PLASMA BIOMARKERS ASSOCIATED WITH ALS AND THEIR RELATIONSHIP TO IRON HOMEOSTASIS

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ABSTRACT: Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease with complicated pathogenesis with variable presentation and disease progression. There is a critical need for a panel of biomarkers to provide clinicians and researchers with additional information. In this study, multiplex immunoassays were used to screen a number of cytokines, growth factors, and iron-related proteins. ALS patients had significantly higher plasma levels of L-ferritin and lower concentrations of transferrin when compared to healthy controls and together classified a test group of subjects with 82% accuracy. Duration of ALS symptoms correlated positively with levels of monocyte chemoattractant protein 1 (MCP-1) and negatively with levels of granulocyte-macrophage colony stimulating factor (GM-CSF). The biomarker profile suggests iron homeostasis is disrupted in ALS patients, and changes in ferritin and transferrin (Tf) appear to be indicators of ongoing inflammatory processes. The data demonstrate a plasma biomarker profile in ALS patients that may differ from published reports of cerebrospinal fluid biomarkers.

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Amyotrophic lateral sclerosis (ALS) is characterized by degeneration of both upper and lower motor neurons.[1] Its heterogeneity with regard to region of onset and rate of progression complicates research into mechanisms and treatment, as well as 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.

Previous attempts to identify biomarkers characteristic of ALS patients and disease processes have highlighted the lack of information provided by single biomarkers due to considerable overlap in expression levels between groups.[2] Several studies have examined proteomic profiles of ALS patients compared to various control groups and suggested biomarkers that may aid classification of subjects.[3–5] Unlike many previous approaches, we have chosen to focus on low-abundance cytokines and trophic factors with an antibody-based approach for appropriate antigen specificity and efficiency of measurement. Previously we used this multiplex immunoassay approach to identify a profile of biomarkers in cerebrospinal fluid (CSF) that were altered by ALS.[6] The availability of a biomarker assay for blood rather than CSF would greatly facilitate diagnosis and monitoring of disease progress. The first question addressed in this study was whether a panel of biomarkers could be identified in the plasma that would support the clinical diagnosis of ALS. Second, we determine the relationship, if any, between the plasma and previously reported CSF biomarker panels to determine whether the plasma may provide insights into mechanisms of disease pathogenesis. Finally, multiple studies have demonstrated that the *H63D HFE* gene variant is associated with sporadic ALS,[7–10] since ≈25% of subjects possess at least one *H63D* allele. The expression of variant forms of the HFE protein affects a range of cellular functions[11] including innate immunity processes[12,13] as well as iron homeostatic mechanisms that are supposedly part of the disease process in ALS.[14,15] Thus, we determined whether the *H63D HFE* gene variant is associated with altered profiles in the panel of biomarkers.

MATERIALS AND METHODS

Patients and Samples. Blood samples were obtained by venipuncture from ALS patients who attend a single ALS clinic and from normal controls between 8 AM and 12 noon. All patients met revised El-Escorial criteria for clinically definite, probable, probable laboratory-supported, or possible ALS.[1] The normal controls consisted of spouses and nonrelated caregivers of ALS patients and volunteers from the community. Although individual ethnicity data were not available for the study subjects, the majority of subjects were Caucasian. After genotyping samples for the *H63D* and *C282Y* HFE variants, the study subjects were then

Abbreviations: ALS, amyotrophic lateral sclerosis; CRP, C-reactive protein; CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; FGF, fibroblast growth factor; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN, interferon; IL, interleukin; IP, IFN-γ induced protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PDGF, plately derived growth factor; RANTES, regulated on activation normal T-cell expressed and presumably secreted; Tf, transferrin; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; Wt, wildtype

Key words: amyotrophic lateral sclerosis; iron; HFE; biomarkers; plasma

Additional Supporting Information may be found in the online version of this article.

