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Discovery and Verification of Amyotrophic Lateral Sclerosis Biomarkers by Proteomics

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Abstract

Introduction—Recent studies using mass spectrometry have discovered candidate biomarkers for amyotrophic lateral sclerosis (ALS). However these studies utilize small numbers of ALS and control subjects. Additional studies using larger subject cohorts are required to verify these candidate biomarkers.

Methods—Cerebrospinal fluid (CSF) from 100 patients with ALS, 100 disease control and 41 healthy control subjects were examined by mass spectrometry.

Results—61 mass spectral peaks exhibited altered levels between ALS and controls. Mass peaks for cystatin C and transthyretin were reduced in ALS, whereas mass peaks for post-translational modified transthyretin and C-reactive protein (CRP) were increased. CRP levels were 5.84±1.01 ng/mL for controls and 11.24±1.52 ng/mL for ALS subjects as determined by ELISA.

Discussion—This study verified prior mass spectrometry results for cystatin C and transthyretin in ALS. CRP levels were increased in the CSF of ALS patients, and cystatin C level correlated with survival in patients with limb-onset disease. Our biomarker panel predicted ALS with an overall accuracy of 82%.

Keywords

amyotrophic lateral sclerosis; mass spectrometry; biomarkers; cerebrospinal fluid; cystatin C

Introduction

Amyotrophic lateral sclerosis (ALS) is a chronic, progressive neurodegenerative disease of motor neurons in the cerebrum, brain stem and spinal cord with concurrent muscle weakness, wasting, and spasticity ¹. ALS is the most common adult-onset motor neuron disease, with an incidence between 1.5 and 2 individuals per 100,000 per year ².

There is currently no rapid in vitro-based diagnostic test for ALS, and diagnosis is determined based on clinical and electrodiagnostic evidence. Clinical diagnosis often takes several months but may be well over one year³. Biomarkers specific for ALS could assist the neurologist in a more rapid diagnosis. In addition, future studies could determine the ability of the biomarker to function as a prognostic indicator of disease progression or as a surrogate marker of drug effects in clinical trials. Of the biofluids available for biomarker discovery, cerebrospinal fluid (CSF) may best reflect the pathogenic events in the central nervous system (CNS) and contain proteins that are secreted or released by injured/dying cells⁴.

Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS)-based proteomics utilizes modified chip surfaces for protein separation. This proteomic approach rapidly analyzes a large number of samples and generates discriminatory mass spectral features that differentiate sample groups, however it does not determine the identity of the protein. Thus, a disadvantage of this technology is the challenging aspect of protein identification for the discriminatory mass peaks. This technique has previously been used by us and others for biomarker discovery in numerous neurologic and neurodegenerative diseases, including ALS⁵⁻⁹. An alternative proteomic approach that determines the amino acid sequence identity of all proteins/peptides in the sample is liquid chromatography tandem mass spectrometry (LC-MS/MS). This method is not a high throughput approach, and that limits its utility for biomarker discovery experiments.

We previously identified a panel of mass spectral peaks that could distinguish ALS from control subjects using SELDI-TOF-MS⁸. This biomarker panel included the cystatin C, transthyretin and neuroendocrine 7B2 proteins. Another study utilizing the SELDI-TOF-MS platform also identified a panel of biomarkers that could distinguish ALS from control subjects⁷. Both mass spectrometry studies demonstrated reduced cystatin C in the CSF of ALS patients. However the SELDI-TOF-MS platform has recently been shown to exhibit low reproducibility or user bias when making diagnostic classifications of prostate cancer patients in serum^{10,11}. No similar information is currently available concerning intra-session variability for SELDI-TOF-MS when applied to CSF samples. A recent study using a small group of ALS and control subjects also indicated that cystatin C levels were reduced in the CSF of ALS patients by enzyme-linked immunosorbent assay (ELISA)¹².

In this study, we performed SELDI-TOF-MS of CSF from a large group of ALS and multiple control groups to identify mass spectral peaks that differentiate ALS and control groups, validate the previously published SELDI-TOF-MS findings, and determine the intra-session reproducibility of the SELDI-TOF-MS platform for CSF samples. This study represents the next step in the biomarker development process by attempting to verify prior results. Our final biomarker panel provided an overall diagnostic accuracy of 82% for 241 CSF samples. In addition, we identified C-reactive protein (CRP) as a putative CSF biomarker for ALS and verified our findings by ELISA.

