An increasingly varied clinical spectrum of cases with amyotrophic lateral sclerosis (ALS) has been identified, and objective criteria for clinical trial eligibility are necessary.

To develop a cerebrospinal fluid (CSF) biomarker sensitive and specific for the diagnosis of ALS.

A case-control study including 51 individuals with ALS and 23 individuals with a disorder associated with a 4-repeat tauopathy was conducted at an academic medical center.

The CSF level of tau phosphorylated at threonine 181 (p-tau) and ratio of p-tau to total tau (t-tau).

Using a cross-validation prediction procedure, we found significantly reduced CSF levels of p-tau and the p-tau:t-tau ratio in ALS relative to 4-repeat tauopathy and to controls. In the validation cohort, the receiver operating characteristic area under the curve for the p-tau:t-tau ratio was 0.916, and the comparison of ALS with 4-repeat tauopathy showed 92.0% sensitivity and 91.7% specificity. Correct classification based on a low CSF p-tau:t-tau ratio was confirmed in 18 of 21 cases (86%) with autopsy-proven or genetically determined disease. In patients with available measures, p-tau:t-tau in ALS correlated with clinical measures of disease severity, such as the Mini-Mental State Examination (n = 51) and ALS Functional Rating Scale–Revised (n = 42), and regression analyses related the p-tau:t-tau ratio to magnetic resonance imaging (n = 10) evidence of disease in the corticospinal tract and white matter projections involving the prefrontal cortex.

The CSF p-tau:t-tau ratio may be a candidate biomarker to provide objective support for the diagnosis of ALS.

Phosphorylated Tau as a Candidate Biomarker for Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative condition with upper motor neuron and lower motor neuron motor deficits. Patients with ALS experience a rapid rate of decline within 3 to 5 years. Diagnostic evaluation of ALS is aimed typically at exclusion of other disorders. Phenotypic variability has resulted in controversy about clinical stratification strategies: patients may show only lower motor neuron or upper motor neuron disease, may have disease restricted to a particular segment (eg, bulbar) or region (eg, flail arm), or may have disease that is strongly lateralized. In addition, ALS may exhibit nonpyramidal motor system involvement, including cognitive difficulty in 33% to 50% of the cases that extends to frontotemporal degeneration (FTD). Clinically presymptomatic ALS may exist in carriers of genetic mutations, such as TARDBP and C9orf72 hexanucleotide repeat expansion. Given these challenges in an era of disease-modifying therapies, it is critical to identify objective biomarkers of ALS during life.

Amyotrophic lateral sclerosis is considered part of the frontotemporal lobar degeneration (FTLD) spectrum of disorders. Approximately 95% of individuals with ALS have transactive DNA-binding protein of approximately 43 kDa (TDP-43) at autopsy, and TDP-43 is also the histopathologic feature in half of the FTLD cases. Most of the remaining patients with FTLD have hyperphosphorylated tau. Perhaps the most common tauopathy is associated with 4-repeat tau (4R-tau) in pro-
Phosphorylated Tau as a Biomarker for ALS

Mercredi disease; pathologically confirmed FTLD-tau, such as several conditions involving tau pathology, including Alzheimer disease.

Elevated CSF ptau is found in another CSF study. We excluded patients with a 3-repeat tauopathy from analysis. The present study evaluated the possibility that CSF ptau is a candidate ALS biomarker. We also assessed the relationship between CSF ptau and clinical markers of disease burden, such as the ALS Functional Rating Scale—Revised (ALSFRS-R).

White matter neuroimaging may be valuable diagnostically in ALS and PSP. To evaluate the extent of white matter disease and relate this to CSF ptau, we obtained fractional anisotropy (FA) measures of white matter disease in ALS.

