamate transport rate in C282Y cells. Minocycline treatment increased glutamate uptake in vector-transfected (69%), but not in the H63D or C282Y cells. Minocycline treatment significantly decreased glutamate uptake in Wt HFE cells by 77% *. **, and *** indicate p < 0.05, 0.01, and 0.001, respectively, compared to untreated cells. n = 5.
Discussion

The results of this study demonstrate that HFE polymorphisms are associated with significant differences in cellular handling of calcium and parameters associated with glutamate regulation. In particular, the results reveal that cells expressing H63D HFE are associated with higher cytosolic levels of calcium, greater secretion of glutamate and reduced uptake of glutamate compared to the Wt or C282Y forms of HFE. It is the H63D allele that is found in increased frequency in ALS and is reportedly also associated with Alzheimer’s disease and ischemic stroke. Thus these observations are consistent with the conceptual framework we propose for this line of research, which is that H63D gene variants provide a permissive milieu at the cellular level for pathways that cause neurodegenerative disease. It is noteworthy that the effect of therapeutic agents such as the antioxidant Trolox and minocycline are also influenced by the HFE polymorphisms. These data provide a compelling argument that a potential mechanism by which the H63D allelic variant of the HFE gene is associated with adult-onset neurodegenerative diseases is through promotion of glutamate excitotoxicity, although other mechanisms likely exist and continue to be explored. The data also suggest that HFE polymorphisms may influence the response to glutamatergic therapies.

Multiple factors may be contributing to the differences in net glutamate secretion in association with the HFE gene variants. Neurons derive a large portion of glutamate by deamination of glutamine to glutamate, catalyzed by glutaminase. Here we show small but significant differences in glutaminase expression which may result in differing
production of glutamate. Although such small differences may not account for the total
difference in glutamate secreted, they may contribute in part to the milieu for glutamate
toxicity. Glutamate secretion in the neuroblastoma cell lines was induced by the addition
of thapsigargin which elevates cytosolic calcium levels, demonstrating that at least some
 glutamate secretion was due to calcium-dependent mechanisms. Baseline and
thapsigargin-induced intracellular calcium levels are higher in H63D HFE cells compared
to Wt HFE cells, which is consistent with the higher rates of glutamate secretion.

Although cytosolic calcium plays a direct role in glutamate secretion, iron may
also be involved. Using retinal pigment epithelial cells and neurons, McGahan et al.
reported that increasing levels of cellular iron resulted in increased glutamate production
and subsequent glutamate secretion through modulation of aconitase activity (McGahan,
Harned et al. 2005). The presence of the Wt HFE protein is associated with decreased
iron uptake in multiple cell systems (Feder, Penny et al. 1998; Chitambar, Kotamraju et
al. 2006) including the neuroblastoma cells used in this study (Lee, Patton et al. 2006),
compared to cells expressing H63D HFE. The effects of the HFE polymorphisms on
 glutamate secretion could be mimicked in the control (vector-only transfection)
neuroblastoma cells by chelating iron or adding exogenous iron. The ability to mimic the
changes in glutamate metabolism by changing iron status indicates the changes in
 glutamate can at least be partly explained by iron regulation. The data herein suggest the
likely effect of the H63D variant on glutamate secretion is the combination of increased
intracellular iron and increased cytosolic calcium. These observations indicate
fundamental differences in cellular mechanisms beyond iron regulation in the presence of HFE variants.

While differences in net glutamate secretion may result from different rates of secretion, the SH-SY5Y and astrocytoma cells also take up glutamate from the medium. Indeed, H63D cells showed less glutamate uptake than Wt cells and C282Y cells, further supporting the notion that the H63D variant promotes glutamate toxicity. Differences in net glutamate accumulation in the medium may result from different rates of glutamate uptake. However, glutamate secretion was measured in the presence of THA, an inhibitor of all sodium-dependent excitatory amino acid transporters, suggesting that differences in uptake do not account for the differences in secretion.

The differences in baseline calcium levels between SH-SY5Y cells, and the differences after thapsigargin treatment, indicate that differential regulation of calcium may be one mechanism by which HFE variants affect glutamate regulation. It is not known exactly how HFE may alter calcium handling, although several possibilities are suggested from previous studies. For example, cytosolic calcium is buffered by a number of mechanisms including uptake into endoplasmic reticulum (Pereira, Ferreira et al. 1996) and mitochondria (Hajnoczky, Csordas et al. 2006) and extrusion from the cell (Pereira, Ferreira et al. 1996). A study using vascular smooth muscle cells demonstrated functional inactivation of SERCA by superoxide, an effect which was inhibited by the addition of superoxide dismutase (SOD) (Suzuki and Ford 1991). Pereira et al. (Pereira, Ferreira et al. 1996) demonstrated oxidative stress increased cytosolic calcium in
synaptosomes, coincident with decreased activity of SERCA as well as the plasma membrane Ca\textsuperscript{2+} ATPase and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger responsible for calcium extrusion. In astrocytes, we have previously demonstrated oxidative stress-induced increases in intracellular calcium, apparently from extracellular stores, and iron chelation inhibited all but a transient increase in calcium (Robb, Robb-Gaspers et al. 1999). The H63D HFE cells used in the current study are characterized by elevated levels of protein oxidation and decreased expression of SOD1 (Lee, Patton et al. 2006), both of which are consistent with potentially deficient calcium buffering mechanisms.

Another possible explanation for our higher levels of cytosolic calcium associated with the H63D HFE variant involves the role of mitochondria as a buffer for excessive cytosolic calcium (Hajnoczky, Csordas et al. 2006). Uptake of calcium by mitochondria is dependent on the mitochondrial membrane potential (MMP) and the MMP is decreased in the presence of the H63D HFE variant, as we have previously reported (Lee, Patton et al. 2006). The decrease in MMP in the H63D cells is consistent with the increase in intracellular calcium measured in these cells and a reduced capacity of mitochondria in these cells to buffer cytosolic calcium.

The increased oxidative stress associated with the H63D allelic variant could influence glutamate handling by the cell through inactivation of EAATs. Previous studies have demonstrated that EAATs are susceptible to oxidative modification which can render them inactive. Sala et al. (Sala, Beretta et al. 2005) proposed that oxidative inactivation of transporters was responsible for different rates of glutamate uptake in SH-
SY5Y cells expressing different variants of SOD1. Our previous data shows oxidative
stress levels in the SH-SY5Y cells in the order of H63D > Wt > C282Y (Lee, Patton et al.
2006), consistent with our current data showing glutamate uptake rates in the order
C282Y > Wt > H63D.

Because of the evidence indicating that oxidative stress could be a factor in the
 glutamate phenotype associated with HFE polymorphisms, we examined two antioxidant
 compounds. Trolox increased glutamate uptake in both Wt and H63D cells, though
glutamate uptake was decreased in cells expressing C282Y HFE. The increased uptake of
glutamate in the H63D cells in the presence of the antioxidant Trolox supports the role of
oxidative stress in limiting cellular glutamate uptake in these cells. The mechanism
underlying the Trolox effects in the C282Y cells are currently under investigation, but
these results are a clear example of how the different HFE alleles have significant and
different effects on cellular behavior consistent with our previous reports (Lee, Patton et
al. 2006).

Minocycline had no significant effect on glutamate uptake in the H63D cells but
dramatically decreased uptake in the Wt cells. In addition to its antioxidant activity
(Kraus, Pasieczny et al. 2005) minocycline also has a variety of other cellular effects
including anti-inflammatory (Yrjanheikki, Tikka et al. 1999) and mitochondria-protective
properties (Mansson, Hansson et al. 2007). Minocycline also has been identified as an
iron chelator (Grenier, Huot et al. 2000). Chelation in the iron-poor Wt HFE cells may
have reduced iron to sub-toxic levels, while chelation in the iron-replete H63D and
C282Y HFE cells may have had little impact on pathways involving glutamate uptake. We did not determine iron levels in cells after treatment with minocycline, but our data suggest that chelation of iron by minocycline may be a property of this agent worth considering in future studies. Despite these properties it was not significantly effective in the H63D cells, although mean glutamate uptake was increased by 29%. Minocycline was also not effective in treating human ALS patients, (Gordon, Moore et al. 2007) even though minocycline had beneficial effects in rodent ALS models (Kriz, Nguyen et al. 2002; Van Den Bosch, Tilkin et al. 2002; Zhu, Stavrovskaya et al. 2002; Zhang, Narayanan et al. 2003). It is likely that multiple effects of minocycline are influencing pathways involved in ALS pathogenesis and the effects on glutamate uptake we observed. The data from our cell models suggest that response to minocycline could be influenced by HFE status. Published studies on HFE gene status in ALS consistently report approximately 30% of ALS patients have the H63D allele (Wang, Lee et al. 2004; Goodall, Greenway et al. 2005; Restagno, Lombardo et al. 2007; Sutedja, Sinke et al. 2007), but pharmacogenetics were not considered in the minocycline trial, and the human subjects in this trial almost certainly were heterogeneous with respect to HFE genotype.

The similar increase in glutamate uptake in the Wt and H63D HFE cells in the presence of Trolox and the differential effect of minocycline in these cells demonstrates that the differences between these cells may not only reflect oxidative stress levels. Other explanations for differences in glutamate uptake include differences in mitochondrial function, as already mentioned, and differential subcellular localization of glutamate transporters. Although the vector-transfected cells and cells expressing Wt and H63D
HFE showed similar total expression of the glutamate transporters, it is not clear what portion was located at the plasma membrane in position to transport glutamate. Additionally, cells expressing C282Y HFE showed higher expression levels of EAAT3, but again the subcellular localization of the transporters is not known.