Material and Methods

Subject consent and CSF

The study population comprised 100 ALS, 18 multiple sclerosis (MS), 53 Alzheimer's disease (AD), 29 other neurologic disease (DC), and 41 healthy control (HC) subjects. Revised El Escorial criteria were used to diagnose all ALS subjects¹³, with 17% diagnosed as definite ALS, 40% probable ALS, 21% probable/lab supported ALS, and 22% possible ALS. Healthy controls were typically spouses or family friends of the ALS patients. Of the ALS patients, 15 had a family history of ALS, and 3 had SOD1 mutations. ALS subjects

were grouped together for all analyses. For patient demographics see Table 1. The average age for the ALS subjects was statistically different from that of the healthy controls and AD groups. Further studies would be necessary to generate age-matched group comparisons. The DC group consisted of 7 neuropathy, 4 pure lower motor neuron (LMN) disease (no upper motor neuron symptoms at time of lumbar tap), 2 CNS metastasis, 2 neuralgia, 2 demyelinating myelopathy, 2 frontotemporal dementia, 2 stroke, 1 normal pressure hydrocephalus, 1 superficial siderosis, 1 cerebral amyloid angiopathy, 1 Lyme disease, 1 viral encephalitis, 1 Parkinson disease, 1 psychiatric patient with mental status change, and 1 conversion disorder patient. The neuropathy subjects included 3 idiopathic sensorimotor polyneuropathy, 1 small fiber neuropathy, and 1 demyelinating neuropathy. All 4 LMN subjects exhibited only lower motor neuron symptoms at the time of the lumbar spinal tap. CSF samples (between 3-10 mL per subject) were obtained by lumbar puncture from 241 subjects at either the University of Pittsburgh Medical Center (UPMC) or Massachusetts General Hospital (MGH) upon informed patient consent. The study was approved by both UPMC and MGH institutional review boards. All samples were spun at 3000 rpm at 4°C for 10 minutes to remove any cells and debris, aliquoted in small volumes and stored in low bind Eppendorf tubes at -80°C within 2 hours from harvesting. Only CSF samples without visible blood were processed by centrifugation, and hemoglobin levels in all final CSF samples were measured by ELISA to eliminate those with evidence of released hemoglobin denoting blood contamination.

SELDI-TOF-MS and spectra analysis

For biomarker discovery 20 µL CSF samples were incubated in 10µL Urea/CHAPS buffer (9M urea, 2.5% CHAPS) for 10 minutes, mixed with 170 µL HEPES, pH 7.5 buffer. We loaded 150µL of the sample onto HEPES buffer pre-equilibrated spots of Q10 Protein-Chips (Bio-Rad Laboratories, Hercules, CA) and incubated for 60 minutes to bind proteins to chip surface. Alternatively the CSF samples were loaded on Zn-IMAC30 Protein-Chips (Bio-Rad Laboratories) by incubating 20µL CSF in 10µL Urea/CHAPS buffer for 10 minutes, mixed with 170µL phosphate buffered saline (PBS), pH 7.4 for 60 minutes incubation on 100 mM Zinc sulfate pretreated IMAC30 Protein-Chips. Protein-Chips were rinsed with HPLC water 4-5 times, air dried and 50% saturated sinapinic acid (Sigma-Aldrich, St. Louis, MO) was added to each spot.

Spectra were generated on a calibrated ProteinChip reader by focusing at three different mass ranges, each in duplicate. For the low mass range (1,000 m/z to 10,000 m/z) we used a detector sensitivity of 8, intensity of 198 and a deflector setting of 250 Da. For mid mass range (10,000 m/z to 20,000 m/z) we used detector sensitivity of 9, intensity of 198 and 500 Da deflector setting, and for high mass range (20,000 m/z to 150,000 m/z) detector sensitivity of 10, intensity of 210 and 500 Da deflector setting.