Methods

Clinical Evaluation

Participants

We studied 51 patients with ALS and 23 patients likely to have 4R-tau pathology recruited from the ALS Center and the Penn Frontotemporal Degeneration Center at the University of Pennsylvania. Experienced neurologists diagnosed ALS according to El Escorial revised criteria, with the initial evaluation showing 7 individuals with definite ALS; 18, probable; 18, possible; and 5, suspected. Three additional individuals had ALS-FTD, with co-occurring FTD diagnosed according to published clinical criteria.

Onset was bulbar in 10 patients, cervical in 15, thoracic in 1, and lumbar in 22 (onset was unknown in patients with FTD). Five patients had autopsy confirmation, 5 had a C9orf72 (OMIM: 61426) expansion, and 1 had a pathogenic TARDBP mutation (OMIM: 605078; p.N390S) consistent with TDP-43 pathology. The 4R-tauopathy cohort comprised 15 patients with clinically diagnosed PSP, which is highly associated with 4R-tau pathology at autopsy, autopsy-confirmed 4R-tau (corticobasal degeneration CBD, 3; PSP, 2), and pathogenic mutations consistent with 4R-tau (2 with MAPT E10 + 16 (OMIM: 157140), with 1 confirmed at autopsy, and 1 with MAPT P301.L). A subset of 43 ALS and PSP patients participated in another CSF study. We excluded patients with a 3-repeat tauopathy to define a homogeneous contrast group. Cerebrospinal fluid was also available in 23 healthy elderly individuals (seniors) screened for dementia using a Mini-Mental State Examination (MMSE) score greater than 28 of 30, for AD pathology using an autopsy-validated tau to β-amyloid ratio (<0.34), and for no neurologic or psychiatric history.

Another cohort of 28 demographically matched healthy elderly individuals (age, educational level, and sex; all P > .05). No neurologic or psychiatric history were recruited as neuroimaging controls. All individuals participated in a written informed consent procedure with their caregivers, when appropriate, that was approved by the institutional review board at the University of Pennsylvania. Individuals serving as controls received compensation.

Functional Measures

As summarized in the Table, patients with ALS, those with 4R-tau, and the healthy seniors were evaluated clinically with the MMSE (n = 78). A subset of ALS patients (n = 42) was additionally evaluated with the ALSFRS-R.

Lumbar Puncture Procedure and Analysis

Lumbar Puncture

Samples of CSF were obtained during routine diagnostic lumbar puncture, as previously described. Briefly, lumbar puncture was performed at the L3/L4 lumbar space using a 20-gauge needle to collect 15 mL of CSF in polypropylene tubes (Corning Life Sciences). Samples were aliquoted and immediately stored at −80°C until analysis. Sample collection, storage, and analysis were performed according to published standard operating procedures.

CSF Analysis

Samples were analyzed using a Luminex xMAP platform (INNOBIA AlzBio3; Innogenetics) (n = 52) or an enzyme-linked immunosassay (ELISA) (INNOTEST; Innogenetics) (n = 13), as described.

Table. Demographic and Functional Measures for ALS, 4R-Tau, and Healthy Seniors

<table>
<thead>
<tr>
<th>Functional Measure</th>
<th>ALS (n = 51)</th>
<th>4R-Tau (n = 23)</th>
<th>Healthy Seniors (n = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex M/F, No.</td>
<td>35/16</td>
<td>14/9</td>
<td>12/11</td>
</tr>
<tr>
<td>Age, y</td>
<td>54.88 (10.6)</td>
<td>65.4 (9.4)*</td>
<td>59.9 (6.0)</td>
</tr>
<tr>
<td>Educational level, y</td>
<td>14.86 (3.9)</td>
<td>14.55 (3.4)</td>
<td>15.9 (4.3)</td>
</tr>
<tr>
<td>MMSE score (adjusted)</td>
<td>27.53 (3.3)</td>
<td>24.45 (5.13)*</td>
<td>29.39 (0.7)</td>
</tr>
<tr>
<td>Disease duration, mo</td>
<td>24.72 (14.1)</td>
<td>54.87 (23.4)*</td>
<td>...</td>
</tr>
<tr>
<td>ALSFRS-R score (n = 42)</td>
<td>37.74 (7.9)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Progression rate (n = 42)</td>
<td>0.46 (0.4)</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Abbreviations: ALS, amyotrophic lateral sclerosis; ALSFRS-R: ALS Functional Rating Scale; MMSE, Mini-Mental State Examination; 4R-tau, 4-repeat tauopathy.