Despite similar effects on cellular iron levels, the H63D and C282Y HFE variants had profoundly different effects on glutamate regulation. Differences in calcium handling may explain the differences in glutamate secretion observed, although the C282Y HFE-expressing cells were characterized by reduced expression of glutaminase and lower intracellular glutamate levels compared to all other cell types, potentially indicating lower levels of glutamate synthesis. We have previously demonstrated that expression of C282Y HFE resulted in reduced protein and lipid oxidation (Lee, Patton et al. 2006), which could be due to an upregulation of antioxidant defenses. This reduced oxidative stress associated with the C282Y variant may be the cause of higher rates of glutamate uptake, but the effects of greater expression of EAAT3 in particular cannot be excluded. These reduced levels of oxidative stress may also underlie the decreased glutamate uptake after Trolox treatment in these cells, which could reduce reactive oxygen species in these cells to borderline insufficient levels. Thus, the differences in glutamate regulation observed between cells expressing H63D and C282Y HFE may extend beyond the influence of HFE on cellular iron regulation.

In conclusion, our results indicate altered cellular glutamate regulation dependent upon variants of HFE. These effects are consistent with a phenotype contributing to
glutamate excitotoxicity in the presence of the H63D HFE variant compared to Wt HFE, and are consistent with the population data that H63D HFE is a risk factor or modifier for adult onset neurodegenerative diseases. The mechanism underlying the potential for increased glutamate toxicity associated with H63D appears related to oxidative stress effects on the cell that are generated by elevated intracellular iron. Specifically, glutamate secretion is likely elevated because of increased intracellular calcium stemming from loss of buffering capacity of the mitochondria and ER. There is a further contribution to glutamate toxicity through decreased uptake of glutamate that may result from oxidative stress-related effects on EAATs. Association of the C282Y HFE allele with neurodegenerative diseases is largely unknown due to the low allelic frequency of this polymorphism. The few reports in the literature are contradictory, finding both a protective (Buchanan, Silburn et al. 2002) and risk-associated (Dekker, Giesbergen et al. 2003; Robson, Lehmann et al. 2004; Guerreiro, Bras et al. 2006) roles. At least with respect to glutamate excitotoxicity, our data suggest the C282Y HFE variant may be protective due to reduced secretion of glutamate and greater rate of glutamate uptake. Our data support our hypothesis that one aspect of the biological basis of the relationship between H63D HFE expression and neurodegenerative diseases could be altered glutamate homeostasis.

These findings may have significant clinical relevance. They support the view of ALS as a clinical syndrome with pathogenetic heterogeneity and a common final pathway of motor neuron and (to a lesser degree) more widespread neuronal loss. Therapeutic
agents for subsets of patients with ALS may be identified by stratifying clinical trials on
the basis of \textit{HFE} genotype.

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histological outcome in the hippocampus of neonatal rats after hypoxia-ischemia."


Chapter 6

Influence of HFE Variants and Cellular Iron Regulation on Monocyte Chemoattractant Protein-1

Abstract

Polymorphisms in the hemochromatosis (HFE) gene have been proposed as genetic modifiers of several neurodegenerative disease characterized by excessive neuroinflammation. Variants of this gene are relatively common and may result in subclinical cellular iron loading even in the absence of overt clinical iron overload. The effects of the variants as well as the resulting cellular iron dyshomeostasis potentially impact a number of disease-associated pathways. We tested the hypothesis that the two most common HFE variants, termed H63D and C282Y, would affect cellular secretion of cytokines and trophic factors. Our results demonstrate that HFE polymorphisms influence the synthesis and release of MCP-1, a potent chemoattractant for monocyctic-lineage cells, via a mechanism at least partly involving iron regulation. The effects of HFE on regulation of MCP-1 appear to extend beyond cellular iron acquisition as these two HFE variants affected MCP-1 through alternate mechanisms. Minocycline, a multifaceted antibiotic explored for use in treating a number of neurologic conditions associated with inflammation, differentially decreased MCP-1 secretion depending on HFE genotypes.
Introduction

Iron accumulation in various brain regions is associated with the pathogenesis of several neurodegenerative diseases. Iron loading is likely to influence diseases through generation of oxidative stress in many cases; however, iron potentially influences a number of disease-associated pathways including the post-transcriptional regulation of amyloid precursor protein (Rogers, Randall et al. 2002). In contrast, iron deficiency during neurodevelopment has been associated with cognitive dysfunction, possibly due to deficient myelination or monoamine synthesis (reviewed in (Beard and Connor 2003)). Brain iron deficiency, particularly in the substantia nigra and putamen has also been associated with restless legs syndrome (Earley, Allen et al. 2000). The degree to which loss of iron homeostasis contributes to cell dysfunction and loss is not clear. Cellular iron homeostasis involves the complex interaction of numerous proteins, and genetic variants in genes encoding these proteins may influence this process and hence pathogenesis and disease progression.

Polymorphisms in the *hemochromatosis* (*HFE*) gene have been proposed as genetic modifiers of amyotrophic lateral sclerosis (ALS) (Wang, Lee et al. 2004), Alzheimer’s disease (AD) (Connor and Lee 2006), and Parkinson’s disease (PD) (Dekker, Giesbergen et al. 2003). The HFE protein is primarily known for regulating transferrin-mediated cellular iron intake through its interaction with the transferrin receptor (Feder, Penny et al. 1998). The H63D HFE variant appears deficient in reducing the affinity of transferrin receptor for transferrin, while the C282Y HFE variant
aggregates in the endoplasmic reticulum (Waheed, Parkkila et al. 1997; Feder, Penny et al. 1998). HFE variants are thought to have low penetration rates (Waalen, Nordestgaard et al. 2005) but there is little disagreement that HFE variants impact iron status at the cellular level (Waheed, Parkkila et al. 1997; Feder, Penny et al. 1998; Lee, Patton et al. 2006; Gao, Zhao et al. 2008), and influence distribution of iron through regulation of hepcidin synthesis (Bridle, Frazer et al. 2003). Thus, the paradigm in which clinical scientists work to reconcile these differences is that individuals with \textit{HFE} gene variants “subclinically load iron.” It is our contention that the “subclinical loading of iron” over a 40-50 year period or more as a person ages will establish a cellular environment that will more readily enable oxidative stress-based diseases in these individuals (Connor and Lee 2006).

In addition to its direct contribution to oxidative stress, iron dysregulation has the potential to indirectly impact numerous disease-associated pathways. Previous studies have demonstrated that cellular iron regulation may impact the secretion of soluble mediators of inflammatory reactions (Zhang, Surguladze et al. 2006; Natarajan, Fisher et al. 2007). Iron dysregulation in neuronal cells induces toxicity (Nunez-Millacura, Tapia et al. 2002), potentially resulting in recruitment of inflammatory cells (Baron, Bussini et al. 2005). One factor proposed to contribute to the relentless progression associated with many neurodegenerative diseases is the recruitment and activation of microglia in the regions of neuronal death (Boillee, Yamanaka et al. 2006). As mediators of innate immunity in the central nervous system, microglia respond to a variety of cytokines and
chemokines and in turn secrete a variety of inflammatory mediators and trophic factors which likely contribute to neurodegenerative disease pathogenesis.

Monocyte chemoattractant protein -1 (MCP-1), is known to recruit and enhance the activation of cells of the monocyte lineage (Gunn, Nelken et al. 1997). Higher levels of MCP-1 have been found in CSF and plasma in association with a number of diseases characterized by excessive inflammation including ALS, AD and PD. Additionally, previous studies from our group and others (Mitchell, Freeman et al.; Inadera, Egashira et al. 1999) have determined a positive correlation between age and expression of MCP-1, which may play a role in the higher incidence of neurodegenerative diseases at advanced ages.

By influencing cellular iron homeostasis, we hypothesize that HFE variants differentially impact secretion of cytokines and trophic factors, which may be a factor in the association with these alleles with various diseases. In the current study we determine the influence of HFE variants and iron regulation on cellular secretion of factors relevant to neurodegenerative disease and explore mechanisms underlying these relationships.
Methods

Materials

SH-SY5Y human neuroblastoma cell lines and human astrocytoma cell lines U251 and U138 MG, were obtained from American Type Culture Collection (Manassas, VA). Mouse microglial BV-2 cells were a kind gift of Dr. Steven W. Levison (University of Medicine & Dentistry of New Jersey). Cell culture reagents including Dulbecco’s Modified Eagle’s Medium (DMEM), DMEM/F12, penicillin/streptomycin, penicillin/streptomycin/glutamine and Geneticin, as well as calcein-acetoxymethyl (AM) ester were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Gemini Bio-Products (West Sacramento, CA). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) reagent was purchased from Promega (Chatsworth, CA). PCR primers for MCP-1 were purchased from SuperArray (Frederick, MD). The DNeasy Tissue kit and RNeasy Mini kit were purchased from Qiagen (Valencia, CA). The DC protein assay and the Bio-Plex Human 27-plex panel of cytokines and growth factors were obtained from Bio-Rad (Hercules, CA). Anti-heat shock protein (HSP)-70 and anti-Hsp90 antibodies were purchased from Stressgen (Ann Arbor, MI). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).
Cell Culture

As previously reported (Wang, Lee et al. 2004), we created stably-transfected human neuroblastoma SH-SY5Y cell lines expressing FLAG-tagged wildtype (Wt), H63D, and C282Y forms of HFE. This cell line was chosen because it lacks endogenous HFE expression (Wang, Lee et al. 2004; Lee, Patton et al. 2006). As a control, we also transfected cells with vector alone. Transfected cells were maintained in DMEM/F12 medium supplemented with 10% FBS, 1% penicillin/streptomycin/glutamine, 1x nonessential amino acids, 1.8 g/L sodium bicarbonate, and 500 μg/mL Geneticin. Cell culture medium contained 20 μM glutamine. Prior to use, cells were differentiated for four to six days in 10 μM all-trans retinoic acid and the genotype of each cell line was confirmed by sequencing.

Astrocytoma cell lines were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. BV-2 cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Cells were generally used after seven to ten (maximum) passages.

