Urinary p75^{ECD}

A prognostic, disease progression, and pharmacodynamic biomarker in ALS

OPEN

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ABSTRACT

Objective: To evaluate urinary neurotrophin receptor p75 extracellular domain (p75^{ECD}) 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 p75^{ECD} was measured using an enzyme-linked immunoassay 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 p75^{ECD} were examined by mixed model analysis, and the prognostic value of baseline p75^{ECD} was explored by survival analysis.

Results: Confirming our previous findings, p75^{ECD} 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 p75^{ECD} showing stability across repeated freeze-thaw cycles, at room temperature and 4°C for 2 days, and no diurnal variation. Urinary p75^{ECD} correlated with the revised ALS Functional Rating Scale at first evaluation ($r = -0.44$, $p = 0.008$) and across all study visits ($r = -0.36$, $p < 0.0001$). p75^{ECD} 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 p75^{ECD} (HR 1.3, $p = 0.0004$) were predictors of survival.

Conclusions: The assay for urinary p75^{ECD} is analytically robust and shows promise as an ALS biomarker with prognostic, disease progression, and potential pharmacodynamic application. Baseline urinary p75^{ECD} 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; **p75^{ECD}** = 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[1,2] and the absence of therapeutic options for this fatal disease[3] has fueled interest in the prospect that biomarkers may hold great promise for advancing therapy development efforts.[4,5] 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.

Supplemental data at Neurology.org

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Among the biological fluid–based biomarker candidates, the cytoskeletal proteins neurofilament light (NfL) and phosphorylated neurofilament heavy (pNfH) 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 ($p75^{ECD}$) is present at elevated levels in the urine of patients with ALS compared to healthy individuals[9] and that urinary p75 increases in the *SOD1*^{G93A} mouse as disease unfolds,[10] we investigated the potential of urinary $p75^{ECD}$ 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 Kessenich 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 criteria[11] 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 $p75^{ECD}$ 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/d or tracheostomy with initiation of invasive ventilation. Urine samples were collected and stored in accordance with the Urine & Kidney Proteome Project Standards[13] 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 $p75^{ECD}$ measurement. A sandwich ELISA was used to quantify $p75^{ECD}$ as previously described.[9] Briefly, ELISA plates (96-well, Costar Corning [Manassas, VA]) were coated with mouse anti-human p75 MLR1 antibody[14] 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 $p75^{ECD}$ standard (amino acids 29–250; R&D Systems, Minneapolis, MN) were diluted in sample buffer and incubated for 20 hours at room temperature. Goat anti-mouse $p75^{ECD}$ (Sigma-Aldrich, St. Louis, MO) and bovine anti-goat immunoglobulin G horseradish peroxidase

Table 1 Participant characteristics

	Patients with ALS		Controls
	All (n = 54)	Longitudinal subset (n = 31)	All (n = 45)
Age at diagnosis, y, mean ± SD (range)	63.6 ± 13.3 (39.2–86.3)	65.7 ± 12.3 (41.9–86.3)	—
Male, n (%)	28 (52)	16 (52)	22 (49)
Known to be familial, n (%)	12 (22)	5 (16)	—
Bulbar onset, n (%)	16 (30)	10 (32)	—
Months from onset to diagnosis, mean ± SD (range)	9.8 ± 7.3 (1.7–41.6)	8.8 ± 6.1 (1.8–34.1)	—
Months from diagnosis to first collection, mean ± SD (range)	6.6 ± 10.1 (0–57.4)[a]	4.1 ± 4.5 (0–19.8)[a]	—
Age at first collection, y, mean ± SD (range)	64.1 ± 13.2 (39.7–86.4)	66.0 ± 12.3 (42.0–86.4)	50.0 ± 13.0 (24–72)
ALSFRS-R at first collection, mean ± SD (range)	38.8 ± 5.7 (22–47)	40.6 ± 3.7 (31–46)	—
ΔFRS at first collection, mean ± SD (range)	0.8 ± 0.5 (0.01–2.7)	0.7 ± 0.4 (0.1–0.7)	—
Death or PAV by end of study, n (%)	44 (81)	26 (84)	—
Disease duration, mo,[b] median (IQR)	18.4 (11.1–32.4)	20.4 (13.9–35.6)	—
Longitudinal subset: no. of collection time points, median (range)	—	3 (2–6)	—

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).
[a] Four had first collection on day of diagnosis; their months from diagnosis to first collection = 0.
[b] Disease duration since diagnosis.