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separated into four groups: *Wt/Wt* ALS patients, *H63D/Wt* ALS patients, *Wt/Wt* controls, and *H63D/Wt* controls. Group sizes were approximately matched according to the number of *H63D/Wt* ALS patients, and the most recently obtained consecutive samples within each group were chosen. All samples used in this analysis were collected between 2004 and 2008. Subjects who carry *C282Y HFE* polymorphisms were excluded from the analysis due to the small numbers with this gene variant. 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, Missouri) was added, 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, California). All patients were genotyped for the *H63D* and *C282Y HFE* polymorphisms by restriction fragment length analysis as previously reported.[10]

Multiplex Cytokine Bead Assay. Multiplex analysis was performed on plasma samples diluted 1:3 using the Bio-Plex Human 27-plex panel of cytokines and growth factors (Bio-Rad, Hercules, California). The proteins measured in this panel are shown in Supporting Information Table 1. The assay was performed according to the manufacturer's protocol, as previously reported.[6] Each sample was analyzed twice, and the coefficient of variance was less than 10% for each sample.

Immunoassays. In addition to the proteins in the Bio-Plex panel, we determined other proteins of interest as follows. Plasma levels of β2-microglobulin (US Biological, Swampscott, Massachusetts), transferrin (Tf) (Bethyl Laboratories, Montgomery, Texas), C-reactive protein (CRP) (R&D Systems, Minneapolis, Minnesota), and pro-hepcidin (DRG International, Mountainside, New Jersey) were assayed by enzyme-linked immunosorbent assay (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 antirecombinant H-ferritin antiserum as previously reported[16] and goat antirabbit secondary antibody. L-ferritin was measured by immunoradiometric assay (Siemens Medical Solutions, Malvern, Pennsylvania) 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. The standard curve for monocyte chemoattractant protein 1 (MCP-1) in the multiplex assay was not usable, and thus each sample was analyzed separately for MCP-1 expression by anti-human MCP-1 ELISA (GE Healthcare, Piscataway, New Jersey).

Iron Measurement and Calculation of Tf Saturation. The total amount of iron in the plasma was determined by digesting the plasma in ultrapure nitric acid (JT Baker, 9598-00; Phillipsburg, New Jersey), 1:4 v/v, and samples were heated to 60°C for 24 h. The digested samples were diluted 1:100 in ddH_2O, and then analyzed on a Perkin Elmer Atomic Absorption Spectrometer 600 series (Waltham, Massachusetts). Tf saturation was calculated as Tf saturation (%) = plasma iron (mol/L)/[2 × Tf (mol/L)] × 100. Replicate sample variation with this approach is <5%, and an external standard was included in every set of analyses.

Statistical Analysis. Normal distribution of analyte expression was assessed with the Kolmogorov–Smirnov test. Biomarker concentrations were compared between groups via *t*-test or Mann–Whitney *U*-test, as appropriate. Correlations between markers and duration of symptoms and age were assessed by Spearman's or Pearson correlation coefficients, as appropriate. The chi-square test was used to assess frequency differences between groups. Biomarker expression levels were adjusted for age differences by linear regression.

To determine the ability of the biomarkers to distinguish ALS patients from control subjects, each of the samples was randomly separated into either a training or a test group. The training group was designed to include 2/3 of ALS patients and 2/3 of controls. The test group was designed to include 1/3 of ALS patients and 1/3 of controls. The training set of subjects was then used to create a logistic regression model according to the pattern of biomarker expression. Classification of samples in the test set was performed incorporating expression of L-ferritin and Tf, the two markers with the lowest *P*-values, plus gender and *HFE* genotype into the logit function. Statistical analyses were performed using SigmaStat 2.03 (SPSS, Chicago, Illinois) and GraphPad Prism 4.03 (GraphPad Software, San Diego, California). *P*-values were considered to be significant at the 0.05 level.

Table 1. Subject demographics given as median (interquartile range).