HFE Genotyping

Genomic DNA was extracted from astrocytoma cell lines using the DNeasy Tissue kit according to the manufacturer's instructions. The PCR-amplified DNA samples were digested by restriction enzymes to determine the HFE genotype (Merryweather-Clarke, Pointon et al. 1997). DNA (50 ng) was amplified by PCR using the primers
forward: 5’-ACA TGG TTA AGG CCT GTT GC-3’, and reverse: 5’ CTT GCT GTG GTT GTG ATT TTC C-3’ for detection of the H63D allele, and primers forward: 5’ CAA GTG CCT CCT TTG GTG AAG GTG ACA CAT-3’, and reverse: 5’ CTC AGG CAC TCC TCT CAA CC-3’ for the C282Y allele. The PCR reaction was performed in a total volume of 50 μL containing 100 pmol of each primer in a PTC-200 Peltier Thermal Cycler (MJ Research, Woburn, MA). The PCR reaction was initiated at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 45 s, annealing at 58 °C for 45 s, and extension at 72 °C for 1 min 30 s. A 294 bp product was obtained for the H63D polymorphism. Following digestion with MboI, the 63D allele gave restriction fragments of 237 and 57 bp, while the H63 allele resulted in three fragments (138, 99 and 57 bp).

For the C282Y polymorphism, the primers resulted in a 343 bp product. Following digestion with RsaI, the 282Y allele resulted in products of 203, 111 and 29 bp, whereas the C282 allele yielded restriction fragments of 203 and 140 bp. Polymorphisms were detected by restriction fragment length analysis in 5% TBE polyacrylamide gel.

*Cell Viability Assays*

Differentiated SH-SY5Y cell lines were grown in 96-well plates until 70-80% confluent. Cells were treated with various concentrations of desferroxamine (DFO) or ferric ammonium citrate (FAC). Cell viability was assessed with the colorimetric agent MTS and compared to untreated cells. Toxicity of Trolox and minocycline were previously assessed, as described in Chapter 5.
**Western Blotting**

SH-SY5Y cells were grown as separate cultures until approximately 80% confluent. Cells were lysed with a RIPA buffer supplemented with 1% Triton X-100 and protease inhibitor cocktail. Forty micrograms total protein was separated by electrophoresis in a 4-20% Criterion polyacrylamide gel. Protein was then transferred to a nitrocellulose membrane and blocked for 1 hr at room temperature in TBS-T with 5% nonfat milk. Membranes were then probed with anti-FLAG (1:2000), anti-Hsp70 (1:1000) and anti-Hsp90 (1:1000) primary antibodies in TBS-T with 5% nonfat milk overnight at 4°C. HRP-conjugated secondary antibodies (1:5000) were then added in 5% nonfat milk for 1 hr at room temperature. Signals were obtained by chemiluminescence and visualized by CCD camera. Each blot shows results obtained from three independent cell cultures.

**Intracellular Labile Iron Measurement**

Intracellular labile iron was determined in the SH-SY5Y cells using the cell-permeable dye calcein-AM. Cells were grown in 24-well plates to 80% confluence. Calcein-AM was then added in medium at a final concentration of 0.25 μM and cells were incubated at 37°C for 30 minutes. Cells were then washed three times in ice-cold Hanks’ balanced salt solution and lysed in 200 μL RIPA buffer plus 1% Triton X-100. The fluorescence of the lysates was measured using an excitation wavelength of 485 nm and an emission wavelength of 538 nm. The fluorescence was then normalized to the
protein content of the lysate, determined by BioRad DC protein assay, after subtracting a blank consisting of cells incubated with medium lacking the dye.

*Multiplex Cytokine Bead Assay*

We performed multiplex analysis on undiluted cell culture supernatants using the Bio-Plex Human 27-plex panel of cytokines and growth factors (Bio-Rad; Hercules, CA). The proteins in this panel included eotaxin, fibroblast growth factor basic (FGF basic), granulocyte colony stimulating factor (G-CSF), granulocyte-monocyte colony stimulating factor (GM-CSF), interferon-gamma (IFN-γ), IFN-γ induced protein-10 (IP-10), interleukin-1beta (IL-1β), IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-8, IL-10, IL-12(p70), IL-13, IL-15, IL-17, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1β (MIP-1β), platelet derived growth factor (PDGF) bb, regulated on activation normal T-cell expressed and presumably secreted (RANTES), tumor necrosis factor-alpha (TNF-α), and vascular endothelial growth factor (VEGF). Briefly, cells were plated in 24-well plates and grown to approximately 70% confluency. Medium was then changed, and cells were incubated for 48 hours. Cells were lysed in RIPA buffer after samples of media were removed and analyzed by a multiplex antibody-based assay. Fifty μL of each sample or standard was added in duplicate to a 96-well filter plate and mixed with 50 μL of antibody-conjugated beads for one hour at room temperature. After one hour, wells were washed and 25 μL of detection antibody was added to each well. After a 30 minute incubation, wells were washed and 50 μL of streptavidin-PE was added to
each well and incubated for 10 minutes. A final wash cycle was then completed and 125 μl of assay buffer was added to each well. The plate was then analyzed using a Bio-Plex 200 workstation (Bio-Rad). Analyte concentration was calculated based on the respective standard curve for each cytokine.

*Measurement of MCP-1 Release*

Cells were plated in 24-well plates and growth to ~70% confluency. The medium was replaced and cells were incubated for 48 hours, after which time medium was collected, cells were washed two times in Hanks’ balanced salt solution, and cells were lysed in RIPA buffer plus 1% Triton X-100. Protein content of the cell lysate was determined by DC protein assay. MCP-1 concentrations in the conditioned medium were determined using commercially available anti-human (SH-SY5Y or astrocytoma cells) or anti-mouse (BV-2 cells) MCP-1 ELISA (GE Healthcare, Piscataway, NJ), and reported as MCP-1 normalized to the protein content of the cell lysate. The impact of iron loading and iron deprivation on MCP-1 release was determined in each cell line. Each cell line was treated with varying concentrations of ferric ammonium citrate (FAC) or desferroxamine (DFO) at levels below toxic concentrations, determined by MTS cytotoxicity assay. Additionally, MCP-1 release was determined in transfected SH-SY5Y cells treated with 50 or 100 μM Trolox or 25 μM minocycline, all concentrations determined to be below toxic levels. For FAC, DFO, Trolox, or minocycline treatment, agents were added to cell culture medium when medium was changed after cells reached ~70% confluency, resulting in treatment for 48 hours.
Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

We performed qRT-PCR for MCP-1 and GAPDH mRNA expression in SH-SY5Y cells using PCR primers commercially available from SuperArray. Total RNA was extracted from five independent cell cultures using the RNeasy Mini kit (Qiagen) and 5 μg of total RNA was used in a 20 μL cDNA reaction mixture using the ReactionReady First Strand cDNA synthesis kit (SuperArray). PCR was performed on cDNA using the RT² Real-Time SYBR Green assay (SuperArray) with an ABI 7300 (Applied Biosystems, Foster City, CA) real-time PCR system. The reaction was initiated at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. Expression levels of MCP-1 were normalized to expression of GAPDH mRNA.

NF-κB

Nuclear fractions were isolated from four independent cultures of each stably-transfected SH-SY5Y cell line using a nuclear extraction kit (Millipore, Billerica, MA). Nuclear extracts were analyzed for NF-κB (p50) content by commercially-available colorimetric assay (Cayman Chemical, Ann Arbor, MI), and results are expressed as optical density per milligram of nuclear protein.
Statistical Analysis

Data are shown as mean ± SEM, with each n representing an independent cell culture. The data were analyzed by one-way ANOVA with Tukey’s post-hoc analysis or Student’s t test, as appropriate. Differences among means were considered statistically significant when the p-value was < 0.05.

Results

Expression of FLAG-tagged HFE was confirmed in the cells prior to use, as shown in (Figure 6-1A). Relative iron levels were determined in cells using the fluorescent dye calcein-AM whose fluorescence is quenched by binding iron. Consistent with the known functions of the HFE protein, we determined intracellular levels of labile iron in these cells in the pattern of C282Y (p<0.001 vs. Wt) > H63D (p<0.05 vs. Wt) > Wt (Figure 6-1B). These data demonstrate that the cells in our study had differences in the labile iron pool associated with the different polymorphisms and are generally consistent with our previous report (Lee, Patton et al. 2006).
Figure 6-1: **Confirmation of cell transfection.** (A) A representative western blot is shown demonstrating FLAG-tagged HFE expression in transfected SH-SY5Y cells (B) Intracellular labile iron was determined in the differentiated SH-SY5Y cells using the cell-permeable dye calcein-AM, as described in Methods. Fluorescence of calcein is quenched in the presence of iron, thus fluorescence is inversely proportional to labile iron levels. Intracellular labile iron levels were in the order C282Y > H63D = Vector > Wt. Results are reported here as the average ± SEM of three independent assays. * and *** indicate p<0.05 and p<0.001, respectively, compared to Wt.
The effect of HFE variants on the release of soluble mediators was determined using the transfected SH-SY5Y cells. A multiplexed antibody-based bead assay was used to screen a prospective panel of analytes consisting of inflammatory and anti-inflammatory cytokines and trophic factors. IL-1 receptor antagonist, IL-7, IL-9, IL-12(p70), IL-13, IFN-γ, MCP-1, RANTES, Eotaxin, and VEGF were measured in detectable quantities in cell-conditioned medium from vector-transfected cells or cells expressing Wt and/or H63D HFE. Of these, only the difference in MCP-1 reached statistical significance between cells expressing Wt HFE and those expressing H63D HFE (p<0.001) (Table 6-1).
Table 6-1

