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Urinary P75: a promising biomarker for amyotrophic lateral sclerosis

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ABSTRACT

Background Amyotrophic lateral sclerosis (ALS) is a progressive and fatal disease. The urinary neurotrophin receptor p75 extracellular domain ($p75^{ECD}$) has previously been reported as a potential disease biomarker for diagnosis, severity assessment and monitoring therapeutic response.

Methods This study measured urinary $p75^{ECD}$ using an enzyme-linked immunoassay and normalised the results against urinary creatinine. Participants were recruited via A Multicentre Biomarker Resource Strategy in ALS (AMBroSIA) programme. Study participants included 97 ALS patients, 24 of whom were studied longitudinally, and 27 healthy controls. The study focused on urinary $p75^{ECD}$ and its potential association with different subtypes of ALS, change over time, disease progression, severity of symptoms and survival from symptom onset.

Results Confirming previous findings, urinary $p75^{ECD}$ levels were significantly higher in patients with ALS (median 6.78 ng/mg, 95% CI (5.12 to 9.23)) compared with controls (4.57 ng/mg, 95% CI (3.35 to 5.89)) at first study visit. There was a significant negative correlation between absolute change in the Revised ALS Functional Rating Scale score and $p75^{ECD}$ levels (Spearman's rho=−0.371, $p \leq 0.0004$, 95% CI (−0.543 to −0.169)), indicating that an increase in the severity of motor neuron injury correlated with an increase in $p75^{ECD}$ levels. There was a significant increase in $p75^{ECD}$ between first and second samples in the same participants, indicating an increase in the level of this biomarker longitudinally during the disease course (moderate effect size of −0.3).

Conclusions Urinary $p75^{ECD}$ is a promising candidate as a biomarker, which increases with disease progression and has the potential to serve as a pharmacodynamic biomarker.

WHAT IS ALREADY KNOWN ON THIS TOPIC?

⇒ There is no specific test for diagnosing amyotrophic lateral sclerosis (ALS), and the delay between symptom onset and diagnosis averages 12–18 months, limiting timely intervention and access to clinical trials. The urinary neurotrophic receptor p75 extracellular domain ($p75^{ECD}$) has emerged as a promising biomarker, particularly due to its role in neuronal injury, and it has been shown to be upregulated in ALS patients in some small-scale studies.

WHAT THIS STUDY ADDS?

⇒ Previous studies have been limited by small cohort sizes and inconsistent findings, necessitating further validation in larger, well-defined patient populations.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY?

⇒ This study validates the use of urinary $p75^{ECD}$ as a biomarker for ALS in a larger UK-based cohort. It allows for more timely diagnosis, earlier interventions and enhanced patient stratification for trials with a potential for accelerating the development of effective treatments.

BACKGROUND

Amyotrophic lateral sclerosis (ALS), also known as motor neuron disease (MND), is a progressive and fatal disease, causing progressive failure of the neuromuscular system, limb muscle weakness, wasting and compromise of the bulbar and respiratory muscles. It is currently incurable, with an average life expectancy of 2–4 years from onset.[1] There is no specific test to diagnose ALS, with a delay of up to 15 months between symptom onset and diagnosis.[2]

Biomarkers for ALS have been researched thoroughly, as they represent an objective characteristic for diagnosis, monitoring disease severity, progression and therapeutic response.[2 3] The lack of a specific biomarker impedes patients and hinders drug trials by failing to detect therapeutic responsiveness quickly. A urinary sample would represent a non-invasive approach for biomarker detection. Previous reports of urinary biomarkers for ALS have shown inconsistent results in small cross-sectional studies.[4–6] In comparison to blood, certain substances in urine can be detected earlier and more sensitively.[7] A previous publication reported that urinary neurotrophic receptor p75 extracellular domain (ECD) ($p75^{ECD}$) increased as the disease progressed (2.3 ng/mg creatinine/year). This provides a prognostic advantage over clinical parameters of change in the Revised ALS Functional Rating

Scale (ALSFRS-R) alone.[8] The present report builds on previous studies by analysing a larger cohort (n=97), with a specific focus on a UK-based cohort, which has not been studied before, to explore the potential use of $p75^{ECD}$ as a biomarker. Identifying predictive biomarkers, such as $p75^{ECD}$, could revolutionise how we identify ALS patients most likely to benefit from experimental therapies.

p75 is a common neurotrophic receptor. It is highly expressed by motor neurons during embryonic development, then down-regulated postnatally and re-expressed during injury.[9] Upregulation of p75 during neuronal injury leads to growth cone collapse and axon growth inhibition and mediates apoptosis of injured neurons.[10] In ALS, it is upregulated, with the ECD cleaved from injured neurons and glia, and expressed by motor neurons in postmortem Central Nervous System (CNS) tissue.[9] The ECD of p75 is cleaved after binding of a pro-apoptotic ligand and can be detected in human urine under different conditions. Several studies have shown that it is shed and excreted in urine after neuronal injury.[9 11]

