Urinary p75ECD
A prognostic, disease progression, and pharmacodynamic biomarker in ALS

ABSTRACT

Objective: To evaluate urinary neurotrophin receptor p75 extracellular domain (p75ECD) levels as disease progression and prognostic biomarkers in amyotrophic lateral sclerosis (ALS).

Methods: The population in this study comprised 45 healthy controls and 54 people with ALS, 31 of whom were sampled longitudinally. Urinary p75ECD was measured using an enzyme-linked immunosassay and validation included intra-assay and inter-assay coefficients of variation, effect of circadian rhythm, and stability over time at room temperature, 4°C, and repeated freeze-thaw cycles. Longitudinal changes in urinary p75ECD were examined by mixed model analysis, and the prognostic value of baseline p75ECD was explored by survival analysis.

Results: Confirming our previous findings, p75ECD was higher in patients with ALS (5.6 ± 2.2 ng/mg creatinine) compared to controls (3.6 ± 1.4 ng/mg creatinine, p < 0.0001). Assay reproducibility was high, with p75ECD showing stability across repeated freeze-thaw cycles, at room temperature and 4°C for 2 days, and no diurnal variation. Urinary p75ECD correlated with the revised ALS Functional Rating Scale at first evaluation (r = −0.44, p = 0.008) and across all study visits (r = −0.36, p < 0.0001). p75ECD also increased as disease progressed at an average rate of 0.19 ng/mg creatinine per month (p < 0.0001). In multivariate prognostic analysis, bulbar onset (hazard ratio [HR] 3.0, p = 0.0035), rate of disease progression from onset to baseline (HR 4.4, p < 0.0001), and baseline p75ECD (HR 1.3, p = 0.0004) were predictors of survival.

Conclusions: The assay for urinary p75ECD is analytically robust and shows promise as an ALS biomarker with prognostic, disease progression, and potential pharmacodynamic application. Baseline urinary p75ECD provides prognostic information and is currently the only biological fluid–based biomarker of disease progression. Neurology® 2017;88:1137-1143

GLOSSARY
ALS = amyotrophic lateral sclerosis; ALSFRS-R = revised ALS Functional Rating Scale; CI = confidence interval; CV = coefficient of variation; HR = hazard ratio; NfL = neurofilament light; p75ECD = extracellular domain of p75; PAV = permanent assisted ventilation; PBS = phosphate-buffered saline; pNfH = phosphorylated neurofilament heavy.

Frustration over the continued failure of amyotrophic lateral sclerosis (ALS) clinical trials and the absence of therapeutic options for this fatal disease has fueled interest in the prospect that biomarkers may hold great promise for advancing therapy development efforts. Prognostic biomarkers, which aid in predicting the future course of disease, might be used to identify more homogeneous subsets of patients at the time of trial enrollment. Pharmacodynamic biomarkers, which have the potential to show that a biological response has occurred in a patient who has received an experimental therapeutic, may help in assessing the efficacy of drugs selected in phase II to advance to phase III clinical trials. Disease progression biomarkers (i.e., those that show a change over time as disease advances) may also serve as markers of pharmacodynamic effect.

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Among the biological fluid–based biomarker candidates, the cytoskeletal proteins neurofilament light (NFL) and phosphorylated neurofilament heavy (pNHF) show great promise as prognostic markers and potential pharmacodynamic biomarkers. However, since neurofilament levels remain largely stable over time,6–8 they do not reflect disease progression. Therefore, we currently lack any biological fluid–based biomarkers of disease progression. This has led us to focus on the common neurotrophin receptor (p75) as a biomarker of motor neuron degeneration. Based on preliminary observations that the extracellular domain of p75 (p75ECD) is present at elevated levels in the urine of patients with ALS compared to healthy individuals9 and that urinary p75 increases in the SOD1G93A mouse as disease unfolds,10 we investigated the potential of urinary p75ECD as a potential disease progression and prognostic biomarker.

METHODS Standard protocol approvals, registrations, and patient consents. This was a prospective cohort study in which urine samples from patients with ALS and controls were collected from the South Australian MND Clinic (Adelaide, Australia) and the Kessenerich Family ALS Center at the University of Miami (Miami, FL).