Table 6-1: Expression of secreted factors in cell-conditioned medium from stably-transfected SH-SY5Y cells. Values given as mean (SEM) in units pg/mL/mg protein. ND = not detected.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Vector</th>
<th>Wt</th>
<th>H63D</th>
<th>C282Y</th>
<th>H63D vs. Wt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>257.02 (27.74)</td>
<td>81.70 (9.87)</td>
<td>73.80 (9.19)</td>
<td>ND</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>280.68 (4.30)</td>
<td>225.75 (7.99)</td>
<td>212.60 (5.28)</td>
<td>ND</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-7</td>
<td>121.29 (4.48)</td>
<td>60.85 (3.99)</td>
<td>63.68 (2.30)</td>
<td>ND</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>IL-9</td>
<td>31.32 (1.71)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>IFN-gamma</td>
<td>380.22 (17.28)</td>
<td>161.88 (19.75)</td>
<td>136.34 (19.26)</td>
<td>ND</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>MCP-1</td>
<td>368.28 (21.99)</td>
<td>238.57 (5.90)</td>
<td>406.65 (5.03)</td>
<td>138.90 (13.85)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RANTES</td>
<td>40.34 (2.55)</td>
<td>23.88 (2.54)</td>
<td>21.40 (2.11)</td>
<td>ND</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>eotaxin</td>
<td>92.30 (14.14)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>VEGF</td>
<td>254494 (24952)</td>
<td>85155 (6444)</td>
<td>77234 (3948)</td>
<td>8751 (580)</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>
Over 48 hours, cells expressing H63D HFE released 71% more MCP-1 than cells expressing Wt HFE (p<0.001), while cells expressing C282Y released 42% less MCP-1 than Wt-expressing cells (p<0.001), as shown in Figure 6-2A. Similar results were also obtained using undifferentiated cells (Figure 6-2B).
**Figure 6-2**: MCP-1 secretion in SH-SY5Y cells. (A) MCP-1 concentrations were measured in conditioned medium from differentiated SH-SY5Y cells and normalized to the protein content of the cell lysis solution and reported here as the average ± SEM of five independent assays. Cells expressing H63D HFE released 71% more MCP-1 than cells expressing Wt HFE, while cells expressing C282Y HFE released 72% less MCP-1 than Wt-expressing cells. C282Y cells released significantly less MCP-1 than all other cell lines. (B) Similar results were obtained using undifferentiated cells. **, and *** represent $p<0.01$, and $p<0.001$, respectively, compared to Wt HFE cells.
The mechanism by which HFE variants may differentially regulate synthesis of MCP-1 was explored at the transcription level. RNA was isolated from each SH-SY5Y cell line for measurement of MCP-1 mRNA expression. After normalizing to the expression of GAPDH mRNA, MCP-1 mRNA expression was 3.0- (p<0.05), 3.2- (p<0.05), and 4.1-fold higher (p<0.01) in vector-transfected, H63D HFE, and C282Y HFE cells, respectively, compared to cells expressing Wt HFE (Figure 6-3A). Between cells expressing Wt HFE and those expressing H63D HFE, MCP-1 mRNA expression followed the pattern of MCP-1 secretion, indicating the difference between these cells lines is likely due to regulation at the transcription level. Despite less secretion of MCP-1 associated with the C282Y HFE variant compared to H63D HFE, MCP-1 mRNA expression was not significantly different between the two cell lines. This may potentially indicate differences in post-transcriptional or post-translational regulation between these cells.

NF-κB is known to have a prominent role in the transcriptional regulation of many inflammatory mediators including MCP-1 (Inadera, Sekiya et al. 2000). Analysis of the nuclear content of NF-κB in the SH-SY5Y cell lines, however, revealed no differences associated with any of the HFE variants (Figure 6-3B).
Figure 6-3: MCP-1 mRNA expression and nuclear NF-κB. (A) RNA was isolated from five separate cultures of differentiated SH-SY5Y cells and used for quantitative RT-PCR. Expression of MCP-1 mRNA was normalized to the expression of GAPDH mRNA. MCP-1 mRNA expression was 3.0-, 3.2-, and 4.1-fold higher in vector-transfected, H63D HFE, and C282Y HFE cells, respectively, compared to cells expressing Wt HFE. (B) Nuclear fractions were isolated from four independent cultures of stably-transfected SH-SY5Y cell lines. Nuclear content of NF-κB (p50) was assessed by colorimetric assay and reported as optical density per milligram of nuclear protein. Results are expressed as mean ± SEM. No differences in NF-κB (p50) were found between any of the SH-SY5Y cell lines. n=4. * and ** represent p<0.05 and p<0.01, respectively, compared to Wt HFE cells.
Heat stress, likely mediated through induction of heat shock proteins, reportedly decreases production of MCP-1 (Urs and Heidemann 2004). SH-SY5Y cells were assessed for expression of the heat shock proteins Hsp70 and Hsp90. Cells expressing C282Y HFE were characterized by increased expression of Hsp70 without an increase in Hsp90 expression. Other cell lines showed no differences in expression of either protein (Figure 6-4).
Figure 6-4: **Heat shock protein expression.** Expression of Hsp70 and Hsp90 were determined in differentiated SH-SY5Y cell lines by western blotting, demonstrating increased expression of Hsp70 in C282Y HFE cells. Samples from three separate cell cultures for each cell line were analyzed on the same gel.
Because the role of HFE is thought to involve regulation of cellular iron uptake, the hypothesis that cellular iron status may influence the synthesis and release of MCP-1 was tested. Toxicity of the iron chelator, desferroxamine (DFO), and ferric ammonium citrate (FAC) were assessed at varying doses in each stably-transfected SH-SY5Y cell line (Figure 6-5). These results showed no toxicity of DFO up to 10 μM, or FAC up to 90 μM, in any cell line.
Figure 6-5: **Cell viability assays.** Cell viability of stably-transfected SH-SY5Y cells was assessed after treatment with various concentrations of DFO and FAC for 48 hours, as reported in methods. $n = 4$ for each. *, **, and *** represent $p<0.05$, 0.01, and 0.001, respectively, compared to control.
Each cell line was treated with non-toxic doses of DFO and FAC to determine the effect of exogenous cellular iron modulation on MCP-1 release (Figure 6-6). DFO treatment resulted in dose-dependent decreased MCP-1 release in vector (maximum 75%, p<0.001), Wt HFE (maximum 68%, p<0.001), and H63D HFE cells (maximum 64%, p<0.001), at 5 μM and 10 μM, but had no effect in C282Y HFE cells at these concentrations. Treatment with 90 μM FAC resulted in significantly more MCP-1 secretion in Wt HFE cells (maximum 76%, p<0.001), but had no effect at any concentration in other cell lines.
Figure 6-6: Impact of iron on MCP-1 secretion. Vector-transfected cells (A) and cells expressing Wt HFE (B), H63D HFE (C), or C282Y HFE (D) were treated with 10 uM, 5 uM, or 1 uM desferrooxame (DFO) or 10 uM, 30 uM, or 90 uM ferric ammonium citrate (FAC) for 48 hours and the secretion of MCP-1 was determined by ELISA and normalized to cellular protein content. n = 4. *, **, and *** indicate p < 0.05, 0.01, and 0.001 compared to control. NS = not significant.
In addition to neurons, within the CNS astrocytes and microglia are also sources of MCP-1 secretion. (Baron, Bussini et al. 2005). Therefore, as an additional model, we employed the use of two human astrocytoma cell lines, U251 and U138 to study the effect of iron modulation on MCP-1 secretion. Both cell lines were genotyped for the $H63D$ and $C282Y HFE$ variants (ms under review). The $Wt/Wt$ $HFE$ U251 cells were characterized by higher intracellular labile iron levels ($p<0.01$) and higher constitutive MCP-1 release ($p<0.001$) compared to the $H63D/Wt$ $HFE$ U138 cells (Figure 6-7A). Altering cellular iron levels with DFO and FAC, with doses obtained from previous reports (Saleppico, Boelaert et al. 1999; Lee and Connor 2005; Raicevic, Mladenovic et al. 2005; Elstner, Holtkamp et al. 2007), had a similar effect in both cell lines. The secretion of MCP-1 was reduced by a maximum of 77% ($p<0.001$) in U138 cells (Figure 6-7B) by DFO at a concentration of 50 $\mu$M, while secretion of MCP-1 was reduced by a maximum of 66% ($p<0.05$) in U251 cells (Figure 6-7C) at this concentration of DFO. Maximum increase of MCP-1 secretion in U138 cells was 41% ($p<0.01$) with 100 $\mu$M FAC, while this concentration of FAC had no effect in U251 cells. Maximum increase of MCP-1 secretion in U251 cells was 107% ($p<0.001$) with 200 $\mu$M FAC. Additionally, this effect was studied in mouse microglial BV-2 cells (Figure 6-7D). Iron chelation with 20 $\mu$M DFO produced a maximal inhibition of MCP-1 release of 22% ($p<0.01$), and treatment with 100 $\mu$M FAC produced a maximal increase of MCP-1 release of 22% ($p<0.05$).
Figure 6-7: MCP-1 secretion in astrocytoma and microglial cells. Secretion of MCP-1 in U138 (H63D/Wt) and U251 (Wt/Wt) human astrocytoma cell lines and mouse microglial BV-2 cells was determined with various concentrations of DFO or FAC, as reported in Methods. MCP-1 concentration in medium was determined, relative to the protein content of each well, and was normalized to control levels. n = 4. *, **, and *** represent p < 0.05, p<0.01, and p < 0.001, respectively, compared to control.
To further study pathways influencing MCP-1 release, stably transfected SH-SY5Y cells were treated for 48 hours with Trolox, a water-soluble form of vitamin E. Additionally, cells were treated with minocycline, an antibiotic with iron chelation, antioxidant, anti-inflammatory, and mitochondria-protective properties (Yrjanheikki, Tikka et al. 1999; Grenier, Huot et al. 2000; Kraus, Pasieczny et al. 2005; Mansson, Hansson et al. 2007), which has been explored for the treatment of neurodegenerative diseases. These doses and duration of treatment were chosen based on cell viability assays described in Chapter 5. Trolox at either concentration had no significant effect on MCP-1 release in any of the SH-SY5Y cell lines in this study. Minocycline treatment resulted in a 76% decrease (p<0.01) and 56% decrease (p<0.001) in MCP-1 release in vector-transfected and H63D HFE cells, respectively, but no significant effects in cells expressing Wt or C282Y HFE (Figure 6-8).
Figure 6-8: Trolox and minocycline effects on MCP-1 secretion. Stably transfected SH-SY5Y cells were treated for 72 hours with Trolox or 48 hours with minocycline to determine the impact of these agents on MCP-1 release. Trolox at either concentration had no significant effect of MCP-1 release in any of the SH-SY5Y cell lines in this study. Minocycline treatment resulted in a 76% decrease (p<0.01) and 56% decrease (p<0.001) in MCP-1 release from vector and H63D cells, respectively, but no significant effects in Wt or C282Y cells. n = 4. **, and *** indicate p<0.01, and p<0.001, respectively, compared to baseline MCP-1 secretion for each respective cell line.
Discussion