A recent meta-analysis highlighted that $p75^{ECD}$ levels were significantly higher in patients with ALS than in control subjects, and the ALSFRS-R was inversely associated, highlighting it as a useful biomarker for disease progression and diagnosis.[7] This project aims to validate this finding and assess p75 as a urinary biomarker for ALS, using a larger patient cohort of ALS patients, assessing $p75^{ECD}$ at diagnosis and with survival data of up to 4 years postdiagnosis.

METHODS

Patient recruitment and sampling

Participants were recruited via A Multicentre Biomarker Resource Strategy in ALS (AMBroSIA) programme, funded by the Motor Neurone Disease Association.[12] Urine, blood, skin biopsy and cerebrospinal fluid samples were obtained from consented participants. The samples were collected in Sheffield Teaching Hospitals National Health Service (NHS) Foundation Trust at the point of diagnosis and anonymised. The participants were diagnosed with ALS by experienced ALS specialist neurologists after appropriate neurological investigations and classified using the standardised El Escorial Criteria.[13] Exclusion criteria included age <18 years, pregnancy, significant bleeding diathesis, sepsis and comorbidity that would have interfered with the interpretation of the results. Healthy controls were also recruited via AMBroSIA, often spouses, relatives or friends of the ALS participants. All participants provided written informed consent. Research nurses recruiting participants were unaware of the specific study or diagnosis. The samples used in this study were collected as part of the MND Association-supported AMBRoSIA biosampling project. Patient and Public Involvement and Engagement (PPIE) advice about this programme was sought from the Sheffield MND Research Advisory panel, and helpful feedback was obtained.

Urine samples were collected and stored in accordance with the Human Tissue Act (2004). A urine pot was provided to the participants to fill with a midstream urine sample. This was then used to fill 5× yellow capped 0.7 mL FluidX tubes and stored in vapour phase nitrogen. Samples were stored at the University of Sheffield Medical School Biorepository, a Human Tissue Authority (HTA)-licensed facility.[14 15] Samples were subsequently transported to the collaborating laboratory at Flinders University frozen on dry ice and then thawed and centrifuged for testing.

Urinary $p75^{ECD}$ measurement

Urinary $p75^{ECD}$ was quantified using an automated sandwich ELISA consisting of a Hamilton Starlet, integrated with a Molecular Devices (MD) reader and Biotek 405 washer, as previously described.[16 17] Briefly, monoclonal MLR1 was used as capture (8.0 µg/mL), and biotinylating (Thermo Fisher Scientific Australia, #A39257), Nerve Growth Factor Receptor (NGFR) (2.0 µg/mL) was used as detection. The enzyme reaction was using streptavidin horseradish peroxidase (Jackson ImmunoResearch Laboratories, #JIO16030084) diluted to 1.0 µg/mL and colour developed using 3,3′,5,5′-Tetramethylbenzidine (TMB) (A:B; BioRad Australia #1721067). Researchers conducting the assays were blinded to sample type. Creatinine $p75^{ECD}$ measurements were corrected for urine dilution using urinary creatinine measured using Enzo Life Sciences Creatinine kits (ADI-907–030A) as per the manufacturer's instructions and expressed as ng $p75^{ECD}$/mg creatinine.[16 17] Osmo1 Single-Sample Micro-Osmometer (Advanced Instruments) was used to measure urinary osmolality as per the manufacturer's instructions. Supercooling and freeze-induction of 1:3 diluted urine samples were performed in triplicate, providing an osmolality output in mOsm/kg H_2O.

Statistical analysis

Urinary $p75^{ECD}$ levels were compared between patients with ALS and controls by a Mann-Whitney U test. The relationships between progression ratio, $p75^{ECD}$, ALSFRS-R and onset until death (months) were assessed using Spearman correlation analysis. A Wilcoxon signed-rank test was used to compare first and second visit ALS participants. Cox regression analysis was used to assess multivariate survival and graphically illustrate on a Kaplan-Meier survival curve, dividing the ALS participants into those above and below the median $p75^{ECD}$ level at baseline. A p value of <0.05 was considered significant. Analyses and graphs were performed initially using SPSS Statistics (IBM 28.0.1.0 (142)), then reanalysed using GraphPad Prism (V.10.5.0 (673)).