Written informed consent was obtained from all participants following ethics approval from the Flinders University of South Australia Southern Adelaide Clinical Human Research Ethics Committee and the University of Miami Human Subject Research Office. Patients with ALS (recruited between March 2011 and March 2015) were diagnosed according to the revised El Escorial criteria11 by experienced ALS neurologists (Australia: D.S.; United States: M.B.). Healthy controls (recruited between June 2008 and February 2015) were typically spouses and friends of patients and exclusion criteria included any neurologic condition or illness that affects kidney function. Sample size was driven by pragmatic considerations related to the availability of funding for this pilot project. Clinical information was collected by investigators blinded to urinary p75ECD results, including the revised ALS Functional Rating Scale (ALSFRS-R), and latencies from symptom onset and diagnosis to baseline assessment and sample collection. The estimated monthly decrease in ALSFRS-R by time of first assessment (baseline) was calculated as ΔFRS = (48 – ALSFRS-R at baseline)/number of months between symptom onset and baseline.12 Permanent assisted ventilation (PAV) was defined as the use of noninvasive ventilation for at least 23 h/day or tracheostomy with initiation of invasive ventilation. Urine samples were collected and stored in accordance with the Urine & Kidney Proteome Project Standards13 and coded to ensure anonymity. Samples collected in Miami were shipped on dry ice and all samples were stored at ~80°C until analysis. Urinary creatinine and osmolarity measurements were performed using a Roche (Basel, Switzerland)/Hitachi (Tokyo, Japan) modular analyzer.

Urinary p75ECD measurement. A sandwich ELISA was used to quantify p75ECD as previously described.14 Briefly, ELISA plates (96-well, Costar Corning [Manassas, VA]) were coated with mouse anti-human p75 MLR1 antibody14 for p75 capture and incubated for 18 hours at 4°C in bicarbonate buffer, pH 9.6. Wells were then blocked with sample buffer, phosphate-buffered saline (PBS) containing 2% bovine serum albumin, and 0.01% thimerosal, pH 7.4, for 1 hour at 37°C. Urine samples and recombinant human p75ECD standard (amino acids 29–230; R&D Systems, Minneapolis, MN) were diluted in sample buffer and incubated for 20 hours at room temperature. Goat anti-mouse p75ECD (Sigma-Aldrich, St. Louis, MO) and bovine anti-goat immunoglobulin G horseradish peroxidase

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Participant characteristics</th>
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<tr>
<td></td>
<td>Patients with ALS</td>
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<tr>
<td>Age at diagnosis, y, mean ± SD (range)</td>
<td>63.6 ± 13.3 (39.2–86.3)</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>28 (52)</td>
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<tr>
<td>Known to be familial, n (%)</td>
<td>12 (22)</td>
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<td>Bulbar onset, n (%)</td>
<td>16 (30)</td>
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<tr>
<td>Months from onset to diagnosis, mean ± SD (range)</td>
<td>9.8 ± 7.3 (1.7–41.6)</td>
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<tr>
<td>Months from diagnosis to first collection, mean ± SD (range)</td>
<td>6.6 ± 10.1 (0–57.4)</td>
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<tr>
<td>Age at first collection, y, mean ± SD (range)</td>
<td>64.1 ± 13.2 (39.7–86.4)</td>
</tr>
<tr>
<td>ALSFRS-R at first collection, mean ± SD (range)</td>
<td>38.8 ± 5.7 (22–47)</td>
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<tr>
<td>ΔFRS at first collection, mean ± SD (range)</td>
<td>0.8 ± 0.5 (0.01–2.7)</td>
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<tr>
<td>Death or PAV by end of study, n (%)</td>
<td>44 (81)</td>
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<td>Disease duration, mo, median (IQR)</td>
<td>18.4 (11.1–32.4)</td>
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<tr>
<td>Longitudinal subset: no. of collection time points, median (range)</td>
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Abbreviations: ΔFRS = rate of progression (average change in revised ALS Functional Rating Scale [ALSFRS-R] per month) from onset to baseline; IQR = interquartile range (25th–75th percentile); PAV = permanent assisted ventilation (tracheostomy or ≥23 hours/day on noninvasive ventilation). *Four had first collection on day of diagnosis, their months from diagnosis to first collection = 0. **Disease duration since diagnosis.
Urinary p75ECD in healthy controls and patients with ALS. Among healthy controls, urinary p75ECD correlates with age (Pearson r = 0.31, p = 0.04) but increases only by 0.32 ng/mg creatinine for each advancing decade. Although the control group was younger than the ALS population, the potential utility of p75ECD as a disease progression or prognostic marker is independent of comparison to the control group and therefore not affected by this age difference. Sex is not a significant determinant of urinary p75ECD.

Confirming our previous findings,7 urinary p75ECD levels are higher in patients with ALS (5.6 ± 2.2 ng/mg creatinine) at first study visit (baseline) compared to controls (3.6 ± 1.4 ng/mg creatinine, p < 0.0001, figure 1). Baseline urinary p75ECD levels do not differ significantly between patients with limb vs bulbar onset disease, even after controlling for baseline disease severity.