The results of this study demonstrate that cells expressing H63D HFE are associated with higher intracellular iron levels, greater expression of MCP-1 mRNA, and greater secretion of MCP-1 compared to cells expressing Wt HFE. The vector-control cells which had labile iron levels similar to the H63D-expressing cells also had similar findings with respect to MCP-1 regulation. Chelating iron or adding exogenous iron to Wt HFE cells decreased and increased, respectively, MCP-1 secretion, indicating at least a strong indirect relationship between intracellular iron availability and MCP-1 secretion. Chelating iron in H63D HFE cells reduced MCP-1 secretion, but the addition of iron did not further increase MCP-1 secretion. These data are consistent with a role for iron in regulating MCP-1 secretion and suggest the effect of iron on MCP-1 release in the H63D HFE cells is at a maximum. To demonstrate that the effects of iron modulation on MCP-1 secretion were not cell-specific, two astrocytoma cell lines and a microglial cell line were examined and similar results were found for all cells examined. That is, addition of iron is associated with increased MCP-1 secretion and chelation of iron decreases MCP-1 secretion. Treatment with the antioxidant Trolox did not affect MCP-1 secretion in any SH-SY5Y cell lines, but the multifaceted antibiotic minocycline reduced MCP-1 secretion in cells expressing H63D HFE. These data may have significant clinical implications as discussed below. The effects of HFE variants on MCP-1 regulation appear to extend beyond their effects on iron regulation. The C282Y HFE variant, which was also characterized by higher intracellular iron levels and greater expression of MCP-
mRNA, secreted lower amounts of MCP-1 compared to Wt HFE cells. Additionally, cells expressing C282Y HFE were insensitive to the effects of iron modulation on MCP-1 secretion and any effects of Trolox or minocycline on MCP-1 secretion.

Consistent with levels of secreted protein, MCP-1 mRNA expression was greater in the H63D HFE cells, indicating that regulation was likely occurring at the transcriptional level. The best known transcriptional regulator of MCP-1, NF-κB, does not appear to be involved here, suggesting roles for other transcriptional regulators of MCP-1. Our findings were consistent with a previous report that iron status did not affect baseline nuclear NF-κB in microglial cells (Zhang, Surguladze et al. 2006). Also similar to our findings, hemin has been found to increase secretion of the chemokine IL-8 in microvascular endothelium via a mechanism independent of NF-κB activation (Natarajan, Fisher et al. 2007).

MCP-1 may also be regulated by hypoxia-inducible factor-1 (HIF-1). Iron chelation generally has similar effects as hypoxia in regulating HIF-1 target gene expression (Mojsilovic-Petrovic, Callaghan et al. 2007), and hypoxia has been shown to inhibit MCP-1 mRNA expression and MCP-1 protein secretion in a mouse macrophage cell line (Bosco, Puppo et al. 2004). However, hypoxia can upregulate MCP-1 gene expression and protein release in conjunction with an increase in HIF-1α in human and mouse astrocytes (Mojsilovic-Petrovic, Callaghan et al. 2007). Thus regulation of MCP-1 by HIF-1 remains unclear and may be cell-type specific. Gene regulation by HIF-1 was not analyzed in the present study, but secretion of vascular endothelial growth factor, a
protein under the control of HIF-mediated gene transcription, was not different between the Wt and H63D HFE genotypes.

While the H63D and C282Y HFE variants have similar effects on cellular iron acquisition, cellular localization of the mutant proteins may have a role in the differing phenotypes observed in the current study as well as multiple previous studies. Wildtype and H63D HFE bind beta-2-microglobulin (β2M) which is needed for transport to the plasma membrane (Waheed, Parkkila et al. 1997). C282Y HFE does not bind β2M and is retained in the endoplasmic reticulum in aggregates which induce an ER overload response and unfolded protein response (Lawless, Mankan et al. 2007). The increased expression of Hsp70 associated with C282Y HFE likely serves as a compensatory response to protein aggregation in the ER. Both Hsp70 and Hsp90 are localized to the ER and cytosol and function in a range of protein folding and stress signaling pathways (Saibil 2008). This induction of heat shock proteins may function to reduce MCP-1 secretion. In vivo, preheating rats prior to LPS treatment reduced secretion of MCP-1 in bronchoalveolar lavage specimens, despite increasing MCP-1 mRNA expression, (Urs and Heidemann 2004). This effect from heating was likely due to induction of heat shock proteins, and may also explain our finding of reduced MCP-1 secretion characteristic of the C282Y HFE cells. It must be noted, however, that a previous study demonstrated that cells expressing C282Y HFE secreted more MCP-1 than Wt HFE-expressing cells (Lawless, Mankan et al. 2007). These results were obtained using transiently-transfected HEK-293 cells, and thus, differences in methodology and cell models may explain the conflict with our results.
A likely mechanism by which cellular iron status would affect cell stress and MCP-1 regulation is through the formation of reactive oxygen species. In the present study the antioxidant Trolox had no significant effect on MCP-1 secretion in any of the SH-SY5Y cell lines. It is possible that the length of treatment (72 hours) was not sufficient to impact MCP-1 synthesis or release, or that oxidative stress was not a major factor in the effects of iron levels on MCP-1. Longer duration of treatment showed evidence of cellular toxicity, however, thus limiting the length of treatment (Chapter 5). Minocycline has iron chelation (Grenier, Huot et al. 2000), antioxidant (Kraus, Pasieczny et al. 2005), anti-inflammatory (Zemke and Majid 2004), caspase-inhibitory (Zemke and Majid 2004), and mitochondria-protective properties (Fernandez-Gomez, Galindo et al. 2005). In the present study, effects of treating cells with minocycline varied with HFE genotype, significantly decreasing MCP-1 release in the two SH-SY5Y cell lines with the highest MCP-1 secretion. These data suggest that minocycline, and presumably other pharmacological treatment strategies, should consider HFE genotype in evaluating treatment efficacy.

The multifaceted nature of minocycline causes uncertainty of the specific contribution of each mechanism of action. Minocycline, like other tetracyclines, has iron chelation properties (Grenier, Huot et al. 2000). The ability to reduce intracellular iron levels may explain the benefit of minocycline in reducing MCP-1 secretion in the relatively iron-laden H63D HFE cells. The lack of effect seen in Wt HFE cells, which responded to iron chelation with DFO, may result from other effects of minocycline, or
simply reflect the relative differences in chelator strengths between DFO and minocycline. An alternative explanation for the differential effects of minocycline involves its actions on mitochondria. Minocycline has been shown to have mitochondrial protective properties, including reduction of mitochondrial swelling and permeability transition pore opening (Fernandez-Gomez, Galindo et al. 2005). We have previously demonstrated mitochondrial dysfunction in the presence of the H63D HFE variant (Lee, Patton et al. 2006). Additionally, we have determined that the H63D HFE variant is associated with elevated cytosolic calcium levels (ms under review), that may result from altered mitochondrial function. Mitochondrial dysfunction and elevated calcium levels are mechanisms proposed to induce transcription of MCP-1; however, the association of mitochondrial function with MCP-1 regulation is mostly regulated through NF-κB (Kim, Kim et al. 2007). Thus, in contrast to its other actions, the data herein suggest that the effects of minocycline on MCP-1 secretion involve iron chelation.

Similar findings to our cell culture based studies have been reported in population studies. Hemochromatosis patients homozygous for the C282Y polymorphism had lower plasma levels of MCP-1 than H63D homozygous patients and Wt/Wt healthy controls (Lawless, White et al. 2007). We recently demonstrated higher levels of MCP-1 in the cerebrospinal fluid of ALS patients compared to a neurological disease control group (Mitchell, Freeman et al.). The potential contribution of MCP-1 to this disease is likely mediated by its role in recruiting and activating microglia and macrophages. The exact role of microglia in neurodegenerative diseases is unknown, as both protective and injurious effects have been proposed (Boillee, Yamanaka et al. 2006; Galimberti,
Fenoglio et al. 2006), although evidence increasingly suggests microglial activation contributes to neuronal destruction. Reducing expression of mutant SOD1 selectively in microglia was shown to delay late-stage disease progression in an ALS mouse model (Boillee, Yamanaka et al. 2006). Anti-inflammatory therapies including minocycline have shown benefit in treating ALS animal models (Zhu, Stavrovskaya et al. 2002), but efficacy in humans has not been demonstrated (Gordon, Moore et al. 2007). Patient heterogeneity is likely to contribute to this therapeutic failure. Here we have demonstrated the effects of minocycline may vary by HFE genotype. Studies have consistently demonstrated that approximately 30% of ALS patients carry at least one H63D or C282Y HFE allele (Wang, Lee et al. 2004; Sutedja, Sinke et al. 2007), which may affect the power of a clinical trial to detect a benefit or harm from this drug.

In conclusion, our results demonstrate that HFE polymorphisms influence the synthesis and release of MCP-1, a potent chemoattractant for monocytic-lineage cells, via a mechanism at least partly involving iron regulation. However, the mechanisms of HFE influencing MCP-1 release extend beyond the effects of HFE on cellular iron acquisition and oxidative stress. MCP-1, as well as inflammatory effects regulated by microglia and macrophages, has been implicated in the pathogenesis of progressive neurodegenerative diseases, several of which have also been associated with HFE polymorphisms. Our results suggest that this association may result partly from the impact of HFE variants on microgliosis and associated increases in neuroinflammation, although future in vivo studies are needed to clarify and expand upon these findings. Minocycline has been explored for use in treating a number of neuroinflammatory conditions including ALS,
AD, PD, multiple sclerosis, and ischemic stroke (Gordon, Moore et al. 2007; 2008; Zhang, Metz et al. 2008) (Choi, Kim et al. 2007) (Yrjanheikki, Tikka et al. 1999). The multiple mechanisms of action of this drug and our evidence of effects dependent on \(HFE\) genotype suggest a pharmacogenetic effect which should be further assessed.