* P < .01.

† P < .001.

‡ P < .05.

§ P < .001.

$ P < .01.
Phosphorylated Tau as a Biomarker for ALS

Results were summarized in the Table. For each correlation, we used an assay sensitive to phosphorylation at threonine 181 because this is the Alzheimer’s Disease Neuroimaging Initiative standard for which the highly reliable Luminex method is available. Biomarker analytes were detected using reporting monoclonal antibodies 3D6 (Aβ1-42) and HT7 (ttau and ptau). Some older samples were analyzed with an ELISA method; monoclonal antibodies for capturing and reporting ttau and ptau were AT120/HT7 and BT2, and HT7/AT270, respectively. As described previously, 26 ELISA values for Aβ also were measured using an in-house method with the capturing monoclonal antibody BAN-50 and the reporting monoclonal antibody BC-05. Using an autopsy-validated formula, 27 a linear regression model converted natural log–transformed raw CSF values from ELISA to an xMAP equivalent.

Statistical Analysis
We evaluated overall group-level differences for raw ptau and ttau levels and for ptau:ttau ratio with nonparametric Kruskal-Wallis and post hoc Mann-Whitney tests for descriptive purposes. We also confirmed that a potential covariate for ALS progression rate, defined using previously published criteria 28 (48 ALSFRS-R/disease duration in months) did not contribute to group differences. We used a cross-validation procedure to evaluate ptau, ttau, and the ptau:ttau ratio as candidate biomarkers for individual patient screening. We randomly divided ALS and 4R-tauopathy cohorts into comparably sized training (ALS, 26; 4R-tauopathy, 11) and validation (ALS, 25; 4R-tauopathy, 12) cohorts. The training and validation cohorts are described in the Supplement (eTable 1). Because the ALS and 4R-tauopathy cohorts differed in age at which CSF samples are obtained and disease duration, and because these factors may influence CSF analyte levels, 29 we performed a logistic regression for each CSF analyte that included age and disease duration nuisance covariates. These logistic regressions were completed in the training cohort to generate a probabilistic likelihood of ALS, and these probabilities then were entered into receiver operating characteristic curves. We defined the optimal cutoff to assess sensitivity and specificity at a probability of 0.703 or more, equivalent to the proportion of patients with ALS in the training cohort (26 of 37) and then applied this logistic regression model to the independent validation cohort. We report screening accuracy using a χ² test: patients in the validation cohort whose ALS probability exceeded the 0.703 threshold were predicted as having ALS and otherwise assigned to the predicted 4R-tauopathy group. We performed Pearson correlations between each analyte (ptau:ttau ratio, ptau, and ttau) and functional measures; these results are summarized in the Table. For each correlation, we used the predicted probability of ALS as an age- and disease duration-adjusted proxy for each CSF analyte.

Imaging Procedure and Analysis
Acquisition
Diffusion-weighted magnetic resonance imaging (MRI) results were available for 10 patients with ALS (1 with ALS-FTD) (Siemens 3.0T Trio scanner; Siemens) using an 8-channel coil. Diffusion-weighted images were acquired with a 30-directional sequence involving single-shot, spin-echo, diffusion-weighted echo planar imaging (field of view, 245 mm; matrix size, 128 × 128; number of slices, 57; voxel size, 2.2 mm isotropic; repetition time, 6700 milliseconds; echo time, 85 milliseconds; and fat saturation). We acquired 30 volumes with diffusion weighting (b = 1000 s/mm²) along 30 noncollinear directions per patient and either 1 (n = 2) or 4 (n = 17) without diffusion weighting (b = 0 s/mm²). When 4 volumes were collected without diffusion weighting, these volumes were averaged to increase the signal to noise ratio. Reasons for exclusion included health and safety (eg, difficulty breathing while supine, metallic implants, shrapnel, and claustrophobia) and lack of interest in an imaging study. Diffusion-weighted images were also available for 9 patients with 4R-tau. The T1-weighted MRI volumes were acquired in the same scanning session with magnetization-prepared acquisition variables: repetition time, 1620 milliseconds; echo time, 3 milliseconds; slice thickness, 0.9 mm; flip angle, 15°; matrix, 192 × 256; and in-plane resolution, 0.9 × 0.9 mm.