The data were compared as values of ng $p75^{ECD}$/mL and normalised to creatinine (ng $p75^{ECD}$/mg). The results were normalised to creatinine to allow for urinary dilution, as is routinely done. Creatinine was a reliable measure of urinary dilution, based on the strong correlation with urine osmolarity (Spearman's rho=−0.783,

Table 1 Participant characteristics

Averages	ALS (n=97)	2nd visit (n=24)	Healthy controls (n=27)
Age at collection (±SD)	62.58 (±12.28)	60.04 (±8.22)	56 (±14.60)
Male, n (%)	59 (60.8%)	13 (54%)	11 (41%)
Familial, n (%)	5 (5.15%)	3 (12.5%)	
ALSFRS-R (±SD)	36.48 (±7.58)	37.95 (±7.56)	
Urine creatinine	1.00 (±0.52)	0.95 (±0.41)	0.94 (±0.48)
Osmolality (mOsm/kg H_2O)	521.76 (±195.62)	553.54 (±221.06)	471.72 (±177.66)
Predicted disease duration at the time of recruitment (months)	21.9 (±19.99)		
Death by end of the study (%)	62 (63.91%)	9 (37.5%)	0 (0%)

ALS, amyotrophic lateral sclerosis; ALSFRS-R, Revised ALS Functional Rating Scale.

p≤0.001, 95% CI 0.701 to 0.845). The normal range in healthy subjects for creatinine is 0.3–3.0 mg/mL±0.3, and osmolality is 200–1000 mOsm/kg H_2O.[18] Samples with urinary creatinine below 0.3±0.03 mg/mL or above 3.0±0.3 mg/mL were rejected, as per the WHO guidelines. Urine samples with extremely low creatinine concentrations are too dilute and may distort detection of low levels of analyte measurement, while extremely high creatinine concentrations indicate dehydration, which could have changed the kidney's processing of the analyte.[18] Therefore, these exclusion criteria meant that four participants were excluded for high creatinine levels and 14 for low.

RESULTS

Study population

The study population includes 97 ALS patients, 24 of whom had two visits, and 27 healthy controls (table 1). 62 of these ALS participants had died by the end of the study (63.91%).

Urinary $p75^{ECD}$ is significantly elevated in ALS versus healthy controls

There was no correlation between $p75^{ECD}$ concentration and age in the ALS population (Spearman's rho=0.165, p=0.107, 95% CI (−0.0417 to 0.358) normalised to creatinine and (Spearman's rho=−0.032, p=0.757, 95% CI (−0.238 to 0.176)) not normalised, and controls (Spearman's rho=0.125, p=0.526, 95% CI (−0.2708 to 0.4849)) normalised to creatinine and (Spearman's rho=0.0816, p=0.680, 95% CI (−0.311 to 0.451)) not normalised (online supplemental figure 1). There was also no significant correlation between $p75^{ECD}$ when normalised against osmolality and age (Spearman's rho=−0.202, p=0.048, 95% CI (−0.004 to 0.392)).

Urinary $p75^{ECD}$ was significantly higher in males (n=71, mean 8584.92, median 7049.74 (3906.25–9890.18) pg $p75^{ECD}$/ml) than in females (n=55, mean 6428.81, median 3840.82, (2274.36–7737.46) pg $p75^{ECD}$/ml) (p=0.002, Mann-Whitney U 1328, Z score −3.072). When compared against creatinine in males (n=71, mean 7.98, median 6.47 (5.10–9.15) ng/$p75^{ECD}$/ml) and against females (n=55, mean 6.87, median 5.69 (3.98–7.83) ng $p75^{ECD}$/ml), this significance was not found (p=0.074, Mann-Whitney U 1589.50, Z score −1.79). When normalised to osmolality, $p75^{ECD}$ was found to be significantly higher in males (mean 15.49, median 12.32 (8.83–15.95) mg $p75^{ECD}$/mOsm/kg H_2O) than in females (mean 12.68, median 9.64 (6.64–14.08) mg $p75^{ECD}$/mOsm/kg H_2O) (p=0.044, Mann-Whitney U 2250).

In confirmation of previous findings,[8 9 16] urinary $p75^{ECD}$ levels were significantly higher in patients with ALS (mean 7740.26, median 6780.05 (5124.45–9234.43) pg $p75^{ECD}$/ml) than in controls (mean 4594.06, median 3617.94 (2218.54–7251.13) pg $p75^{ECD}$/ml) at the first study visit (p=0.0045, Mann-Whitney U 883). This significance was also found when using the data normalised to creatinine levels in ALS (mean 7.5, median 6.78 (5.12–9.23) ng $p75^{ECD}$/ml) and controls (mean 4.62, median 4.57 (3.35–5.89) ng $p75^{ECD}$/ml) at the first study visit (p≤0.0001, Mann-Whitney U 596.5) (figure 1). There was also a significant difference in $p75^{ECD}$ when normalised against osmolality values in ALS (mean 14.41 median 12.74, (12.72–12.72) mg $p75^{ECD}$/mOsm/kg H_2O) in comparison to healthy controls (mean 9.00, median 8.53, (6.16–12.50) mg $p75^{ECD}$/mOsm/kg H_2O) (p≤0.001, Mann-Whitney U 751.00).