	Control		ALS		
rs1799945 genotype	Wt/Wt	H63D/Wt	Wt/Wt	H63D/Wt	p
n	19	17	18	11	
age (y)	54 (47-75)	58 (50–70)	61 (48–75)	60 (48–72)	0.98
gender (male:female)	12:7	10:7	11:7	7:4	0.99
onset (limb:bulbar)			12:6	6:5	0.39
duration of disease (mo.)			19 (12–28)	11 (9-19)	0.14
clinically definite (n)			4	4	0.07
clinically probable (n)			8	2	
clinically probable-lab. supported (n)			6	2	
clinically possible (n)			0	3	

P-values indicate comparisons for all groups.

RESULTS

Control subjects and ALS patients did not differ in either age or gender distributions. No differences were found between ALS patients for region of onset, duration of symptoms, or El-Escorial classification based on *HFE* genotype (Table 1). The correlation of analyte expression with age was determined independent of ALS disease (Table 2). A number of inflammatory cytokines increased with increasing age as did β2M 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.

Biomarkers were screened for associations with ALS, region of onset, and disease progression independent of *HFE* genotype (Table 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 granulocyte-macrophage colony stimulating factor (GM-CSF), were associated with duration of symptoms; MCP-1 levels increased, whereas GM-CSF concentrations decreased with longer duration. For classification of subjects by presence or absence of ALS, all samples were randomly separated into a training set and a test set and matched for disease status and *HFE* genotype, as described in Materials and Methods (Table 4). No differences were found between the two groups based on age, gender, duration of symptoms, *HFE* genotype, or El-Escorial classification of the ALS patients. The training set was used to determine a logistic regression function characteristic of disease status. Only L-ferritin and Tf had *P*-values < 0.1 (L-ferritin: $P = 0.031$; Tf: $P = 0.063$). These two markers were used to classify the test set with 82% accuracy, 80% sensitivity, and 83% specificity.

The secondary aim of this study was to assess biomarkers associated with the *H63D HFE* polymorphism. All subjects were initially grouped according to genotype (*Wt/Wt* vs. *H63D/Wt*) regardless of ALS status (Table 5). L-ferritin and Tf were both increased in the plasma of *H63D* carriers, and interferon gamma (IFN-γ), G-CSF, and regulated on activation normal T-cell expressed and presumably secreted (RANTES) were decreased in the plasma of these subjects. The expression of these five proteins was then assessed by considering ALS patients and controls separately. Within the healthy control group, *HFE* genotype had no impact on the expression of any markers. Within the ALS patient group, those patients with an *H63D* allele had higher plasma levels of L-ferritin, and lower levels of IFN-γ, G-CSF, and RANTES compared to the ALS *Wt/Wt* group. The presence of the *H63D* allele negated the increase in Tf associated with ALS, and thus Tf levels were not different between the ALS *Wt/Wt* and ALS *H63D/Wt* groups.

A number of findings in this study suggest disrupted iron regulation in association with either ALS or the *H63D HFE* allele. Within the control group, increasing plasma iron levels were associated with increasing levels of IP-10 ($r = 0.403$, $P = 0.020$). Within the ALS group, increasing iron levels were associated with increasing levels of MIP-1α

Table 2. Correlations of analytes with subject age.

	Age Correlations	
Marker	r	p
B2M	0.645	<0.001
MCP-1	0.437	<0.001
Eotaxin	0.377	0.002
VEGF	0.373	0.002
IL-7	0.348	0.005
MIP-1b	0.346	0.005
IP-10	0.342	0.006
IL-12 (p70)	0.341	0.006
IL-10	0.340	0.006
IFN-gamma	0.329	0.008
IL-1ra	0.324	0.009
IL-6	0.290	0.020
pro-hepcidin	−0.281	0.026
IL-13	0.251	0.046
PDGF	0.248	0.048

Table 3. Biomarkers affected by disease status.