Preprocessing
Whole-brain MRI volumes were preprocessed using PipeDream (https://sourceforge.net/projects/neuropipedream/) and Advanced Normalization Tools (ANTS; http://www.nicel.upenn.edu/ANTS/), as described. 46 Briefly, PipeDream deformed each data set into local template space in a canonical stereotactic coordinate system. Each participant’s T1 image was warped to the template via the symmetric diffeomorphic procedure in ANTs. For diffusion-weighted images, motion and distortion artifacts were removed by affine coregistration of each image with diffusion weighting to the unweighted (b = 0) image. Diffusion tensors were computed using a linear least-squares algorithm 44 implemented in the Camino program, 45 and tensors were reoriented using the preservation-of-principal-directions algorithm. 46 Fractional anisotropy was computed from the diffusion tensor image for each participant. Distortion between T1 and diffusion tensor images was corrected by registering the FA image to the T1 image. The diffusion tensor image was then warped to template space by applying both the FA-to-T1 and T1-to-template warps for each participant. The FA images were smoothed using a 4-mm full-width half-maximum isotropic gaussian kernel.

Statistical Analysis
Analyses of FA were performed in SPM8 (http://www.fil.ion.ucl.ac.uk/spm/software/spm8/) using the 2-samples t test module. The FA volumes were analyzed using an explicit mask (FA, ≥0.25) to constrain comparisons with white matter regions. Comparisons of ALS patients with healthy seniors used a height threshold of q < 0.05 with false–discovery rate correction for multiple comparisons, and comparisons of 4R-tau with healthy seniors used a height threshold of q < 0.005 with false–discovery rate correction. Both comparisons used an extent threshold of 200 voxels. Regression analyses related FA to the adjusted ptau:ttau ratio at P < 0.05 (uncorrected) with a 50-voxel extent. Regression analyses were constrained to white matter fibers with reduced FA using explicit masks generated elsewhere. 41 Briefly, the xMAP platform used capture monoclonal antibodies 4D7A3 (Aβ1-42), AT120 (ttau), and AT270 (ptau) bound to color-specific beads. We used an assay sensitive to phosphorylation at threonine 181 because this is the Alzheimer’s Disease Neuroimaging Initiative standard for which the highly reliable Luminex method is available. Biomarker analytes were detected using reporting monoclonal antibodies 3D6 (Aβ1-42) and HT7 (ttau and ptau). Some older samples were analyzed with an ELISA method; monoclonal antibodies for capturing and reporting ttau and ptau were AT120/HT7 and BT2, and HT7/AT270, respectively. As described previously, 26 ELISA values for Aβ also were measured using an in-house method with the capturing monoclonal antibody BAN-50 and the reporting monoclonal antibody BC-05. Using an autopsy-validated formula, 27 a linear regression model converted natural log–transformed raw CSF values from ELISA to an xMAP equivalent.
from the results of the direct comparisons with healthy elderly individuals; different thresholds were used for group comparisons to create disease masks of comparable size. Using a deterministic tractography procedure in Camino,\textsuperscript{45} white matter fibers were tracked in a healthy elderly template using the diffusion tensor imaging sequence described above. Fiber tracts that passed through voxels of reduced FA were retained to define the masks for regression analyses. This was done to limit the interpretation of a correlation between white matter and CSF to white matter fibers with disease.