Urinary p75 was significantly correlated with the severity of symptoms

The ALSFRS-R was measured at visit one and subsequent visits. This is a validated rating instrument for monitoring the progression of disability in patients with ALS.[19 20] A Spearman's correlation coefficient showed a weak but significant negative correlation with urinary $p75^{ECD}$ levels (Spearman's rho=−0.371, p≤0.0004, 95% CI (−0.543 to −0.169)) (figure 2), implying that a decrease in ALSFRS-R score is correlated with an increasing pg $p75^{ECD}$ per ml.

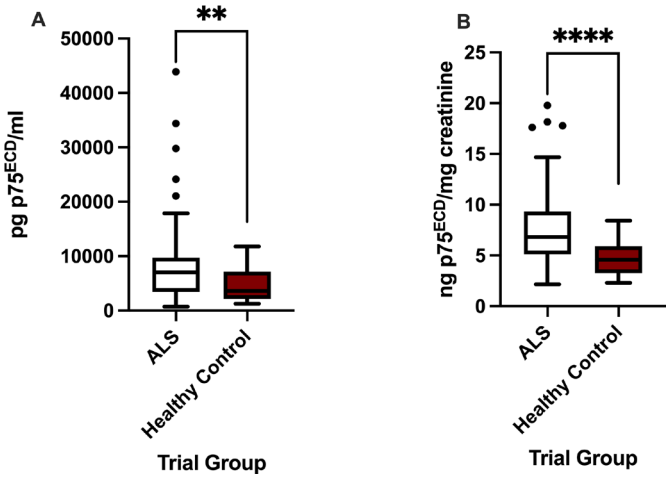

Figure 1 p75^{ECD} in ALS versus controls. Box plot highlighting the amount of p75^{ECD} in ALS participants versus controls, with error bars, highlighting outliers. Circles representing outliers more than 1.5x the IQR away from the quartiles. (A) pg p75^{ECD}/ml of urine and (B) ng p75^{ECD}/mg normalised to creatinine in urine. The urinary p75^{ECD} levels were significantly higher in patients with ALS than in controls at the first study visit (p=0.0045, Mann-Whitney U 883) and when normalised to creatinine levels in ALS and controls (p≤0.0001, Mann-Whitney U 596.5).

Significance highlighted by **=0.01 (highly significant), ****≤0.0001 (extremely significant).
ALS, amyotrophic lateral sclerosis; ECD, extracellular domain.

The lower the ALSFRS-R, the greater the severity of ALS symptoms. This was also seen when the values were normalised against creatinine (Spearman's rho=−0.625, p≤0.0001, 95% CI (−0.741 to −0.473)). This significance was also found when normalising against osmolality (Spearman's rho=−0.380, p=0.001, 95% CI (−0.552 to −0.177)). Therefore, this strong negative correlation indicates that when symptom severity increases, so does the urinary p75^{ECD} concentration.

A significant correlation between disease progression and p75^{ECD} levels

The potential of p75^{ECD} as a biomarker of disease progression was assessed, looking at the progression rate (change in validated ALSFRS-R score per month) with longitudinal data of up to 13 months (ranged from 2 to 13 months) difference in time from first to second consent. The Spearman's correlation coefficient was calculated (r=0.269, p=0.0114, 95% CI (0.0564 to 0.458)) (figure 3), showing a weak but significant correlation between progression rate and pg p75^{ECD} per ml of urine. When normalised against creatinine, this showed a stronger significant correlation (r=0.383, p=0.0002, 95% CI (0.182 to 0.554)). This significance was also found when the results were normalised against osmolality (Spearman's rho=0.295, p=0.006, 95% CI (0.082 to 0.482)).

There was no significant difference in p75^{ECD} levels between subtypes of ALS

A Kruskal-Wallis multivariant analysis showed that there was no significant difference between p75^{ECD} normalised to creatinine in any subset of ALS (ALS (n=80), primary lateral sclerosis (n=6), progressive muscular atrophy (n=10), progressive bulbar palsy (n=1)) (not normalised p=0.926, normalised to creatinine p=0.546 and normalised to osmolality p=0.512) (figure 4).