	Control	ALS	p
L-ferritin (ng/mL)	25.78 (14.64–49.84)	66.17 (26.10–91.23)	0.002
Tf (ug/mL)	3098 (2058–3945)	2262 (1754–3365)	0.052
	ALS Patients		
	Limb	Bulbar	p
Pro-hepcidin (ng/mL)	213.5 (43.5–400.0)	483.3 (330.9–745.2)	0.004
IP-10 (pg/mL)	545.8 (364.0–715.1)	338.8 (234.1–480.1)	0.060
	Duration of Symptoms		
	r	p	
MCP-1	0.500	0.006	
GM-CSF	−0.435	0.018	

Values given as median (interquartile range).

($r = 0.410$, $P = 0.030$), and decreasing levels of RANTES ($r = -0.399$, $P = 0.035$).

A key regulator of iron is hepcidin, an acute phase reactant that is increased in inflammatory states.[17] There was no difference in the plasma concentration of pro-hepcidin, the precursor of hepcidin, between the control group and the ALS patients. Because of the suspected role of inflammatory processes in ALS, the relationship of iron to those processes[13] and the putative regulatory interaction between hepcidin and some cytokines, the association of pro-hepcidin with plasma iron and inflammatory markers was assessed (Table 6, Fig. 1). Control subjects but not ALS patients were characterized by a negative correlation between IL-6 and pro-hepcidin. 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.

DISCUSSION

The results of this study support our hypotheses that there are plasma biomarkers that can distinguish ALS patients from normal control subjects. The panel of distinguishing biomarkers, however, is very different in plasma than that reported in CSF.[6] In this study L-ferritin and Tf were used in a logistic regression model to classify the presence or absence of ALS with 82% accuracy. Our finding of higher L-ferritin in patients with ALS is consistent with a previous study, although that study did not find any changes in Tf levels.[14] In a recent abstract, plasma ferritin concentrations also correlated with disease progression.[15] Values for Tf in our study fell outside the 95% confidence level when they were considered independently, but we included Tf in the logistic regression model to improve the accuracy for identifying ALS patients. Including Tf supports the concept of using a biomarker panel for disease status classification rather than relying on one protein or setting artificial limits for biologically and clinically meaningful expression changes for proteins. In addition to identifying potential biomarkers for the disease, our observation that plasma levels of MCP-1 and GM-CSF levels were associated with duration of symptoms suggest these proteins could be biomarkers for disease progression.

Table 4. Demographics of training and test sets.

	Training Set	Test Set	p
Control (n)	24	12	
ALS (n)	19	10	
clinically definite	5	3	
clinically probable	6	2	
clinically probable-laboratory supported	5	5	0.39
clinically possible	3	0	
onset limb:bulbar	11:8	7:3	0.52
duration of disease (mo.)	18 (11–27)	12 (10–25)	0.60
Age (y)	58 (49–72)	59 (43–77)	0.94
Sex (male:female)	27:16	13:9	0.99
HFE (Wt/Wt:H63D/Wt)	22:21	15:7	0.19

Values given as median (interquartile range).

Table 5. Biomarkers affected by *HFE* genotype.