(Jackson ImmunoResearch, West Grove, PA) antibodies were used for $p75^{ECD}$ detection for 1 hour each at room temperature, and the peroxidase reaction was developed using TMB (Bio-Rad, Hercules, CA) and stopped using 2M sulfuric acid. Plates were washed between steps (PBS, 0.05% Tween 20, 0.01% thimerosal, pH 7.4), and were read using a PerkinElmer (Waltham, MA) Victor-x4 plate reader.

The detection limit and reproducibility of $p75^{ECD}$ measurement was determined by testing recombinant human $p75^{ECD}$ standard (R&D Systems) and 3 urine samples across 8 plates. The lowest detection limit was calculated by plotting the $p75^{ECD}$ standard curve with a $\log_{10}$ x-axis. Intra-assay and inter-assay reproducibility was determined by testing 3 urine samples with low, medium, and high $p75^{ECD}$/mL measurements in duplicate, across 8 plates, to determine the coefficient of variation (CV). In addition, the diurnal fluctuation of urinary $p75^{ECD}$ was determined in healthy individuals by comparing measurements made from a first void sample in the morning, a random spot urine in the afternoon/evening, and a sample collected over 24-hours. $p75^{ECD}$ stability was determined by repeatedly assaying samples stored at room temperature (~23°C) or 4°C for up to 7 days. In addition, samples were subjected to 1–4 freeze-thaw cycles switching between −80°C and room temperature (~23°C) for 15 minutes per cycle and $p75^{ECD}$ measured after each freeze/thaw.

Statistical analysis. Intra-assay and inter-assay reproducibility of ELISA measurements are expressed as CVs. Linear regression was used to describe the linearity of a standard curve for human $p75^{ECD}$. Urinary $p75^{ECD}$ levels were compared between patients with ALS and controls by 2-sample t test. The relationships among baseline age, urinary $p75^{ECD}$, creatinine, urinary osmolarity, and ALSFRS-R scores were assessed by Pearson correlation. The correlation between urinary $p75^{ECD}$ and ALSFRS-R were further assessed by including data from baseline as well as longitudinal follow-ups, with and without adjustment for repeated measures.[15] In the 31 patients with ALS who had longitudinal samples, the rate of urinary $p75^{ECD}$ increase over time—with time defined as months since diagnosis (primary analysis), symptom onset, or baseline (first urine collection)—was ascertained by mixed model analysis; quadratic and interaction terms were considered. In addition, the association between baseline urinary $p75^{ECD}$ levels and survival (time to death or PAV) was evaluated by Cox proportional hazards model, and graphically illustrated by dividing the patients into those with baseline $p75^{ECD}$ above vs below the median value and plotting their Kaplan-Meier survival curves. Summary statistics are presented as mean ± SD, median, range, or frequency and percentage. A p value of <0.05 (2-sided) was considered statistically significant. Longitudinal and survival analyses were performed using SAS 9.3 (SAS Institute, Cary, NC); all other analyses were performed, and figures generated, using GraphPad Prism 6 (GraphPad Software, La Jolla, CA).