All spectra were subjected to baseline subtraction and normalized to total ion content. Spectra with a normalization coefficient either <0.33 or >3 were omitted to exclude artifacts. Peak detection was done at 1.5 signal to noise ratio (S/N), 1.5 valley depth and 20% min peak threshold. Ciphergen Biomarker Pattern Software version 3.0 (BPS) was used to identify peaks with high prediction values for ALS. Peaks with intensity value lower than 0.2 were removed prior to analysis.

The rule induction knowledge-based problem solving Rule Learner (RL) algorithm¹⁴ was used in a 10-fold cross validation study on mass spectra (1.5 – 35 kDa mass range) from all CSF samples to generate a final set of predictive rules. 58,324 total mass peaks from the Q10 and Zn-IMAC30 protein chips were used in this analysis. Sensitivity, specificity and overall accuracy were generated from the 10-fold cross validation results. The results were compared to those we previously published⁸ to evaluate reproducibility across studies.

Putative protein identification of SELDI-TOF-MS peaks was obtained using the Empirical Proteomics Ontology Knowledge Base (<http://www.dbmi.pitt.edu/EPO-KB>)¹⁵.

C-reactive protein ELISA

CRP concentrations in CSF were determined using a human CRP ELISA Kit (Millipore, item # CYT298) in accordance with the manufacturer's instructions. The CSF samples were diluted 1:10 in wash buffer for use in the assay. All ELISA measurements were performed in duplicate, and each experiment was repeated at least twice. The absorbance for each well was measured at 450 nm. Coefficient of variation between experiments with this ELISA kit was 5-10%.

Statistics

For SELDI-TOF-MS data a significance level at $p < 0.01$ was used. For all other data analysis we set a significance level of $p < 0.05$. Data are expressed as mean $\pm$ S.E.M. For group comparisons, Student's t-test and one-way ANOVA were used to determine statistical significance. For comparison of individual mass peaks across groups we used non-parametric Kruskal-Wallis ANOVA, followed by the Mann-Whitney U-test for pair wise comparisons. Pearson's test was used for testing correlation.

Results

The demographics of the subject groups are shown in Table 1. After normalization, alignment and clustering of the spectra, 187 unique peaks above the chosen threshold could be detected in the IMAC dataset and 179 unique peaks in the Q10 dataset. We first identified individual mass peaks that differentiated ALS from each control subject group and then used a learning algorithm to generate a panel of candidate biomarker peaks that differentiate ALS from all combined controls groups. Using a cut-off level of $p < 0.01$, a total of 33 mass peaks were statistically significant between the ALS and HC groups (Supplemental Table 1). 15 of the 100 ALS samples were from individuals with a family history of ALS, and 3 harbored SOD1 mutations, thus representing familial ALS (FALS) subjects. Comparison of these 15 FALS to all other ALS samples yielded no statistically significant peak differences, and therefore all subsequent analysis was performed by combining all ALS sample data. ALS versus DC revealed 15 statistically significant mass peaks (Supplemental Table 2). Database search using the Empirical Proteomics Ontology Knowledge Base¹⁵ indicated the putative protein identity for many mass peaks (Supplemental Tables 1 – 2).

Biomarker peaks that predict ALS from control subjects

We performed a univariate statistical analysis of the SELDI-TOF-MS mass peaks across all subject groups to identify biomarker mass peaks that distinguish subject groups with high predictive value. We initially compared the ALS to healthy control (HC) subjects. The mass peak with the highest predictive value for separating ALS from HC subjects was a Q10 mass peak at 23,030 Da, with an overall accuracy of 69% (sensitivity of 65% and specificity of 79%) using a cut-off peak intensity value of 1.59 (Fig. 1A). A SELDI mass peak of this size was previously shown to be C-reactive protein (CRP)^{16,17}. We confirmed this mass spectrometry result using a commercial CRP ELISA and CSF from 41 ALS and 20 age-matched HC subjects used in the SELDI-TOF-MS analysis (Fig. 1B). The CRP protein levels were 5.84 ± 1.01 ng/mL for controls and 11.24 ± 1.52 ng/mL for the ALS group ($p = 0.02$). CRP ELISA results provided an overall accuracy of 62% (sensitivity of 51% and specificity of 85%) to discriminate ALS from HC using a cut-off value of 9 ng/mL. Within the ALS subjects, we did not observe any correlation between CRP levels above or below the cut-off value to patient age, gender, or site of disease onset. However the CRP mass peak

did exhibit 78% sensitivity for definite ALS cases versus 60% for all other ALS diagnostic groups as defined by El Escorial criteria.