A further multivariate analysis of variance (ANOVA) was conducted to assess whether a different location of onset, including limb, bulbar, cognitive, trunk or respiratory, had an impact on p75^{ECD} levels. There was no

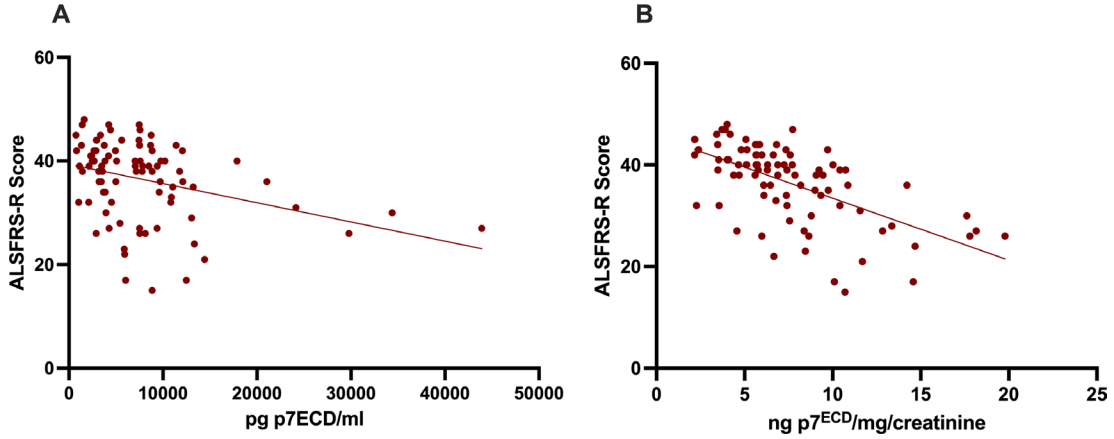

Figure 2 Severity of symptoms. The correlation between p75^{ECD}/ml of urine and the Revised ALS Functional Rating Scale (ALSFRS-R). An increase in ALSFRS-R highlights a decrease in severity of symptoms. It focuses on physical symptoms, such as one's ability to swallow, climb stairs, breathe and use of utensils. Each function is rated from 0 (no ability) to 4 (normal for the participant). (A) pg p75^{ECD}/ml or urine (r=−0.371, p≤0.0004, 95% CI (−0.543 to −0.169), equation of the line Y=−0.000371*X+39.36 and R^2=0.119). (B) ng p75^{ECD}/mg normalised to creatinine. (r=−0.625, p≤0.0001, 95% CI (−0.741 to −0.473), equation of the line Y=1.221*X+45.67 and R^2=0.365).

ALS, amyotrophic lateral sclerosis; ECD, extracellular domain.

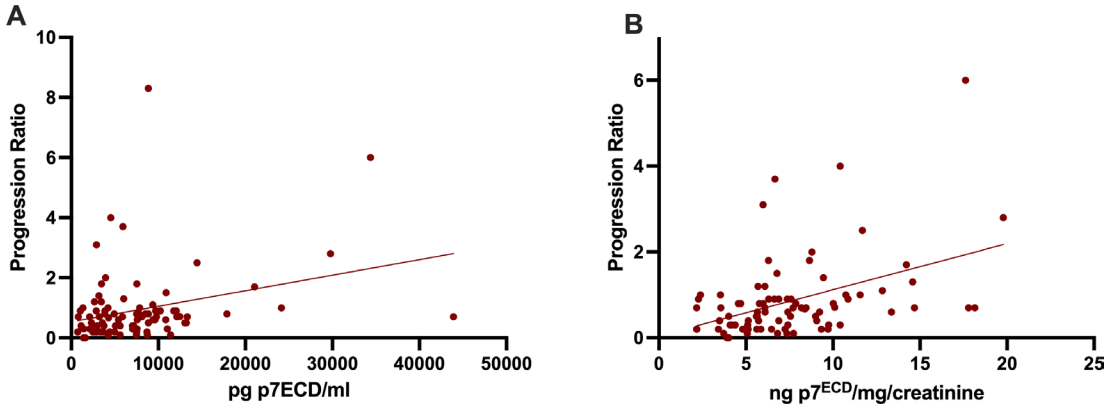

Figure 3 Disease progression. The correlation between progression ratios (change in validated ALSFRS-R score per month) in the ALS participant group in relation to urinary $p75^{ECD}$. (A) pg $p75^{ECD}$/ml of urine, (r=0.269, p=0.0114, 95% CI (0.0564, 0.458), equation of the line Y=5.170e-005*X+0.5351 and R^2=0.0885). (B) ng $p75^{ECD}$/mg normalised to creatinine in urine, (r=0.383, p=0.0002, 95% CI (0.182, 0.554), equation of the line Y=0.1074*X+0.04647, R^2=0.185).