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Chapter 7

The \textit{H63D HFE} Allele as a Risk Factor for ALS and Pharmacogenetic Considerations of HFE Polymorphisms

Introduction

The complex pathogenesis of ALS resulting in relatively selective degeneration of motor neurons has hampered the elucidation of disease risk factors, causes, and mechanisms of pathogenesis, as well as the identification of effective treatments. Despite numerous studies, few genetic risk factors for ALS have been identified, validated, and studied at the functional level (Schymick, Talbot et al. 2007). This thesis provides extended analysis at the genetic and functional levels of the association of the \textit{H63D HFE} allele with ALS and the mechanisms underlying this relationship. By demonstrating the association of the \textit{H63D HFE} allele with an increased risk of ALS, these studies suggest improper regulation of iron is involved in the pathogenesis of ALS and suggest environmental and other genetic factors affecting bodily iron may also be involved.

Genetic Association

Early studies assessing the \textit{H63D HFE} allele as a risk factor for sporadic ALS largely supported the association of this allele with an increased risk of ALS (Wang, Lee et al. 2004; Yen, Simpson et al. 2004; Goodall, Greenway et al. 2005; Restagno,
Lombardo et al. 2007; Sutedja, Sinke et al. 2007). These studies were limited by small numbers of ALS patients, geographically and/or ethnically limited study populations, and assessment of single nucleotide polymorphisms in isolation. We addressed some of these issues by genotyping the largest number of ALS patients to date and using samples from the NINDS Neurogenetics Repository, a collection of DNA samples from across the United States. In aggregate, these studies suggest the \(H63D\) \(HFE\) allele is associated with an increased risk of ALS in a dose-dependent manner.

Whether the \(H63D\) \(HFE\) allele is linked to another allele that may also increase risk of ALS is not known, but this is a possibility. The \(H63D\) \(HFE\) allele could even be merely tagging the true disease-associated allele in the region, but it is unlikely that the \(H63D\) allele is only tagging another allele without contributing to ALS pathogenesis. Surrounding genes that may be implicated by association with the \(H63D\) \(HFE\) allele consist mostly of histone genes with little to no known association with diseases (Nayernia, Meinhardt et al. 2003; Bogni, Cheng et al. 2006; Parssinen, Alarmo et al. 2008). Thus, in this region, \(HFE\) variants have been most widely associated with disease and are most likely to be associated with ALS. A mere tagging function of the \(H63D\) \(HFE\) allele is also unlikely because functional assessment of this genetic variant in numerous studies (Waheed, Parkkila et al. 1997; Feder, Penny et al. 1998; Lee, Patton et al. 2006) has elucidated a number of ways the H63D HFE protein alters pathways implicated in ALS pathogenesis. Thus, the \(H63D\) \(HFE\) allele could be associated with another risk-associated locus, but also appears to independently contribute to disease pathogenesis.
Identifying the $H63D$ $HFE$ allele as a risk factor for ALS implies that the incidence of ALS may be increased in populations with greater frequencies of this allele. Although large-scale and properly-controlled studies are few, it has been suggested that the incidence of ALS is greater in Caucasians (Cronin, Hardiman et al. 2007). The frequency of the $H63D$ $HFE$ allele is also highest in Caucasians (Merryweather-Clarke, Pointon et al. 1997), and this may be a factor in the ethnic variance. The frequencies of the $H63D$ and $C282Y$ alleles vary by geographic region, with the $C282Y$ variant being most common in northern Europe and the $H63D$ variant being more common in Mediterranean regions (Merryweather-Clarke, Pointon et al. 2000). The incidence of ALS is nearly equal in Irish and Italian populations (Traynor, Codd et al. 1999; Beghi, Millul et al. 2007; Bonvicini, Vinceti et al. 2008), however, possibly suggesting either that the study populations have been inadequate to detect any difference or that other genetic variants may compensate for the $HFE$ allelic differences. Alternatively, differing environmental influences between these geographic regions may compensate for genetic differences. A better assessment of the impact of the $H63D$ and $C282Y$ alleles at the population level may compare their frequencies in ALS patients within the same geographic region.

Based on the existing evidence, our conclusion is that the $H63D$ $HFE$ allele is the best established genetic risk factor for sporadic ALS. The genetic association of the $H63D$ $HFE$ polymorphism with ALS does not identify the mechanisms by which it contributes to disease or how treatments may target these processes. Subsequent studies
included in this thesis sought to define the pathogenetic mechanisms and the roles of the H63D HFE variant in particular, as well as the C282Y HFE variant, in the pathways associated with ALS.

**Biomarker Identification**

We took the novel step of considering the impact of *HFE* variants, particularly the *H63D* allele, on the expression of biomarkers in cerebrospinal fluid (CSF) and plasma because of the association of the *H63D* allele with an increased risk of ALS. The hypothesis behind this line of reasoning was that an allele that alters cellular pathways involved in disease pathogenesis would likely impact the expression of biomarkers associated with the disease. A relatively novel approach of simultaneously measuring numerous low-abundance cytokines and trophic factors was also utilized. To some degree, both broad and targeted screening approaches have previously been used to assess biomarkers associated with ALS and a number of other diseases. However, no single method is best for screening protein biomarkers as each method inherently targets different protein subsets. For example, the use of proteomic technologies has likely excluded the identification of extremely low-abundance biomarkers, such as the cytokines indentified in our study.

The combined approaches of multiplex biomarker measurement and multifactorial analysis were used for the aims of this thesis since we proposed that a single biomarker would not achieve desired levels of accuracy, sensitivity and specificity in classifying
subjects by disease status. In agreement with this hypothesis, we determined that a panel of five CSF biomarkers provided the best accuracy in distinguishing ALS patients from a group of neurological disease controls. This study also identified altered expression of several markers dependent on \( HFE \) gene. Thus we accomplished our goal of identifying biomarkers capable of distinguishing the ALS patient group from the control group, and demonstrated the impact of HFE variants on biomarker expression. The identification of efficiently-measured cytokines capable of identifying ALS patients suggests this approach may be clinically applicable if the data hold in additional clinical trials.

Although the CSF analyses provided potentially useful clinical biomarkers, identification of biomarkers in plasma would be advantageous because of the inherent difficulties and risks in obtaining CSF. For these reasons, analysis of plasma biomarkers associated with ALS is warranted, and was our next step.

We analyzed roughly the same panel of biomarkers used for the CSF analysis in comparing ALS patients versus normal controls. The use of normal controls allowed better age and sex matching of patients and controls. Additionally subjects were matched for possession of the \( H63D \) \( HFE \) allele for the plasma analysis. The iron homeostasis proteins, L-ferritin and transferrin, were altered by disease status, and could be used to classify subjects, demonstrating the potential involvement of iron misregulation in ALS pathogenesis. Additionally, as suggested in Chapter 4, the identification of lower Tf expression in ALS patients compared to controls may indicate hepatic dysfunction accompanying disease pathogenesis. Over 20 years ago hepatic abnormalities either at the microscopic or functional levels were identified in the majority of ALS patients (Nakano,
Thus plasma Tf levels may reflect insufficient protein synthesis in the liver. The role of hepatic function in ALS pathogenesis appears to be a line of research worth pursuing because of the liver’s critical role in metabolism, iron regulation, and inflammation, which all likely contribute to ALS pathogenesis. Plasma biomarkers altered by the presence of the $H63D$ allele, including L-ferritin and Tf, which were both elevated in subjects with one $H63D$ allele, suggest potential iron loading of parenchymal cells, as opposed to the macrophages which typically store iron (Ganz 2005; Nemeth and Ganz 2006). The impact of parenchymal iron loading may exacerbate pathways associated with ALS to increase risk of developing ALS in the $H63D$ carriers.

Another protein associated with iron homeostasis, pro-hepcidin, may have important implications in ALS pathogenesis. Pro-hepcidin was elevated in ALS patients with bulbar onset compared to patients with limb onset of symptoms, and pro-hepcidin levels also negatively correlated with age. As a precursor of hepcidin, a systemic regulator of iron absorption and distribution (Nemeth, Tuttle et al. 2004), deficient levels of pro-hepcidin may contribute to cellular iron redistribution and excessive gut iron absorption. The synthesis of pro-hepcidin is increased in response to increasing levels of IL-6 (Wrighting and Andrews 2006). However, we found a negative correlation between plasma IL-6 levels and expression of pro-hepcidin in control subjects, but not ALS patients. Additionally, IL-1β and IL-10 both negatively correlated with levels of pro-hepcidin in control subjects. In ALS patients, increased IL-1β was associated with increased pro-hepcidin levels. These results demonstrate a potential influence of ALS disease on the relationship between inflammation and iron regulation. It should be noted,
however, that baseline influences of inflammatory cytokines on pro-hepcidin have not been well studied, and none of these markers differed between ALS patients and controls.

Biomarkers associated with disease progression and/or disease severity may ultimately be used for assessing prognosis and response to treatment. In CSF samples from ALS patients, we identified a negative correlation between expression of the chemokine IL-8 and revised ALS Functional Rating Scale score. This scale represents a measure of disease progression and severity, and thus, IL-8 may be a potential biomarker of disease progression. Two markers in plasma samples, monocyte chemoattractant protein-1 (MCP-1) and granulocyte-macrophage colony stimulating factor (GM-CSF), were correlated with duration of symptoms, suggesting potential biomarkers of disease progression. Changes in biomarker expression throughout the course of the disease may also indicate changing cellular pathways. The biomarkers we identified suggest increasing inflammation with disease progression. Declining plasma levels of GM-CSF with duration of symptoms may also be a factor in the higher levels of GM-CSF we found in the CSF of ALS patients and may indicate a greater influence of this cytokine in the CNS later in the disease course.