The CRP mass peak provided an overall accuracy of 62% (sensitivity of 65% and specificity of 60%) for differentiating ALS from all non-ALS subjects. The drop in specificity across all groups was due to increased CRP levels in the CSF of DC and MS subject groups (Fig. 2A). The Mann-Whitney U-test for pair-wise comparisons identified statistically significant CRP mass peak alterations between ALS and HC, ALS and AD, and DC and HC (Fig. 2A). There was no significant alteration in CRP levels between the ALS and MS or DC groups.

Both cystatin C and transthyretin were identified in prior mass spectrometry-based proteomic studies as candidate ALS biomarkers ^{7,8}. In this study, the cystatin C peak at m/z 13,380 had an overall accuracy of 67% (sensitivity of 60% and specificity of 71%) for distinguishing ALS across all subject groups. The cystatin C mass peak exhibited statistically significant alterations between the ALS and HC groups, and between the ALS and DC groups (Fig. 2B). This particular mass peak also was significantly reduced in AD subjects when compared to HC subjects (Fig. 2B). The sensitivity of cystatin C was identical across all ALS diagnostic groups defined by El Escorial criteria.

Significantly reduced levels of native and double-charged native transthyretin were found in ALS, whereas mass peaks corresponding to oxidative modified forms of transthyretin were increased in ALS (Supplemental Table 1). Mass peaks at 6,959 Da and m/z 7,060 Da, which represent double-charged species of different conjugated species of transthyretin, were significantly increased in ALS. The 6,959 Da mass peak exhibited the best transthyretin predictive value with an accuracy of 65% (63% sensitivity and 70% specificity) for distinguishing ALS from the HC group, and an overall accuracy of 54% (sensitivity of 63% and specificity of 47%) for distinguishing ALS across all subject groups. This peak was identified as the cysteinyl-glycine (CysGly) conjugated form of transthyretin ¹⁸. Similar to the CRP peak, the CysGly-transthyretin mass peak exhibited higher diagnostic sensitivity for subjects defined as definite ALS (78%) versus probable or possible ALS (58%) using El Escorial criteria. The 6,959 Da CysGly-transthyretin peak was significantly increased in ALS subjects when compared to the HC or MS subject groups (Fig. 2C). These results verify our prior SELDI-TOF-MS results for cystatin C and transthyretin alterations in ALS patients using a much larger sample size. The alterations in transthyretin mass peaks were also confirmed using matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) to obtain higher resolution protein profiling of samples (data not shown).

We next correlated the mass peak intensity values for CRP, cystatin C, and the 6,959 Da transthyretin peak to ALS disease duration for all ALS subjects. Disease duration was defined as the time from patient-reported symptom onset till the lumbar tap. We observed a non-significant trend towards reduced cystatin C levels during disease duration (Fig. 3). Levels of CRP or transthyretin did not exhibit any correlation with patient disease duration (data not shown). We also correlated mass peak intensity values to patient survival. 50 of the SALS patients had survival information from date of lumbar tap to death. We found that the level of cystatin C correlated with survival time for limb onset patients (Figure 4). Cystatin C level did not correlate with survival in bulbar onset ALS patients, and the levels of CRP or transthyretin did not correlate with patient survival (data not shown).

Biomarker panel for ALS

We next performed a multivariate analysis to generate biomarker panels with predictive value for ALS using the Rule Learner (RL) algorithm. All CSF samples were used in a 10-fold cross-validation study, and the final biomarker panel predicted ALS with 82% overall

accuracy (63% sensitivity, 94% specificity); this was better than any individual biomarker candidate. A total of 41 mass peaks between 1.5 – 35 kDa were used by RL to generate the discriminatory rules (Table 2). The high level of specificity indicates the RL-generated biomarker rules can readily distinguish non-ALS subjects. The lower level of sensitivity likely reflects ALS disease heterogeneity. Another indication of the overall sample heterogeneity is the large number of mass peaks used by RL to create discriminatory rules, reflecting the generation of rules that distinguish small sub-populations of samples. Three of the potential biomarker mass peaks listed in Table 2 are equivalent to those identified in our prior study that utilized only 54 CSF samples ⁸.