RESULTS

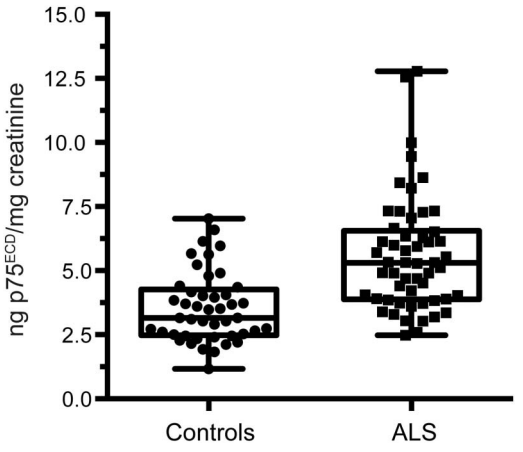
Study population. The study population includes 45 healthy controls and 54 patients with ALS (table 1). In the subset of 31 patients with ALS with longitudinal follow-up, 26 (84%) reached PAV or death. Median disease duration (from diagnosis) among all 54 patients was 18.4 months (25th–75th percentile: 11.1–32.4).

Urinary $p75^{ECD}$ in healthy controls and patients with ALS. Among healthy controls, urinary $p75^{ECD}$ 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 $p75^{ECD}$ 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 $p75^{ECD}$.

Confirming our previous findings,[9] urinary $p75^{ECD}$ 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 $p75^{ECD}$ levels do not differ significantly between patients with limb vs bulbar onset disease, even after controlling for baseline disease severity.

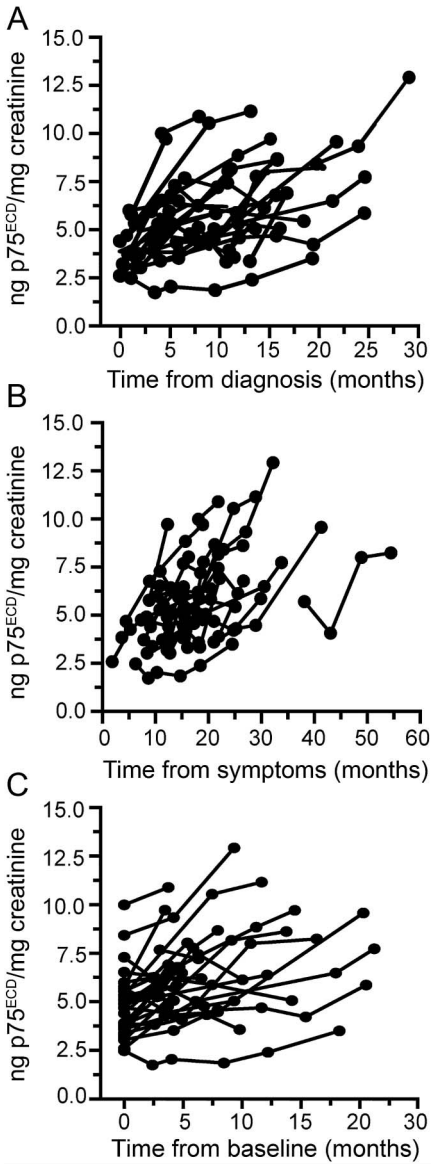
Analytic validation of urinary $p75^{ECD}$. Confirming our previous observation of the linearity of the standard curve (absorbance and human urinary $p75^{ECD}$ concentration) for our in-house ELISA,[9] here we show an assay sensitivity of ~70 pg/mL and a linear standard curve up to 2.5 ng/mL (figure e-1, A–C, at Neurology.org). Our assay for human urinary $p75^{ECD}$ demonstrates reliability with an intra-assay CV of 6.6% and an inter-assay CV of 12.5% (table e-1). Urinary $p75^{ECD}$ levels remained steady after multiple freeze-thaw cycles (figure e-1D). The level of $p75^{ECD}$ 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 $p75^{ECD}$ 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

Figure 1 Urinary extracellular domain of p75 ($p75^{ECD}$) is elevated in amyotrophic lateral sclerosis (ALS)



Urinary $p75^{ECD}$ levels are higher in patients with ALS (n = 54) than in controls (n = 45) at first study visit ($p < 0.0001$, 2-sample t test).