Analytic validation of urinary p75ECD. Confirming our previous observation of the linearity of the standard curve (absorbance and human urinary p75ECD concentration) for our in-house ELISA,9 here we show an assay sensitivity of ~70 pg/ml and a linear standard curve up to 2.5 ng/ml (figure e-1, A–C, at Neurology.org). Our assay for human urinary p75ECD demonstrates reliability with an intra-assay CV of 6.6% and an inter-assay CV of 12.5% (table e-1). Urinary p75ECD levels remained steady after multiple freeze-thaw cycles (figure e-1D). The level of p75ECD detected was also stable in 3 samples after 2-day of storage both at room temperature and at 4°C, although from 2 to 7 days there was some variation from that measured in freshly collected urine (i.e., at time 0; figure e-1E). Diurnal variation in p75ECD was tested in controls (n = 2), between first void in the morning, a spot urine sample in the afternoon, and 24-hour samples. The variation...
between the spot urine and the first void in addition to the spot and the 24-hour samples was modest (below 17%). When urinary dilution was considered by correcting with creatinine (as we routinely do for reporting results), the difference between spot and first void and spot and 24-hour sample decreased to 7% (figure e-1F). In addition, creatinine was a reliable measure of urinary dilution, based on the strong correlation with urine osmolarity (figure e-1G, $r = 0.88, p < 0.0001$).

**Urinary p75ECD as biomarker of disease progression.** The potential utility of p75ECD as a biomarker of disease progression was explored in the 31 patients with ALS with longitudinal data and urine samples (median of 3 time points, range 2–6; table 1). To assess change in p75ECD over time, we considered 3 options for the time base—namely, time since symptom onset, time since diagnosis, and time since the initial evaluation and sample collection (i.e., baseline)—and the limitations of each. Symptom onset relies upon subjective report of symptoms and the decision of which symptoms represent the onset of disease. On the other hand, baseline date is somewhat arbitrary, as patients were enrolled at variable stages in the course of disease. While the delay from symptom onset to diagnosis can be highly variable, the date of diagnosis is reliably obtained from clinic notes, patient self-report, and medical record review. For these reasons, we elected a priori in our longitudinal analyses to use time from diagnosis for primary analysis. Results of our mixed model analysis show that urinary concentration of p75ECD increases linearly and on average by 0.19 ng/mg creatinine each month ($p < 0.0001$; figure 2A). Adjusting for baseline p75ECD does not significantly affect the slope, suggesting a fairly uniform rate of p75ECD change over time, irrespective of concentration at the earliest time point (data not shown). Adjusting for age similarly does not affect the slope of increase of p75ECD, likely because the effect of age is seen over decades rather than years, and median follow-up duration in this study was 10.8 months (range 0.4–43 months). Models using time from symptom onset and time from baseline show similar results (figure 2, B and C).

For comparison, we performed similar mixed model analyses with ALSFRS-R as the outcome measure. In the same 31 patients, average rate of ALSFRS-R decline was 0.82 points/month (95% CI 0.68–0.97, $p < 0.0001$), comparable to reported rate in other longitudinal observational studies and clinical trials. Similar to p75ECD, adjustment for baseline ALSFRS-R had little effect on the slope (data not shown). Moreover, using data from all 115 person-visits, there was a correlation between ALSFRS-R and p75ECD ($r = -0.36, p < 0.0001$), which was essentially unchanged after adjusting for the within-person correlation due to repeated measures (figure 3A). This correlation was also apparent when comparing ALSFRS-R and p75ECD at baseline for each of the 54 patients (figure 3B; $r = -0.44, p = 0.0008$).

**Urinary p75ECD as prognostic biomarker.** To explore the potential utility of baseline p75ECD as a predictor of prognosis, we performed a survival analysis using Cox
proportional hazards model, including data from all 54 patients with ALS. In univariate analyses, neither age at diagnosis nor sex was associated with survival, but in agreement with prior studies,12,17 both bulbar onset (p = 0.0085) and DFRS (p < 0.0001) were predictors of survival. In multivariate models, adjusting for bulbar onset (hazard ratio [HR] 3.0, 95% confidence interval [CI] 1.4–6.3, p = 0.0035) and DFRS (HR 4.4, 95% CI 2.4–8.0, p < 0.0001), baseline p75ECD was also a predictor of survival (HR 1.3, 95% CI 1.1–1.5, p = 0.0004), indicating that baseline p75ECD provides additional prognostic value over and above what can be gleaned from routinely available clinical parameters. To visually illustrate the relationship between baseline p75ECD and survival, we divided the 54 patients into high vs low p75ECD groups (i.e., based on their baseline value being above or below the median), and present their Kaplan-Meier survival plot (figure 4).