Discussion

In this study we performed ALS biomarker discovery and verification on CSF from 241 subjects using the SELDI-TOF-MS platform. Protein mass peaks corresponding to C-reactive protein (CRP), cystatin C and transthyretin provided the best individual diagnostic predictive value for ALS, though the overall accuracy for any one mass peak remained less than 70%. A biomarker panel identified using the RL algorithm generated 82% accuracy in a 10-fold cross-validation study using all CSF samples. Three of the biomarker peaks identified by RL are identical to those we previously reported ⁸. We also performed direct group comparisons and obtained mass peaks that could distinguish between the subject groups. However further studies using additional ALS disease mimics (such as pure upper motor neuron disease, pure lower motor neuron disease, multifocal motor neuropathy) are required to assess the ability of this biomarker panel to differentiate between motor neuron diseases. The current study included 4 LMN subjects, and 3 of the 4 were classified as ALS by the biomarkers. However 2 of these 3 LMN cases later exhibited UMN symptoms and were subsequently re-classified as either definite or probable/lab supported ALS. Therefore the protein biomarkers identified LMN subjects that later developed clinical symptoms for the classification of ALS. Our study did not detect any normal age-related changes in biomarker protein levels, though it was not powered to examine age-associated changes in protein levels. This issue must be explored in future studies.

The 23,030 Da mass spectral peak exhibited the single best sensitivity and specificity for ALS and has been reported to be C-reactive protein (CRP), an acute-phase inflammatory protein ^{17,19}. CRP was not detected as a potential biomarker in our prior study due to the mass range cut-off of 20 kDa used for that study. We observed a statistically significant increase of CRP in ALS subjects when compared to HC or AD subjects but no significant difference to MS or other disease control subjects, indicative of inflammatory processes in these other neurologic disorders. We also failed to detect significant differences for CRP between ALS and the upper and lower motor neuron disease mimics. These results were confirmed by ELISA using a subset of the total subjects used for mass spectrometric analysis (Fig. 1). A recent study found increased blood levels of CRP in ALS patients that correlated with ALS-FRS measurements ²⁰. We also identified alterations in another acute-phase protein, transthyretin, during ALS.

Little is known about the role of CRP in the central nervous system, but it is expressed by neurons ²¹ and has been observed in neurofibrillary tangles during AD ²². The mean levels of CRP in control CSF have been reported to be 3.2 to 8 µg/L ^{23,24}, values similar to those in our study. A 2-fold elevated CSF level of CRP in ALS suggests low level activation of the immune system, consistent with prior studies that indicate increased inflammation in ALS and the potential for a CSF inflammatory profile to be indicative of the disease ^{25,26}. By comparison, this CRP level is much lower than that observed in acute bacterial or viral infections, such as meningitis ²⁷. It would be interesting to determine if CRP levels increase during disease progression within individual patients, suggestive of increased inflammation

during ALS. We found no correlation between the CRP level and the time from symptom onset to lumbar spinal tap used in this study.

Our SELDI-TOF-MS analysis demonstrated reduced levels of native transthyretin and increased levels of CysGly-transthyretin, a modified form generated by oxidative damage^{28,29}. Oxidative modifications to transthyretin were increased in ALS patients, but they also occurred in other neurodegenerative conditions (Fig. 2C). Oxidized transthyretin retains its tetramer structure and is resistant to formation of amyloid fibrils that occur in familial amyloidotic polyneuropathy patients³⁰. Thus oxidation of transthyretin may stabilize transthyretin function during metabolic stress conditions associated with ALS. Statistically significant reductions in cystatin C were also noted in ALS patients. These results confirm prior studies from our lab and others^{7,8,12}. In addition, we identified mass spectral peaks corresponding to a number of proteins that were previously shown to exhibit altered protein levels or contribute to pathogenic mechanisms of ALS, including chromogranin B, neuroendocrine protein 7B2, β 2-microglobulin, and β -amyloid peptide^{8,26,31-33}.