Figure 2 Longitudinal changes in urinary extracellular domain of p75 ($p75^{ECD}$) in amyotrophic lateral sclerosis (ALS) as disease progresses



(A) Individual patient urinary $p75^{ECD}$ trajectories increase since time of diagnosis at an average rate of 0.19 ng/mg creatinine per month (95% confidence interval [CI] 0.15–0.24). (B) $p75^{ECD}$ trajectories also increase since time from symptom onset (0.18 ng/mg, 95% CI 0.13–0.22), and (C) time since the initial evaluation and sample collection (i.e., baseline) (0.20 ng/mg, 95% CI 0.15–0.25). Total 31 patients with ALS, each sampled at least twice.

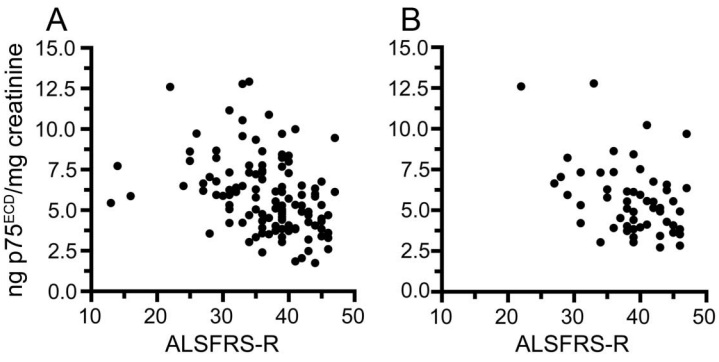
between the spot urine and the first void in addition to the spot and the 24-hour samples was modest (below 17%). When urinary dilution was considered by correcting with creatinine (as we routinely do for reporting results), the difference between spot and first void and spot and 24-hour sample decreased 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 $p75^{ECD}$ as biomarker of disease progression. The potential utility of $p75^{ECD}$ 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 $p75^{ECD}$ 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 $p75^{ECD}$ increases linearly and on average by 0.19 ng/mg creatinine each month ($p < 0.0001$; figure 2A). Adjusting for baseline $p75^{ECD}$ does not significantly affect the slope, suggesting a fairly uniform rate of $p75^{ECD}$ 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 $p75^{ECD}$, 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.[16] Similar to $p75^{ECD}$, 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 $p75^{ECD}$ ($r = -0.36$, $p < 0.0001$), 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 $p75^{ECD}$ at baseline for each of the 54 patients (figure 3B; $r = -0.44$, $p = 0.0008$).

Urinary $p75^{ECD}$ as prognostic biomarker. To explore the potential utility of baseline $p75^{ECD}$ as a predictor of prognosis, we performed a survival analysis using Cox

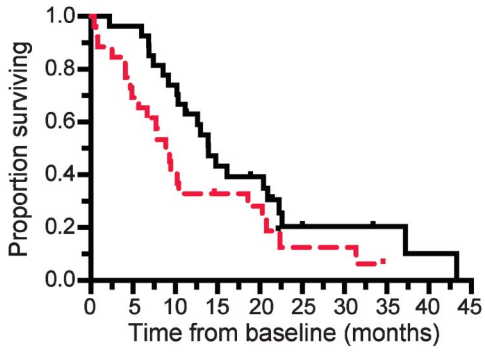
Figure 3 Correlation between urinary extracellular domain of p75 ($p75^{ECD}$) and revised ALS Functional Rating Scale (ALSFRS-R)



(A) Using $p75^{ECD}$ measures from all 115 person-visits, urinary $p75^{ECD}$ correlates with ALSFRS-R ($r = -0.36$, $p < 0.0001$). After adjusting for the within-person correlation that arises from repeated measures of the longitudinal subset, the correlation remains essentially unchanged ($r = -0.37$). (B) Urinary $p75^{ECD}$ also correlates with ALSFRS-R at first study visit for each of the 54 patients with amyotrophic lateral sclerosis in the study ($r = -0.44$, $p = 0.008$).