	Wt/Wt	H63D/Wt	p
	All Subjects		
IFN-gamma (pg/mL)	132.8 (116.6–178.3)	121.3 (100.8–145.0)	0.022
G-CSF (pg/mL)	38.17 (32.41–45.28)	34.80 (31.85–38.54)	0.043
Tf (ug/mL)	2281 (1831–3234)	3098 (2251–4189)	0.045
RANTES (pg/mL)	7394 (4966–9752)	5951 (4675–7753)	0.049
L-ferritin (ng/mL)	27.88 (15.46–67.51)	40.15 (23.04–87.53)	0.050
	Control Subjects		
IFN-gamma (pg/mL)	129.4 (117.6–175.8)	129.4 (108.6–152.4)	NS
G-CSF (pg/mL)	35.94 (31.98–43.97)	35.56 (31.85–39.74)	NS
Tf (ug/mL)	2487 (1952–3408)	3225 (2640–4198)	NS
RANTES (pg/mL)	7305 (5471–9934)	6540 (4446–8899)	NS
L-ferritin (ng/mL)	23.04 (14.60–49.17)	34.22 (15.18–59.82)	NS
	ALS Patients		
IFN-gamma (pg/mL)	167.1 (112.7–221.5)	103.2 (93.83–130.0)	0.033
G-CSF (pg/mL)	39.74 (33.32–46.85)	34.62 (29.05–35.65)	0.028
Tf (ug/mL)	2214 (1433–3428)	2465 (2153–3496)	NS
RANTES (pg/mL)	6851 (4966–10590)	5536 (4964–6925)	0.044
L-ferritin (ng/mL)	41.17 (18.90–79.52)	82.92 (38.78–145.4)	0.029

Values given as median (interquartile range).

Our secondary aim addressed the association of the *H63D HFE* variant with altered expression of biomarkers, particularly those associated with ALS. Five proteins in our panel were associated with the *H63D* allele, and two of them were also in the ALS profile. Thus, the *H63D* allele is associated with protein expression changes, and it can be a confounder in studies aimed at identifying biomarkers for ALS. Similar conclusions were reached in our report on CSF biomarker panels.[6] Of particular note is that ferritin levels in plasma are influenced by HFE genotype, and ferritin, as mentioned, has been reported in two studies[14,15] in addition to this study as being consistently increased in ALS patients. Knowledge of the HFE genotype may be critical to rule out false-positives if ferritin is going to be part of the diagnostic profile in ALS. Low plasma ferritin levels are used in Restless Legs Syndrome to support decisions regarding diagnosis and design treatment strategies (for review see Ref. 18).

Most biomarker studies in ALS, particularly those that focus on biomarker panels, have evaluated CSF.[2,6] Using an approach similar to this study on plasma, we previously identified ALS-associated biomarkers in CSF that were capable of distinguishing ALS patients from a group of neurological disease control subjects.[6] Unlike the finding

Table 6. Correlations of markers with pro-hepcidin.

	Control			ALS		
	Wt/Wt	H63D/Wt	All	Wt/Wt	H63D/Wt	All
Iron	−0.265	−0.412	−0.253	−0.358	−0.297	0.026
	0.322	0.133	0.156	0.145	0.405	0.897
L-ferritin	−0.197	0.039	−0.182	−0.185	0.436	0.113
	0.433	0.881	0.287	0.463	0.180	0.558
H-ferritin	0.156	−0.447	−0.191	0.158	0.612	0.319
	0.564	0.082	0.288	0.531	0.060	0.098
Tf	−0.253	0.206	0.047	0.123	0.318	0.232
	0.345	0.443	0.794	0.657	0.340	0.227
IL-1b	−0.335	−0.287	−0.462	0.579	0.236	0.413
	0.172	0.264	0.005**	0.012*	0.484	0.026*
IL-6	−0.373	−0.360	−0.483	0.162	−0.073	0.019
	0.128	0.155	0.003**	0.521	0.832	0.921
IL-10	−0.300	−0.306	−0.368	−0.040	−0.045	−0.071
	0.212	0.232	0.027*	0.874	0.894	0.713
TNF-alpha	−0.240	−0.230	−0.175	0.340	−0.368	0.007
	0.338	0.376	0.307	0.167	0.266	0.972
CRP	−0.056	−0.429	−0.256	−0147	−0.433	−0.305
	0.837	0.144	0.181	0.587	0.250	0.138

*The correlation value is given for each measurement with the P value listed below. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively.*