We failed to identify a specific protein peak that correlated with disease duration across ALS patients (Fig. 3). A recent study using a small number of subjects also failed to identify a correlation between cystatin C levels and disease duration¹². However data in both studies represents only a single time point within the disease for each patient. Further studies using longitudinal samples collected from individual ALS patients are required to identify candidate prognostic biomarkers of disease progression. Interestingly, cystatin C levels correlated with patient survival in limb-onset ALS. This suggests that cystatin C levels in the CSF may be useful as a marker of survival in specific sub-types of ALS. However bulbar-onset ALS patients in our study exhibited a significantly shorter disease course when compared with limb-onset patients. Therefore levels of cystatin C may not correlate with survival of rapid disease progressors. Further studies are required to explore this possibility. A functional role for cystatin C in ALS remains uncertain but worthy of pursuit. While cystatin C is a known component of Bunina bodies³⁴, reduced cystatin C in CSF indicates a reduction in extracellular cystatin C levels. Extracellular cystatin C is a regulator of cysteine proteases such as cathepsins and calpains. Reduced cystatin C in ALS may enhance cysteine protease-mediated degradation of extracellular matrix proteins and retard regenerative and/or repair events³⁵. Extracellular cysteine proteases also modulate degradation of cell surface proteins and may facilitate localized neuronal damage and death. Reduction of cystatin C in transgenic mice results in enhanced neuronal cell death following focal ischemia³⁶. Cystatin C has also been shown to modulate cerebral amyloidosis, and reduced cystatin C may enhance cerebral vessel damage due to amyloidosis³⁷. Recently, reduced levels of cystatin C were observed in spinal motor neurons and astrocytes in ALS patients and were correlated with formation of TDP-43 inclusions³⁸. Therefore cystatin C exhibits multiple extracellular and intracellular functions that may contribute to the pathogenesis of ALS.

The rules generated by the RL algorithm predicted ALS with a high level of specificity (94%) and 82% overall accuracy. The large cohort size in this study may explain the increased number of mass peaks necessary to distinguish ALS from healthy and other disease controls, when compared to our prior study. The ability to rule out ALS with a high degree of confidence (94% specificity) while predicting ALS with only 63% sensitivity is suggestive of ALS disease heterogeneity. A high degree of specificity would provide diagnostic value to the clinician to rule out ALS, but the lower sensitivity would initially miss many ALS patients. Alternatively, the biomarker peaks identified by SELDI-TOF-MS may not provide the best panel or platform to predict ALS with high sensitivity. To enhance our ability to identify biomarkers with high sensitivity for ALS, future studies will require a larger ALS patient cohort containing sufficient numbers of motor neuron disease subtypes based on clinical parameters, and the use of multiple experimental platforms. A consortium

of clinical sites, each following standard procedures of sample collection and processing, will be required to complete such a study. While we replicated findings for many mass peaks from our prior study, issues of SELDI-TOF-MS reproducibility across laboratories may limit its utility as a diagnostic platform^{10,11,39}. ELISA or more quantitative mass spectrometry techniques will likely be required to obtain the high level of sensitivity and reproducibility across laboratories necessary to generate clinical utility for a diagnostic assay. A recent review article described the biomarker development pathway for ALS⁴⁰. Our current verification study represents a next-step in this biomarker development pathway, but further studies are required to first validate the biomarkers and then ultimately qualify them for ALS. Future prospective studies using CSF collected from subjects at the initial neurology clinic visit are required to first validate any candidate diagnostic biomarkers and demonstrate clinical utility by comparing the biomarker assay predictions to the subsequent clinical diagnosis. Such a prospective study will reduce the time from symptom onset to lumbar tap observed in our current study (Table 1) and determine the ability of the biomarkers to identify ALS soon after clinical symptom onset. Finally, large prospective studies would be required to evaluate the utility of any ALS specific biomarker for use in the general population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
SELDI-TOF-MS	Surface Enhanced Laser Dissociation/Ionization Time-of-Flight mass spectrometry
CNS	Central nervous system
CSF	Cerebrospinal fluid
CRP	C-reactive protein
CysGly	Cysteinyl-glycine
ELISA	Enzyme-linked immunosorbent assay
HC	Healthy control
FALS	Familial amyotrophic lateral sclerosis
MS	Multiple sclerosis
DC	Neurologic disease
RL	Rule Learner
SALS	Sporadic amyotrophic lateral sclerosis

Figure 1.