Overall, our results suggest that measurement of six proteins in CSF and four proteins in plasma provides an efficient assessment of biomarkers that may aid in establishing the diagnosis of ALS and provide a measure of disease severity and progression. Ultimately, sensitive and specific biomarkers for ALS would permit earlier and more accurate diagnosis, and thereby facilitate earlier enrollment of patients into
clinical treatment trials. Clinical trials frequently exclude patients with less definitive diagnoses (e.g. suspected ALS), and thus accurate classification of these subjects may permit inclusion in trials if ALS is confirmed, or prevent exposure to an unnecessary therapeutic agent if ALS is excluded. In addition, the ability to stratify patients into subgroups based on mechanisms of pathogenesis and to correlate biomarker expression with disease progression may allow tailored drug regimens and more sensitive measures of treatment response to improve clinical drug trials for ALS patients. It is likely that panels of biomarkers, possibly including a mix of genetic, protein, and other markers, may be necessary for clinical use.

**Cellular Effect of HFE Variants and A Model for the Impact of H63D HFE on ALS Risk and Pathogenesis**

This thesis increases support for the *H63D HFE* allele as being a risk factor for ALS. By their function in cellular iron regulation which stands in position to negatively impact many disease-associated pathways, HFE variants may greatly influence ALS pathogenesis, as discussed in Chapter 1. The frequency of *HFE* variants in the population places them in position to impact a large number of patients. We have demonstrated that the H63D HFE polymorphism may contribute to generation of glutamate excitotoxicity through both increased secretion and reduced cellular uptake of glutamate. This genetic variant might also be associated with an increased tendency toward neuroinflammation by increasing secretion of monocyte chemoattractant protein-1 (MCP-1). Additionally, the effects of HFE variants on these pathways are likely to be cell-type specific. The
effects of HFE variants and iron on glutamate regulation are mostly confined to neurons and astrocytes, while initiation of inflammation may be most pronounced in neurons. Here we explore a model for the mechanisms by which the H63D variant may increase risk of developing ALS.

Recent data has suggested that a conducive neuronal environment may underlie a predisposition to the onset of ALS (Boillee, Yamanaka et al. 2006). Early processes occurring in neurons prior to disease onset may include an accumulation of labile iron, aggregation of proteins, oxidative inactivation of calcium and glutamate transporters, and mitochondrial dysfunction resulting from oxidative stress. Based on previous data and results presented in this thesis, we propose that H63D HFE contributes to each of these pathways and creates a conducive neuronal environment for the onset of ALS. These concepts will be explored in the subsequent paragraphs.

The H63D form of HFE, as opposed to Wt HFE, likely results in excessive iron accumulation in the brain due to Tf receptor-mediated iron uptake at the blood brain barrier. Wt HFE decreases the affinity of Tf receptor for transferrin, limiting cellular iron acquisition, while the H63D form of HFE is deficient in this function (Feder, Penny et al. 1998). Iron may be distributed to any cells within the CNS, but iron uptake by neurons is probably increased disproportionately due to expression of Tf receptor and HFE in neurons but not other brain cells ((Connor, Milward et al. 2001) and unpublished data). Iron within neurons may generate reactive oxygen species (ROS) via the Fenton reaction, and these ROS are likely to impair a number of proteins involved in disease-relevant
pathways. Aggregation of mutant SOD1 has been proposed to underlie the toxic gain of function associated with dismutase-active SOD1, and oxidative modification of SOD1 may have a similar result. Wang et al. (Wang, Lee et al. 2004) previously demonstrated reduced expression of SOD1 in the presence of H63D HFE, but this may represent only a decrease in soluble SOD1 expression due to oxidation of SOD1 and subsequent aggregation. Hence the H63D HFE variant may impact cellular processes similar to mutant SOD1. Protein aggregates are toxic to cells by sequestering molecular chaperones (e.g. heat shock proteins) and degradative enzymes needed for normal cellular functioning. It should be noted that expression of C282Y was also associated with reduced detection of SOD1 by western blotting (Wang, Lee et al. 2004), possibly for the same reasons as stated for the H63D form of HFE. However, as noted in Chapter 6, expression of C282Y HFE was associated with an upregulation of Hsp70, which may be serving a compensatory response that limits the toxicity of this HFE variant. The lack of upregulation of heat shock proteins in cells expressing H63D HFE would deprive cells of that benefit.

H63D HFE may also create a toxic neuronal environment by damaging mitochondria. A major target of the excess iron may be mitochondria, and resulting damage to the mitochondria may be a central factor in disease pathogenesis. Oxidative damage to proteins of the electron transport chain and lipids of the mitochondrial inner membrane can impair normal ATP synthesis with widespread consequences. A reduced mitochondrial membrane potential resulting from the impaired electron transport chain function would also impair the calcium-sequestering function of mitochondria, thus
predisposing cells to the calcium-mediated damage associated with glutamate excitotoxicity. Direct oxidative inactivation of calcium transporters located on the mitochondrial membranes, endoplasmic reticulum, and plasma membrane may also result in elevated cytosolic calcium levels in the affected neurons.

Glutamate excitotoxicity is considered a likely mechanism involved in the pathogenesis of ALS, but it is unknown whether it contributes to early or late stage disease. Although astrocytes account for the bulk of glutamate uptake from the synaptic cleft (Maragakis and Rothstein 2001), deficient neuronal glutamate uptake may be sufficient to cause excitotoxic extracellular glutamate levels. Our data suggest that neurons expressing H63D HFE secrete more glutamate compared to neurons expressing Wt HFE, and have deficient glutamate uptake. This combination associated with H63D HFE could produce a milieu conducive to the initiation of motor neuron destruction and clarify why the C282Y HFE variant has not been associated with an increased risk of ALS.

We are not suggesting that H63D HFE causes ALS. However, the accumulation of cellular insults resulting from this form of HFE may increase the risk that a disease trigger will initiate the cascade of disease pathogenesis. Continuing with the line of evidence suggesting initial stages of disease pathogenesis involve the motor neurons, any trigger that accentuates neuronal toxicity may initiate the disease cascade. Previous authors have suggested the possibility of an infectious agent triggering the disease. The exact mechanism by which this would occur is unknown, but a susceptibility to the
particular agent(s) may also be conferred by the H63D variant of HFE. Our plasma biomarker study in Chapter 4, as well as previous data, suggest the H63D HFE allele may be associated with a relative shift of iron stores from the reticuloendothelial system to parenchymal cells, particularly hepatocytes. This shift in iron may impair the ability of hepatocytes to properly regulate systemic iron, and may also induce toxicity in hepatocytes that impairs their normal function.

As mentioned, hepatic dysfunction has been associated with ALS before, and may influence the disease by several mechanisms. As part of the normal acute phase response involving hepatocytes, serum ferritin is reduced and hepcidin levels are elevated, resulting in reduced iron available to infectious agents (Kushner and Rzewnicki 1994). Hepatic dysfunction may negatively affect this response, impairing the body’s defense against an infectious agent that may trigger ALS pathogenesis. Abnormally deficient plasma levels of pro-hepcidin, the precursor of hepcidin, are also associated with variants of HFE (Deicher and Horl 2006). Hepatocytes are also the major source of insulin-like growth factor-1 (IGF-1) which may provide trophic support for motor neurons (Dodge, Haidet et al. 2008). Deficiency of IGF-1 may be detrimental to motor neurons, and administration of IGF-1 has shown benefits in mSOD1 rodent models and possible benefit in human trials (Traynor, Bruijn et al. 2006; Dodge, Haidet et al. 2008). Another proposed trigger for the initiation of ALS pathogenesis is an unidentified neurotoxin (Kaneko and Hachiya 2006). Ingested neurotoxins or those produced in the gut would largely be subjected to first-pass metabolism in the liver, and thus hepatic dysfunction may increase the likelihood of a toxin reaching motor neurons.
In addition to potentially contributing to an environment that enables disease onset, hepatic dysfunction may contribute to disease progression. A high fat diet and elevated plasma LDL cholesterol levels have been associated with prolonged survival in ALS patients (Dupuis, Corcia et al. 2008). The hypothesis behind this association is that elevated lipids are beneficial for skeletal muscles, which preferentially use lipids as an energy source (Dupuis, Corcia et al. 2008). Deficient hepatic cholesterol synthesis may therefore deprive skeletal muscles of sufficient nutrition and accelerate muscle atrophy.

Disease progression may also be accelerated by any cellular processes that might transform the disease from a regional event affecting a few motor neurons to a more widespread process that continues to feed forward. MCP-1 secretion from damaged neurons may be a prominent mechanism of inducing the progressive neurodegeneration and microgliosis associated with ALS. The H63D HFE variant, unlike C282Y HFE, may be associated with an increased tendency toward neuroinflammation. Compared to Wt HFE, H63D HFE increased secretion of monocyte chemoattractant protein-1 (MCP-1) in a neuronal cell culture model. Consistent with elevated levels of labile iron in the presence of the H63D HFE variant, treating cells with exogenous iron also increased MCP-1 secretion. Thus, H63D HFE, but not C282Y HFE, may increase the risk of developing ALS due to a tendency toward neuroinflammation by enhancing the recruitment and activation of microglia and macrophages. These affects may also impact later disease progression when microgliosis and inflammation have been proposed to exert a larger influence (Hall, Oostveen et al. 1998; Boillee, Yamanaka et al. 2006).
MCP-1 also reduces expression of the tight junction proteins contributing to the blood brain barrier (BBB) (Stamatovic, Shakui et al. 2005), and thus BBB function may be impaired in the presence of H63D HFE. Focal BBB breakdown may contribute to accumulation of T lymphocytes recruited by the activated microglia and macrophages.