DISCUSSION

Our pursuit of urinary p75ECD as a potential ALS biomarker was informed by the biology of the neurotrophin receptor p75. Rodent motor neurons express p75 during development18 but this disappears soon after birth only to be re-expressed after injury,19,20 including apoptotic motor neurons of SOD1G93A mice.21 p75ECD is cleaved from cell membranes post injury,19 with elevated levels detected 40 days prior to onset of disease in the urine of SOD1G93A mice, progressively rising as symptoms unfold.9,10 p75 is also re-expressed on motor neurons22 and Schwann cells23 in postmortem tissue of patients with ALS. Hence, the presence of p75ECD in urine is indicative of underlying motor neuron degeneration. While ALS is heterogeneous in etiology, biology, and phenotypic manifestations,24 it is always characterized by degeneration and death of motor neurons. As a generic biomarker of motor neuron degeneration, therefore, urinary p75ECD is expected to be useful as biomarker in all forms of ALS, irrespective of etiology. This stands in contrast, for example, to C9RANT dipeptides, which may have utility as a pharmacodynamic biomarker only in patients with ALS due to a C9ORF72 repeat expansion.5

The most striking and important aspect of our findings is that urinary p75ECD changes over time, increasing as disease progresses and motor function declines. This is true even in patients with slowly progressive disease in which the absolute values of p75ECD remain relatively low (figure 2). In this respect, p75ECD is currently the only potential biochemical biomarker of ALS disease progression. In addition, p75ECD may have utility as a potential pharmacodynamic biomarker insofar as showing that an experimental therapeutic that blunts the increase, stabilizes, or reduces urinary p75ECD over time would provide evidence of an underlying biological effect of the treatment.25 Urinary p75ECD therefore joins CSF and blood NfL6,26–34 and CSF pNfH7,33–35 as the lead candidates for further development as pharmacodynamic biomarkers. The important difference is that neurofilament levels, although elevated in ALS compared to controls,6,7,26–34,36 are largely stable over time, with the possible exception of patients with rapidly progressive disease, in whom CSF NfL may increase,6 and levels of blood pNfH may decrease over time.7 These data suggest that NfL and pNfH may also have potential utility as pharmacodynamic biomarkers.
In addition to its value as a disease progression and pharmacodynamic biomarker, urinary p75NECD may have value as a prognostic biomarker. Essential to this claim is the observation that baseline p75NECD informs the probability of survival in a way that supplements the prognostic value of readily available clinical parameters such as site of disease onset and ΔFRS, even though the HR for the effect of p75NECD is modest. While other biochemical biomarkers including pNFH8,34,36 and NfL6,31 levels in blood and CSF have been reported to have prognostic utility, published data have not (yet) demonstrated that they add prognostic value over and beyond what can be learned from clinical parameters.

Moreover, p75NECD is currently the sole ALS biomarker that is measurable in urine, a biological fluid that is readily accessible and which most patients are willing to provide. While blood is also readily accessible, not all patients are willing or able to undergo lumbar puncture to obtain CSF, especially longitudinally. Since patient comfort and compliance are important pragmatic considerations, especially in clinical trials, obtaining urine for p75NECD quantification may be more practical than the more invasive and logistically complex option of obtaining CSF. In addition, the less complex nature of the urinary proteome than that of blood is an advantage in assaying proteins such as p75NECD. Notwithstanding these considerations, we are also exploring measurement of p75NECD in blood, which will enable validation of these findings using banked samples previously collected from patients.

This study is not without its limitations. Most notably, the study population represents a sample of convenience, with the attendant risk that the cohort is biased towards patients with more slowly progressive and perhaps more advanced disease. Moreover, the limited number of samples and assessments available from each patient precluded reliable estimation of the individual rates of ALSFRS-R decline,23 thereby limiting our ability to explore whether baseline p75NECD is useful in predicting prognosis in terms of future rate of ALSFRS-R decline, in addition to survival. These shortcomings, however, will be addressed through an ongoing validation study being performed as part of the Clinical Research in ALS and Related Disorders for Therapeutic Development (CReATe) consortium. In this study (NCT02327845), urine samples are collected every 3–6 months (over an 18- to 24-month period), along with rigorously and prospectively collected deep clinical phenotypic data.

Urinary p75NECD currently stands alone as a biofluid-based biomarker that adds prognostic value to readily available clinical parameters, and changes longitudinally in individual patients with ALS as disease progresses. Urinary p75NECD and blood and CSF quantification of pNFH and NfL represent the most promising potential pharmacodynamic biomarkers available today that are suitable for further investigation in the context of future clinical trials.

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**DISCLOSURE**
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