(**A**) SELDI-TOF-MS relative intensity values of the 23.03 kDa peak for the 100 ALS and 41 HC CSF samples. (**B**) C-reactive protein absolute concentration values (ng/mL) measured by ELISA using 41 ALS and 20 age-matched HC samples. A line in each subject group denotes the mean value.

Figure 2. SELDI-TOF-MS analysis for CRP, cystatin C and CysGly-transferrin levels in ALS, AD, HC, MS and DC subject groups. **(A)** Box plot of CRP levels (Kruskal-Wallis test across all groups, $p = 0.002$). The Mann-Whitney U test for pair-wise comparisons identified significant CRP alterations between ALS and HC (**, $p = 0.002$), ALS and AD (*, $p = 0.012$), and DC and HC (*, $p = 0.014$). **(B)** Box plot of cystatin C (Kruskal-Wallis test across all groups, $p = 0.001$). Cystatin C was significantly reduced in the ALS group when compared with the HC group (*, $p = 0.002$) or to the DC group (*, $p = 0.01$). Cystatin C levels in the AD group were significantly decreased when compared to the HC ($p = 0.004$) or DC ($p = 0.01$) groups. **(C)** Box plot of CysGly-transferrin (Kruskal-Wallis test across

all groups, $p = 0.001$). CysGly-transthyretin was significantly increased in ALS when compared to HC (*, $p = 0.002$) or MS (*, $p = 0.049$) groups. CysGly-transthyretin level in AD was increased over HC ($p = 0.001$). For all panels, the box represents the 25th to 75th quartile, the horizontal bar the median and the whiskers the range.

Figure 3. Cystatin C (13,377 Da) mass peak intensity correlated to ALS disease duration. Pearson correlation coefficient is $r = -0.209$ ($p = 0.06$).

Figure 4. Correlation of the cystatin C mass peak with survival, defined as the time from lumbar tap to patient death. Pearson correlation coefficient is $r = 0.486$ ($p = 0.001$).

Table 1

Subject group demographics

Subject group	Number of subjects	Mean age (years)	Gender M / F	Bulbar onset (%)	Average Disease Duration (Months)
SALS	85	52.6	58 / 27	28.4	21
FALS	15	51.1	9 / 6	21.4	20
AD	53	67.9	22 / 31	N.A.	N.D.
MS	18	46.7	7 / 11	N.A.	N.D.
DC	29	57.4	12 / 8	N.A.	N.D.
HC	41	44.9	16 / 25	N.A.	N.A.

Abbreviations: SALS is sporadic amyotrophic lateral sclerosis, FALS is familial ALS, AD is Alzheimer's disease, MS is multiple sclerosis, DC is disease control consisting of both neurologic and non-neurologic conditions, and HC is the healthy control participants. N.A. is not applicable, and N.D. is not determined. Average disease duration is the time that clinically diagnosed ALS patients retrospectively reported first symptom onset till time of CSF draw.

Table 2
RL Biomarker Panel

Protein Chip Array	m/z peak (kDa)
Q10	3.04, 3.07, 3.14, 3.22, 3.32, 3.41, 3.56, 3.59, 6.38, 13.73 , 15.69, 27.49, 27.52, 27.63, 27.67, 27.68, 27.73, 27.86, 30.26
Zn-IMAC	5.24, 5.26, 5.74, 5.75, 5.77, 6.12, 6.13, 6.81 , 11.71, 11.94, 17.14 , 17.54, 17.56, 19.70, 20.50, 20.70, 20.71, 20.72, 20.73, 20.74, 31.53, 33.80

Set of 41 candidate biomarkers in the rules generated by the RL algorithm for diagnostic predictions. The bold-faced mass-to-charge (m/z) peak numbers correspond to peaks identified by RL in our prior study ⁸.