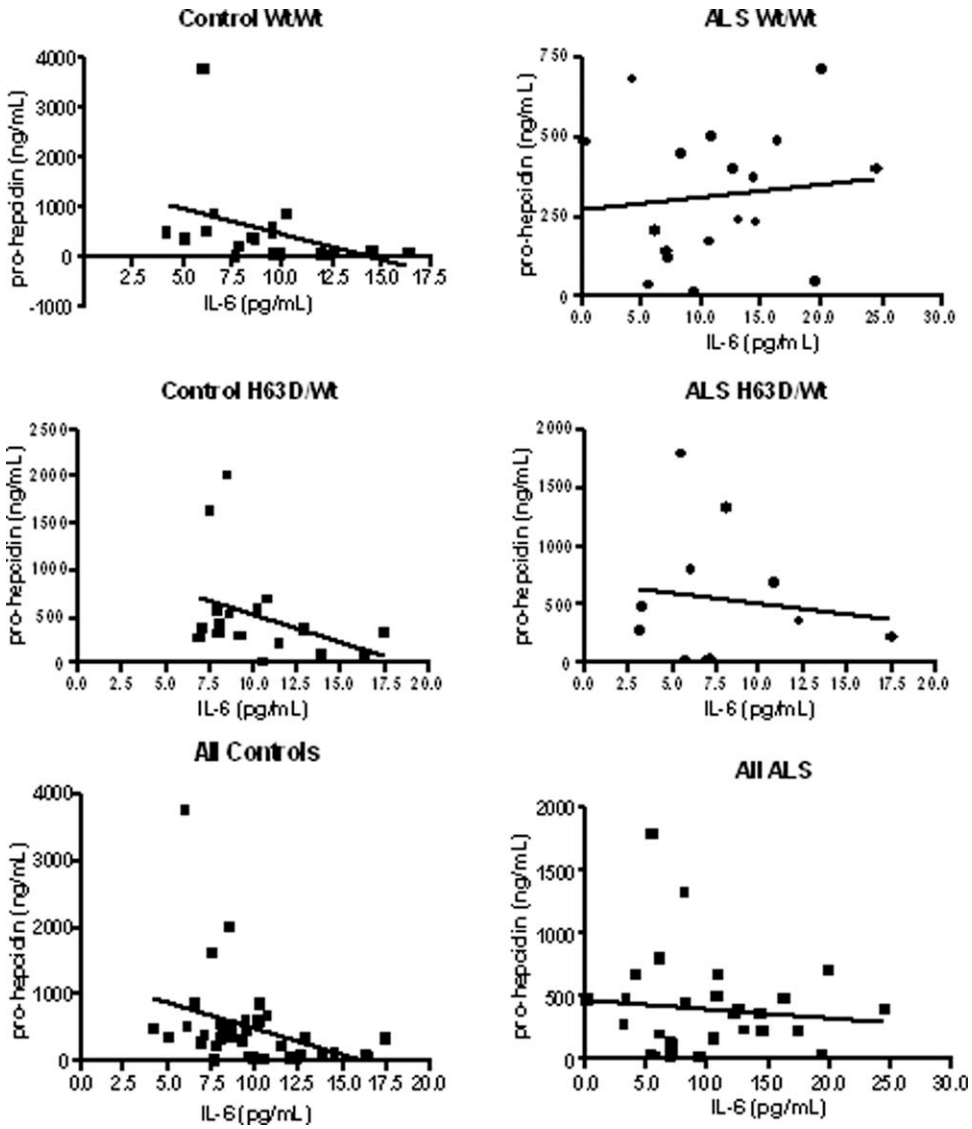

FIGURE 1. Correlation of plasma pro-hepcidin and IL-6 in disease and genotype subgroups. Pro-hepcidin and IL-6 levels were measured in each plasma sample as described in Materials and Methods. The relationship between pro-hepcidin and IL-6 was determined within each group. These two proteins were correlated in control subjects grouped together independent of *HFE* genotype ($r = -0.483$, $P = 0.003$), but not in ALS patients.