ALS, amyotrophic lateral sclerosis; ALSFRS-R, Revised ALS Functional Rating Scale; ECD, extracellular domain.

significant difference in any group (not normalised p=0.993, normalised to creatinine p=0.198 and normalised to osmolality 0.227).

Longitudinal changes in $p75^{ECD}$ concentration

To assess longitudinal changes in $p75^{ECD}$ per ml of urine, we conducted a Wilcoxon signed rank test. There was no significant difference in ALS participants in the first visit (median 5602.82, IQR 2849.60–8555.41) and second visit (median 5769.59, IQR 3750.25–9342.23, p=0.218). The time between the first and second visits was up to 13 months (ranging from 2 to 13 months). There was a significant and moderate difference between the first (median 5.63, IQR 4.52–6.09 ng $p75^{ECD}$/ml) and second visit (median 7.18, IQR 5.37–8.70 ng $p75^{ECD}$/ml) when $p75^{ECD}$ values in ALS participants were normalised against creatinine (moderate effect size −0.3, CI (−2.88 to −0.56), p=0.011). There was no significant difference between the first visit (median 9.94, IQR 7.36–12.39 mg $p75^{ECD}$/mOsm/kg H_2O) and second visit (median 10.90, IQR 9.66–13.69 mg $p75^{ECD}$/mOsm/kg H_2O) when normalised against osmolality levels (p=0.332).

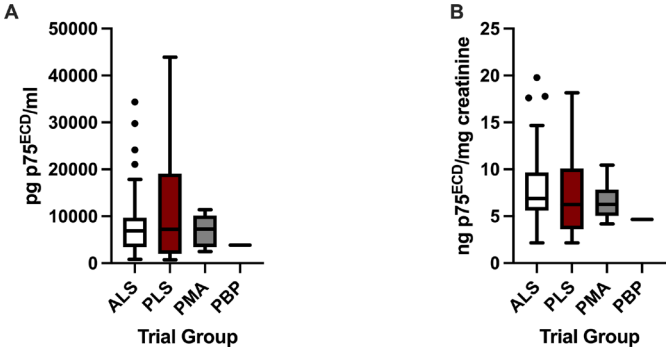

Figure 4 Subtypes of MND. A box plot highlighting the amount of $p75^{ECD}$ in each group, with error bars highlighting the outliers. Circles representing outliers more than 1.5× the IQR away from the quartiles. The groups are amyotrophic lateral sclerosis (ALS), primary lateral sclerosis (PLS), progressive muscular atrophy (PMA) and progressive bulbar palsy (PBP). (A) pg $p75^{ECD}$/ml or urine and (B) ng $p75^{ECD}$/mg normalised to creatinine in urine. There was no significant difference between $p75^{ECD}$ normalised to creatinine in any subset of ALS (not normalised p=0.926, normalised to creatinine p=0.546).

ECD, extracellular domain; MND, motor neuron disease.

Urinary p75 level at baseline does not correlate with survival time from symptom onset

A biomarker can aid with prognostication, and therefore we assessed the onset until death in months with the pg $p75^{ECD}$/ml of urine (figure 5). The Spearman's correlation coefficient showed next to no inverse correlation (Spearman's rho=−0.0459, p=0.723, 95% CI (−0.299 to 0.214)) and when normalised to creatinine, showed a weak but non-significant inverse correlation with onset until death in months (Spearman's rho=−0.202, p=0.115, 95% CI (−0436 to 0.0576)). When normalised against osmolality, there was no significant correlation between death in months and $p75^{ECD}$ (Spearman's rho=−0.138, p=0.294, 95% CI (−0.385 to 0.128)). In a multivariate survival analysis, using Cox regression analysis, the progression ratio (HR) 2.684, 95% CI (1.805 to 3.993), p≤0.001) was significant at increasing hazard of a terminal event. Age (HR 0.97, 95% CI (0.95 to 1.00), p=0.06), ALSFRS-R score (HR 1.05, 95% CI (1.00 to 1.11), p=0.065), ng $p75^{ECD}$/ml (HR 1.05, CI (0.94 to 1.20), p=0.356) and pg $p75^{ECD}$/ml (HR 1.00, CI (1.00 to 1.00), p=0.951) were not significant and showed no increase in risk of a terminal event. When normalised to osmolality, a Cox regression survival analysis also showed that the progression ratio (HR 2.591, 95% CI (1.744 to 3.850), p≤0.001) was significant in increasing the hazard of a terminal event, but age (HR 0.976, 95% CI (0.949 to 1.004), p=0.092), ALSFRS-R score (HR 1.040, 95% CI (0.988 to 1.094), p=0.134) and mg $p75^{ECD}$/mOsm/kg H_2O (HR 1.014, 95% CI (0.985 to