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 ΔFRS ($p < 0.0001$) 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 ΔFRS (HR 4.4, 95% CI 2.4–8.0, $p < 0.0001$), baseline $p75^{ECD}$ was also a predictor of survival (HR 1.3, 95% CI 1.1–1.5, $p = 0.0004$), indicating that baseline $p75^{ECD}$ provides additional prognostic value over and above what can be gleaned from routinely available clinical parameters. To visually illustrate the relationship between baseline $p75^{ECD}$ and survival, we divided the 54 patients into high vs low $p75^{ECD}$ groups (i.e., based on their baseline value being above or below the median), and present their Kaplan-Meier survival plot (figure 4).

Figure 4 Urinary extracellular domain of p75 ($p75^{ECD}$) at baseline predicts future survival



Kaplan-Meier survival estimates comparing 27 patients with amyotrophic lateral sclerosis who had baseline urinary $p75^{ECD}$ above (red dashed line) and 27 with values below (black line) the median (5.3 ng/mg creatinine), illustrates the longer median survival in those with lower compared to higher urinary $p75^{ECD}$ levels (13.9 vs 8.9 months; Wilcoxon test $p = 0.024$).

DISCUSSION Our pursuit of urinary $p75^{ECD}$ as a potential ALS biomarker was informed by the biology of the neurotrophin receptor p75. Rodent motor neurons express p75 during development[18] but this disappears soon after birth only to be re-expressed after injury,[19,20] including apoptotic motor neurons of *SOD1*^{G93A} mice.[21] $p75^{ECD}$ is cleaved from cell membranes post injury,[19] with elevated levels detected 40 days prior to onset of disease in the urine of *SOD1*^{G93A} mice, progressively rising as symptoms unfold.[9,10] p75 is also re-expressed on motor neurons[22] and Schwann cells[23] in postmortem tissue of patients with ALS. Hence, the presence of $p75^{ECD}$ 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 $p75^{ECD}$ 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 $p75^{ECD}$ 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 $p75^{ECD}$ remain relatively low (figure 2). In this respect, $p75^{ECD}$ is currently the only potential biochemical biomarker of ALS disease progression. In addition, $p75^{ECD}$ may have utility as a potential pharmacodynamic biomarker insofar as showing that an experimental therapeutic that blunts the increase, stabilizes, or reduces urinary $p75^{ECD}$ over time would provide evidence of an underlying biological effect of the treatment.[25] Urinary $p75^{ECD}$ therefore joins CSF and blood NfL[6,26–32] and CSF pNfH[7,33–35] 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 $p75^{ECD}$ may have value as a prognostic biomarker. Essential to this claim is the observation that baseline $p75^{ECD}$ 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 $p75^{ECD}$ is modest. While other biochemical biomarkers including pNfH[8,34,36] and NfL[6,31] 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, $p75^{ECD}$ 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 $p75^{ECD}$ 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 $p75^{ECD}$. Notwithstanding these considerations, we are also exploring measurement of $p75^{ECD}$ 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,[25] thereby limiting our ability to explore whether baseline $p75^{ECD}$ 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 $p75^{ECD}$ 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 $p75^{ECD}$ 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.

AUTHOR CONTRIBUTIONS

S.R. Shepheard: experiments, manuscript preparation. J. Wuu: supervision of participant recruitment and sample collection (Miami site), statistical analysis, results interpretation, manuscript preparation. M. Cardoso: experiments. L. Wilkendt: data analysis. P. Dinning: consultant on data analysis. T. Chataway: design of experiments. D. Schultz: participant recruitment and sample collection (Flinders site). M. Benatar: supervision of participant recruitment and sample collection (Miami site), hypothesis generation, results interpretation, manuscript preparation. M.-L. Rogers: design of experiments, data analysis, manuscript preparation.

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DISCLOSURE

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