reported herein on plasma, biomarkers in CSF that were associated with ALS suggested there was a robust inflammatory process associated with the disease. The absence of elevated cytokine expression in plasma is in agreement with most previous studies that compared ALS patients with controls,[2] although some studies identified alteration of individual cytokines in blood.[19] Our panel included not only a range of cytokines but also growth factors, and previous studies have also been inconsistent regarding altered plasma levels of growth factors in ALS.[20,21] Inconsistent observations among previous studies were hypothesized to reflect methodological differences as much as biology, thus our approach using the identical biomarker profile analysis between CSF and plasma suggests that inflammation associated with ALS is limited to the central nervous system (CNS) and is not reflected in the plasma. However, elevated plasma L-ferritin is associated with ALS. Cellular synthesis and secretion of ferritin is upregulated not only by iron loading but also by inflammatory cytokines.[22] To address the possibility that the L-ferritin was associated with active inflammation in ALS patients, we analyzed CRP levels, which suggested no general inflammation. There was, however, no increase in total plasma iron, Tf, or Tf saturation in association with the increased ferritin, suggesting there are not increased iron stores in ALS patients.

There is no evidence for increased liver iron stores in patients with ALS that could account for an increase in plasma ferritin. This provides an opportunity to speculate on the potential for ferritin in plasma to reflect the CNS inflammatory

activity suggested by the CSF biomarker panel. The source of L-ferritin in the blood is generally considered to be macrophages.[23] Thus, the increase in L-ferritin in plasma in ALS could reflect higher activation and iron turnover in the macrophages and perhaps brain microglia. There is little understanding about iron release from microglia in the brain, but it is possible that the increased plasma ferritin could reflect the activity of these cells in the neurodegenerative process. It will be useful to compare plasma ferritin levels in ALS patients to levels of ferritin in patients with other neurodegenerative diseases to determine the specificity of this finding for ALS. We cannot rule out that plasma L-ferritin may also be released from parenchymal cells, particularly hepatocytes.[24] Indeed, the plasma ferritin increase in ALS could reflect altered hepatic protein synthesis in ALS patients, as was reported over 20 years ago.[25] The observation that L-ferritin was even more increased in ALS patients with the *H63D* gene variant is consistent with elevated plasma ferritin in individuals with this gene variant.[26] These observations support our conceptual framework that the presence of this gene variant alters cell function in a manner that could facilitate disease-producing events in ALS.

The other biomarker associated with ALS was the iron transport protein, Tf, which was decreased in ALS patients, but only when patients were grouped together independent of genotype. When *HFE* genotype plus ALS disease status was considered, any differences in plasma Tf concentration between ALS and control were lost. Plasma Tf mostly originates from the liver and typically decreases with elevated iron stores[27] or inflammation.[28,29] Tf levels were not associated with increased CRP or total iron levels, and Tf saturation was not different between ALS and controls. Thus, it seems unlikely that the lower Tf is reflective of iron stores or inflammation. Similar to higher L-ferritin in plasma, lower Tf levels may be an additional indicator of CNS inflammation in ALS, although hepatic dysfunction should also be considered.

Markers Associated with Disease Duration. The plasma concentration of MCP-1, a potent chemoattractant for monocyte lineage cells, increased with longer duration of symptoms. ALS patients have been shown to have elevated CSF levels of MCP-1, although CSF levels of MCP-1 may not correlate with disease progression.[6,30,31] Thus, MCP-1 levels may be useful for distinguishing ALS patients from controls, and plasma levels may be an indicator of disease progression. We have recently demonstrated that cells expressing the H63D HFE polymorphism secrete greater levels of MCP-1 compared to cells expressing either Wt HFE or C282Y HFE.[32] Thus, although not affected by HFE polymorphisms in this study using plasma, the association of the H63D HFE variant with ALS and the association of MCP-1 levels with progression of ALS is worthy of continued monitoring and analysis.