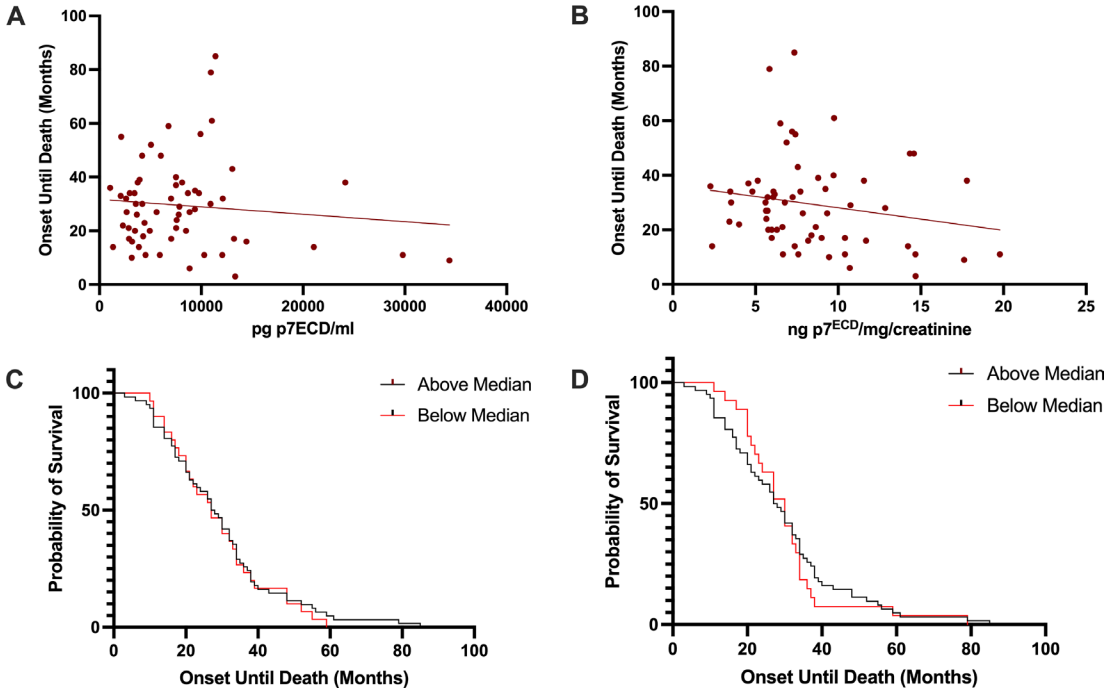

Figure 5 Survival. The correlation between onset until death in months in ALS participants and urinary $p75^{ECD}$. (A) pg $p75^{ECD}$/ml of urine (r=−0.0459, p=0.723, 95% CI (−0.299, 0.214)), equation of the line Y=−0.0002762*X+31.70 and R^2=0.011). (B) ng $p75^{ECD}$/ml of urine, normalised to creatinine, (r=−0.202, p=0.115, 95% CI (−0436, 0.0576), equation of the line Y=−0.8292*X+36.36, R^2=0.0356). (C) Kaplan-Meier survival curve to allow visualisation of survival, using the median value of 6772.54 mg p75ECD/ml urine, with n=49 below median (red) and n=48 above median (black) (Gehan-Breslow-Wilcoxon test χ^2 0.000155, p=0.990). (D) Kaplan-Meier survival curve to allow visualisation of survival, using the median value of 6.82 ng $p75^{ECD}$/ml normalised to creatinine with n=49 below median (red) and n=48 above median (black) (Gehan-Breslow-Wilcoxon test χ^2 0.132, p=0.716).

ALS, amyotrophic lateral sclerosis; ECD, extracellular domain.

1.044), p=0.348) were not significant. When normalised against creatinine, the same was found; the progression ratio (HR 2.624, 95% CI (1.761 to 3.909), p≤0.001) had a significant effect on the terminal event, whereas age (HR 0.973, 95% CI (0.946 to 1.001), p=0.057), ALSFRS-R score (HR 1.051, 95% CI (0.996 to 1.108), p=0.071) and ng $p75^{ECD}$/mg creatinine (HR 1.077, 95% CI (0.969 to 1.197), p=0.168) were not significant in affecting survival (figure 5).