**Results**

Median raw CSF analyte values for ALS, 4R-tau, and the healthy seniors are illustrated in Figure 1. Kruskal-Wallis test results revealed group differences for the ptau:ttau ratio ($\chi^2 = 30.55; P < .001$) and for ptau level ($\chi^2 = 22.80; P < .001$). Planned post hoc Mann-Whitney test results revealed that, relative to 4R-tau, ALS had a reduced ptau:ttau ratio ($Z = 3.74; P < .001$) and reduced ptau levels ($Z = 2.82; P = .005$). Amyotrophic lateral sclerosis also had a reduced ptau:ttau ratio ($Z = 4.92; P < .001$) and ptau levels ($Z = 4.36; P < .001$) relative to the healthy seniors. There was no group effect for ttau level ($Z = 1.73; P > .10$). By comparison, 4R-tau had only a marginally reduced ptau level ($Z = 2.27; P = .05$) relative to the healthy seniors.

Receiver operating characteristic analyses illustrated in Figure 2 showed an area under the curve for the ptau:ttau ratio of 0.916 ($P < .001$). In the training cohort, the probabilistic ALS cutoff achieved 80.8% sensitivity and 90.9% specificity. A cross-validation analysis using the same cutoff in the validation cohort revealed 92.0% sensitivity and 91.7% specificity ($\chi^2 = 24.90; P < .001$). An analysis of ptau level alone was robust (area under the curve = 0.92; $P < .001$). We found 80.8% sensitivity and 81.8% specificity in the training cohort, but the validation cohort achieved high sensitivity (88.0%) with only modest specificity (75.0%) ($\chi^2 = 17.42; P < .001$).

Follow-up analyses of individuals with autopsy confirmation of a genetic mutation ($n = 21$) showed correct classification in 18 of 21 patients (86%) using the most robust analyte: the ptau:ttau ratio. The 3 misclassified cases included 1 patient with the C9orf72 expansion (33%), 1 with the MAPT (E.10 + 16 C>T) mutation (33%), and 1 with autopsy-confirmed ALS (33%).

Correlation analyses in ALS using age- and disease duration–adjusted CSF levels revealed that MMSE is related to the ptau:ttau ratio ($r = 0.342; P = .048$) and ptau level ($r = 0.354; P < .040$), but not to the ttau level; ALSFRS-R is related to the ptau level ($r = 0.448; P < .003$) and ttau level ($r = 0.406; P < .001$).
Cerebrospinal fluid levels of phosphorylated tau were very low in ALS. A cross-validation analysis revealed that the ptau level and ptau:tau ratio appear to distinguish individuals with ALS from those with 4R-tau and from healthy seniors. This was confirmed in the subgroup of patients with known histopathologic features. A lower ptau level and a lower ptau:tau ratio correlated with clinical measures of disease and with MRI measures of reduced white matter FA in the corticospinal tract and prefrontal cortex in ALS subgroups.

The histopathologic abnormality in sporadic ALS is TDP-43, and patients with ALS (except for those with ALS/parkinsonism who are Chamorro from Guam) have negligible brain hyperphosphorylated tau at autopsy. Thus, we predicted low CSF ptau levels in ALS. The ptau:tau ratio was consistently sensitive and specific, generalizing from training to validation cohorts, and thus is a candidate biomarker for screening patients for ALS. Another study of TDP-43 proteinopathies and 4R-tauopathies reported similar findings. Additionally, almost all cases with known TDP-43 pathology had a low ptau:tau ratio. Two of the 3 incorrectly classified cases had genetically determined disease, and we cannot rule out that these patients may have additional abnormalities due to another condition.