The H63D and C282Y HFE variants may also have effects that act to decrease macrophage and microglial activation. The H63D HFE allele was associated with lower plasma levels of RANTES and IFN-γ that could potentially indicate reduced induction of macrophage and microglia activation by T lymphocytes. The H63D and C282Y HFE variants may also decrease iron levels in macrophages and microglia (Wang, Johnson et al. 2008). HFE variants have been associated with decreased storage of iron in macrophages of the reticuloendothelial system and an increase in parenchymal iron stores (Pietrangelo 2006). This shift in iron from macrophages and microglia to parenchymal cells in the presence of H63D or C282Y HFE may decrease the release of inflammatory cytokines from macrophages and microglia (Wang, Johnson et al. 2008), but may subsequently increase parenchymal iron-mediated toxicity. This parenchymal toxicity may then result in recruitment and activation of microglia and macrophages which could override the deficient inflammatory response of the monocyte-lineage cells. Increased levels of MCP-1 associated with the H63D HFE variant may also counteract the reduced iron levels in microglia and macrophages since MCP-1 sensitizes these cells to activating factors (Thompson, Karpus et al. 2008).
We, like others, have found a similar effect of the H63D and C282Y HFE variants on cellular labile iron levels (Feder, Penny et al. 1998; Lee, Patton et al. 2006). Despite this fact, these two variants are associated with divergent effects on many other cellular pathways. Many of the effects we demonstrated to be associated with the H63D HFE variant could be mimicked by modulating cellular iron levels, suggesting the mechanism by which H63D HFE influenced these pathways included iron regulation. Effects of C282Y HFE on the pathways studied were often opposite those of the H63D variant, and suggest another effect resulting from C282Y HFE that counteracts the increased iron levels. It is possible that many of these differences between H63D and C282Y HFE result from the retention of C282Y HFE in the endoplasmic reticulum (ER), resulting in a lack of HFE at the cell surface and protein aggregates in the ER.

Expression of HFE at the cell surface is necessary for interaction with TfR, but may have other effects as well. Cytotoxic T lymphocytes are able to recognize HFE (Rohrlich, Fazilleau et al. 2005), and as the blood brain barrier is disrupted and these cells enter the CNS (Kawamata, Akiyama et al. 1992), they may contribute to the destruction of neurons. Whether H63D HFE is recognized by T lymphocytes is unknown, but lymphocytes would not be able to bind C282Y HFE as it is retained in the ER, thus protecting cells expressing this HFE variant from T lymphocyte-mediated cytolysis. Lymphocyte recognition of HFE and resulting cytolysis may also be a factor in the selectivity of ALS for specific motor neurons. In the brainstems of rats, β2M mRNA is largely absent from neurons innervating ocular muscles (Linda, Hammarberg et al. 1999), and it is expected that HFE expression at the plasma membrane would also be reduced in
these neurons. Impaired lymphocyte recognition of HFE in neurons innervating the ocular muscles may explain why these neurons are typically spared from degeneration in ALS (Linda, Hammarberg et al. 1999).

Retention of C282Y HFE in the ER leading to induction of the ER overload and unfolded protein responses and upregulation of protein chaperones may also explain divergent effects between C282Y and H63D HFE (Lawless, Mankan et al. 2007). The induction of heat shock proteins, in particular, likely has broad effects in altering cellular inflammatory signaling, as suggested in Chapter 6, as well as regulating protein aggregation, a mechanism of ALS pathogenesis discussed in Chapter 1. We identified increased expression of heat shock protein-70 (Hsp70) in cells expressing C282Y HFE compared to cells expressing either Wt or H63D HFE. The heat shock protein coinducer, arimoclomol, is currently being explored as a treatment for ALS (Kieran, Kalmar et al. 2004) and it is possible that this agent would not have any additional effects in ALS patients carrying C282Y alleles due to high baseline expression. A logical next step would be to stratify patients in clinical trials by HFE genotype to assess this potential pharmacogenetic effect, as well as others discussed below.

HFE variants may also affect response to treatments and may be a factor in the failure of some agents to demonstrate efficacy in human trials. Glutamate excitotoxicity may contribute to disease pathogenesis in all patients, but have a greater effect in ALS patients carrying H63D HFE alleles. Treatments targeting glutamate excitotoxicity (e.g. riluzole and ceftriaxone) may be less efficacious in H63D carriers if they only have a
small inhibition of this pathway. Alternatively, if glutamate excitotoxicity is the major mechanism of pathogenesis in the \textit{H63D} carriers, a treatment with a large impact on this pathway may be more efficacious in these patients. Anti-inflammatory therapies (e.g. COX-2 inhibitors, minocycline) for ALS patients are also likely to be impacted by HFE variants. ALS patients expressing C282Y HFE may have less inflammation and anti-inflammatory therapies would be unlikely to benefit them. Patients with the \textit{H63D HFE} allele may also be less responsive to anti-inflammatory therapies if they do not affect pathways involving MCP-1, as discussed above. Additionally, minocycline, which failed to benefit humans in a recent trial (Gordon, Moore et al. 2007) may effectively inhibit microglial activation, but it also may have adverse effects on glutamate uptake and other pathways in patients with Wt HFE, as discussed in Chapters 5 and 6. There has recently been an interest in using G-CSF mobilized bone-marrow cells to provide trophic support to motor neurons (Cashman, Tan et al. 2008), and our results suggest the efficacy of this treatment may be affected by \textit{HFE} genotype. In Chapter 4 we describe decreased plasma levels of G-CSF in association with the \textit{H63D HFE} allele. Administering G-CSF to ALS patients possessing an \textit{H63D HFE} allele may not have the same benefit as treating ALS patients with \textit{Wt HFE} due to this baseline deficiency. These examples demonstrate that including patients of all \textit{HFE} genotypes together in the same clinical trials without considering pharmacogenetic effects may impair the ability to detect efficacy of the treatments.
Figure 7-1: **Timeline of pathways affected by H63D HFE in ALS pathogenesis.** The H63D HFE variant may contribute to a cellular predisposition to the onset of motor neuron degeneration as well as disease progression. Early cellular events that increase the likelihood of developing ALS may include elevated glutamate levels and mitochondrial dysfunction that could be accentuated by the H63D form of HFE. Deficient hepatic protein synthesis or detoxification may directly subject persons to several disease triggering agents and allow initiation of ALS. Hepatic dysfunction and deficient trophic factor synthesis, as well as increased tendencies toward glutamate excitotoxicity, neuroinflammation, and mitochondrial dysfunction may all influence the progression of ALS and affect patient response to therapeutic agents.
Figure 7-2: Schematic of the cellular pathways impacted by HFE. H63D and Wt HFE bind beta-2 microglobulin (β2M) and are transported to the plasma membrane where they interact with transferrin receptor (TfR). C282Y HFE does not bind to β2M, and is mostly retained in the endoplasmic reticulum (ER). Both the H63D and C282Y variants of HFE result in greater cellular iron acquisition compared to Wt HFE. Excess iron levels contribute to the formation of reactive oxygen species (ROS), which oxidatively modify proteins, DNA, and lipids, resulting in cellular dysfunction. Major systemic cell types affected by HFE include hepatocytes, which regulate bodily iron metabolism, and macrophages, which store iron and mediate inflammation. In the central nervous system, HFE is in position to affect brain iron acquisition and cellular distribution of iron between neurons, astrocytes, and microglia. Particularly within neurons, oxidative stress may impair the sequestration of calcium leading to increased release of glutamate. In neurons and glial cells, oxidative modification of glutamate transporters and deficient mitochondrial ATP production could impair removal of extracellular glutamate, further contributing to glutamate excitotoxicity. Increased cellular iron levels may either directly or indirectly increase synthesis and secretion of monocyte chemoattractant protein-1 (MCP-1) with resulting recruitment and activation of microglia and blood brain barrier impairment.
Figure 7-2 continued
Other Risk Factors for Sporadic ALS

Identification of the H63D HFE allele as a risk factor for sporadic ALS suggests other potential risk factors for the disease. Regardless of the effects of HFE variants in cell culture models, *in vivo* iron regulation depends on a large number of other proteins. Bodily iron accumulation and expression of iron homeostasis proteins are also largely dependent on environmental factors. The H63D HFE variant is clearly not causing ALS, as possession of the *H63D* allele is neither necessary nor sufficient for the development of ALS. It is the conclusion of this thesis that the *H63D HFE* allele is involved in a gene-environment or gene-gene interaction that increases the risk of developing ALS.

At the cellular level, many of the effects of this allele appeared to be due, at least in part, to iron misregulation. The incidence of ALS is reportedly higher in males (Traynor, Codd et al. 1999; Beghi, Millul et al. 2007; Valdmanis and Rouleau 2008), who also have higher indices of tissue iron loading, particularly compared to pre-menopausal women (Zacharski, Ornstein et al. 2000). Even after menopause the incidence of ALS continues to be lower in women compared to men (Traynor, Codd et al. 1999; Beghi, Millul et al. 2007), which could result from decades of iron loss through menstruation which confers lifetime protection against ALS. It is an intriguing notion that men may be more susceptible to ALS due to greater iron-mediated toxicity. Increased tissue iron levels may be a risk factor for development of ALS, and H63D HFE may be one of many causes of iron loading.
To take this concept a step further, it is possible that the \textit{H63D HFE} allele is also involved in gene-gene interactions that increase risk of developing ALS. Other genetic polymorphisms affecting iron homeostasis proteins could potentially have additive effects on ALS pathogenesis. Even in the absence of the \textit{H63D HFE} allele, cellular iron homeostasis may be adversely affected by variants in other proteins involved in iron regulation.

The idea that gene-gene interactions are involved in determining the risk of developing ALS has been difficult to study. Genetic association studies have had to assess individual polymorphisms independently due to sample size considerations. Testing large numbers of loci requires large sample sizes and assessing gene-gene interactions exponentially increases the number of tests being performed, which would further increase the required sample sizes. However, statistical power could be increased by limiting the analysis to pathways implicated in ALS pathogenesis and by focusing on loci previously associated with ALS. This thesis provides mechanistic evidence of the association of the \textit{H63D HFE} allele with ALS, and suggests that iron regulatory pathways and variants of genes involved in these pathways warrant further study. Additionally, the impact of iron regulation on other pathways implicated in ALS pathogenesis should continue to be studied. \textit{HFE} allelic variants have also been associated with other conditions including Alzheimer’s disease (Connor and Lee 2006), Parkinson’s disease (Dekker, Giesbergen et al. 2003), and ischemic stroke (Ellervik, Tybjaerg-Hansen et al. 2007). Our results suggest that a similar approach of expanding
upon the genetic association and assessing functional contributions of HFE variants to mechanisms of pathogenesis could determine the nature of these associations as well.

References


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American Academy of Neurology, Society for Neuroscience, American Medical Association, Tau Beta Pi

Publications


Mitchell RM, Lee SY, Simmons Z, Connor JR. HFE Polymorphisms Affect Cellular Glutamate Regulation. Submitted