Plasma GM-CSF levels declined with disease progression, but we previously reported higher CSF GM-CSF levels in ALS patients.[6] GM-CSF crosses the blood–brain barrier[33] and is a potent immune stimulator.[34] Lower GM-CSF in plasma could indicate increased transport into the CSF from plasma, but CNS sources of GM-CSF also exist.[35] Therefore, we cannot unequivocally interpret the higher CSF levels as being directly related to the decreasing plasma levels observed in this study, but such an interpretation would be consistent with increased CNS inflammation in ALS and with GM-CSF as a marker of disease progression. GM-CSF also acts on neurons to upregulate the antiapoptotic molecules Bcl-2 and Bcl-XL.[33] Thus, it is possible that any increased movement of GM-CSF from plasma to brain is part of a neuroprotective effort. However, the observation of increased CNS levels of GM-CSF coupled with an increase in MCP-1 with increasing duration of disease support activation of microglia and macrophages that may contribute to disease progression.[36]

Loss of Correlation between Iron Regulation and Cytokine Expression. Pro-hepcidin expression is used as an indicator of hepcidin, a systemic regulator of iron absorption and distribution.[37] Hepcidin functions to limit the bioavailability of circulating iron. It promotes iron accumulation in macrophages and is probably a key player in the iron withholding defense.[38] The synthesis of pro-hepcidin is reportedly increased in response to increased IL-6,[39] but we found a negative correlation between IL-6 and pro-hepcidin in the plasma of control subjects. This correlation was lost in the ALS patients, suggesting an uncoupling of the tightly controlled relationship between iron and the inflammatory response by macrophages. The effect of this uncoupling could be increased iron release by macrophages that could exacerbate the disease processes and be related to the increased ferritin. Consistent with this concept is the loss of correlation between pro-hepcidin and the other interleukins also measured in this study, and in particular the reversal of the correlation with IL-1β between control and ALS subjects.

Impact of H63D HFE on the Biomarker Profile. Five proteins were associated with the presence of the *H63D HFE* allele. Two of these proteins, L-ferritin

and Tf, were identified as part of the ALS profile, and the impact of the H63D allele on the ALS profile has already been discussed. Three cytokines, G-CSF, RANTES, and IFN-γ, were reduced in ALS patients who carry an *H63D* allele, but they were not part of the profile seen in the ALS patients with *Wt HFE*. The decrease in these cytokines in the presence of the *H63D* allele could have direct relevance to disease pathogenesis and to stratification of treatments based on genotype. G-CSF has been explored for use in ALS patients to induce mobilization of hematopoietic stem cells to cross the blood–brain barrier and potentially provide neuroprotection in regions of motor neuron death.[40] ALS patients who carry the *H63D* allele may be less responsive to G-CSF treatments because of lower baseline levels.

Both RANTES (CCL5) and IFN-γ were lower in ALS patients with the *H63D HFE* allele compared to *Wt/Wt* ALS patients. RANTES recruits and activates monocytic lineage cells and T_H1 lymphocytes.[41] IFN-γ is produced by T_H1 lymphocytes and activates macrophage-family cells inducing them to produce the toxic mediators IL-1, IL-6, and TNF-α.[42] The decrease in RANTES and IFN-γ in the *H63D HFE* ALS patients may reduce activation of microglia and may explain the trend in some studies toward a later onset of disease in *H63D* carriers.[9,10] The interaction between iron and cytokines, however, is complex.

In conclusion, a biomarker panel associated with ALS could be identified in the plasma, but the proteins that constitute the panel differed from previously reported CSF biomarkers. This may suggest plasma biomarkers are of limited use for revealing pathogenic events in ALS but are reflective of the disease process. However, our results suggest plasma expression of L-ferritin and Tf are consistent with the inflammatory profile in ALS identified in a previous publication that used CSF.[6]

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