Some previous work described elevated CSF ttau levels in ALS, and others reported normal ttau levels. Interpretation of inconsistent results should be performed cautiously because of the substantial variability associated with the ELISA method used in those studies. The present study used a more reliable Luminex method to assess most CSF analyte levels. Other studies reported significantly reduced CSF amyloid precursor protein levels in ALS, and elevated CSF Aβ levels were related to shorter survival, possibly reflecting the small number of patients with ALS who have concurrent Alzheimer disease abnormalities. Some studies described abnormal axonal markers that were related to survival and abnormal glial markers that were related to the progression rate, although our observations of reduced ptau were unrelated to survival and the progression rate. Two reports described CSF TDP-43 levels in ALS, although the variability of the results, including substantial overlap with control values, suggests that TDP-43 assays may be premature.

Lower CSF ptau levels and ptau:tau ratios in ALS correlated with clinical measures, and, although there are many measures of clinical functioning, this suggests that the ptau:tau ratio may be a sensitive marker of disease. Amyotrophic lateral sclerosis is associated with cognitive difficulty in many individuals, and we found that the ptau level and the ptau:tau ratio correlate with cognitive functioning. The ALSFRS-R is commonly used to reflect disease severity in ALS, and this correlated with the ptau level. Additional converging evidence suggesting that the ptau:tau ratio may be biologically...
meaningful comes from white matter neuroimaging in anatomic regions known to be compromised pathologically in ALS.77 Because the CSF ptau:tau ratio appears to be related to both clinical and imaging measures, the ratio may be a candidate marker to assess eligibility in clinical trials for disease-modifying treatments of ALS.

Our findings also suggest that a low ptau:tau ratio may be specific for ALS. We demonstrated this by contrasting ALS with individuals highly likely to have 4R-tau histopathologic features. Others52,54 also have reported comparative studies to demonstrate specificity. Although it is not unreasonable to expect elevated CSF ptau levels in this cohort because tau is hyperphosphorylated in autopsy assessments of these conditions, some reports have described elevated levels,14-17 some normal levels,18,25-58 and some reduced levels39 relative to healthy controls. This variability may be the result, in part, of mixed etiology in clinically diagnosed groups and the less-reliable ELISA method used in most prior studies. Regardless of the basis for previous findings, our observations suggest a reliable difference between individuals with ALS and those highly likely to have 4R-tau pathology.

Several caveats should be kept in mind when evaluating our findings. Our cohort was relatively small. Tau is phosphorylated at several sites, and assaying other phosphorylation sites may be informative. Most participants were assessed soon after the onset of typical ALS, and it would be important for future work to assess patients with ALS who have other phenotypic presentations and lengths of disease and evaluate CSF ptau levels in these different presentations. The contrast group consisted of patients with 4R-tau because of limited CSF samples available from individuals with 3-R tau pathology and our desire to have a relatively homogeneous contrast group, and it would be important to evaluate CSF ptau levels in individuals with 3-R tau pathology. Limited MRI assessments were available because of patient limitations, and verification of FTLD-TDP pathology was possible in only a subset of cases.

Conclusions

With the caveats discussed above in mind, our cross-validation prediction design suggests that individual patients with ALS highly likely to be due to FTLD-TDP pathology are characterized by a low CSF ptau:tau ratio relative to individuals highly likely to have FTLD-tau pathology. In addition, a low CSF ptau:tau ratio is associated with several clinical and imaging measures of ALS.

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Acquisition of data: Grossman, Elman, McCluskey, Boller, Shaw, Irwin, Lee, Trojanowski.


Drafting of the manuscript: Grossman, McMillan, Powers, Rascovsky, Lee, Trojanowski.

Critical revision of the manuscript for important intellectual content: Grossman, Elman, McCluskey, McMillan, Boller, Rascovsky, Hu, Shaw, Irwin, Lee, Trojanowski.


Obtained funding: Grossman, Lee, Trojanowski.

Administrative, technical, or material support: Grossman, McCluskey, McMillan, Boller, Rascovsky, Shaw, Lee, Trojanowski.

Study supervision: Grossman, Lee, Trojanowski.

Conflict of Interest Disclosures: Dr Hu has filed a provisional patent on the ptau:tau ratio as a biomarker for FTLD-TDP and ALS through Emory University. No other disclosures were reported.

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