DISCUSSION

This study builds on Shepheard *et al*'s work using $p75^{ECD}$ as a biomarker for ALS.[8 9 16] Previous rodent studies showed that p75 is expressed by motor neurons during development, disappears afterbirth, and is re-expressed during neuronal injury.[8 21–23] ALS involves degeneration of the motor neurons, and p75 is expressed by motor neurons during this process and has been highlighted in the CNS tissue of ALS postmortem cases.[9 24 25] Therefore, it represents a potentially useful biomarker. Detecting $p75^{ECD}$ in urine offers a rapid, accessible biomarker for ALS. Unlike invasive CSF collection, urine sampling is more acceptable for trial participants, and the simpler urinary proteome makes it easier to assay proteins.[9 26]

Key findings show that $p75^{ECD}$ levels are significantly higher in ALS patients compared with controls (figure 1), with a significant negative correlation between ALSFRS-R scores and $p75^{ECD}$ levels (figure 4). This implies that as the severity of the disease increases, the level of $p75^{ECD}$ also increases. We found that it does not correlate with age; however, this may support its utility as a biomarker independent of demographic variation. This aligns with Shepheard *et al*'s findings in an Australian ALS cohort and adds value to previous research, including a Chinese ALS study.[16 27] In the Chinese cohort, $p75^{ECD}$ levels were positively correlated with disease stage (p=0.0309), the ALSFRS-R score was negatively correlated (p=0.022), and urine concentration of $p75^{ECD}$ in a fast-progressing ALS group was significantly higher than in a slow-progressing group (p=0.0026). This report confirms these findings in a larger UK cohort with an up to 4-year follow-up. Variations in baseline $p75^{ECD}$ levels in Chinese ALS patients when compared with Australian/US patients (11.36±5.83 vs 5.6±2.2) highlight the need for larger, multinational studies.[8 27 28] Additionally, a recent meta-analysis (n=251) found that $p75^{ECD}$ levels were inversely associated with ALSFRS-R in ALS patients (r=−0.32, 95% CI (−0.43 to −0.21), p<0.001).[7] These reports imply that $p75^{ECD}$ may represent a useful biomarker of disease progression,

with the potential to transform how we identify ALS patients more likely to benefit from specific experimental therapies.

The present study adds value to a previous report, which also described urinary $p75^{ECD}$ increasing as the disease progressed.[8 16] A limitation of this previous report was that the sample was a sample of convenience, allowing for bias. The sample included participants diagnosed at the South Australian MND Clinic (Adelaide, Australia) and the Kessenich Family ALS Centre at the University of Miami (Miami, Florida). A single assessment was taken from each patient. Due to the delay in diagnosis in most ALS patients, there is a risk that this cohort is biased towards patients with more advanced and slowly progressive disease. Healthy controls were typically patients' spouses and friends, like the AMBroSIA cohort; however, this may have a benefit due to a reduction in any genetic differences in creatinine levels or urinary biomarkers.[28–30] Despite this, convenience sampling is useful in allowing hypotheses to be formed. This research allowed such, highlighting that urinary $p75^{ECD}$ increased as the disease progressed. The samples used in the present study were recruited at the time of diagnosis and followed up for up to 11 months afterwards. This allowed the inclusion of a range of slow and fast progression of ALS participants. These results indicate, with significance, that the faster the progression of the disease, the higher the $p75^{ECD}$ level. This adds value to the report of Shepheard *et al*[8] by highlighting that in a larger sample size (n=97), a different location and different cohort of ALS patients, a similar result was found, linking urinary $p75^{ECD}$ to ALS disease progression.[8]

Regarding survival, the patients were followed up for up to 4 years beyond diagnosis, which is longer than previously. Unfortunately, this study only has a small number (n=24), who were followed up over this time period. This is due to the rarity and rapidly progressive nature of this disease from onset until death, with the average survival from onset being 2–4 years.[1] The use of AMBroSIA allowed for a large multicentre recruitment, improving generalisability; however, it also may have impacted the patient's ease to attend for follow-up. Despite this, the longer follow-up in this study allows for further in-depth analysis of survival, which was a limitation of the Shepheard *et al* study.[8] Their study highlighted that $p75^{ECD}$ was a predictor of survival; however, that was not found in this longer-term study. There was no significant impact of $p75^{ECD}$ levels on survival outcome. This may be due to only 63% of subjects reaching death in this trial; therefore, the results include some censoring. This would need to be studied on a larger scale to allow for this. A more recent study by Shepheard *et al* had a longitudinal analysis of 29 ALS patients.[16] They found that urinary $p75^{ECD}$ significantly and progressively increased from diagnosis (0.19±0.02 ng/mg creatinine per month (p<0.0001)). Therefore, this study is consistent with the findings of our study, further strengthening and increasing validity and confidence in the potential of urinary $p75^{ECD}$ for use as a biomarker in ALS.

The present study is not without limitations. In particular, the number of healthy controls (n=27) and ALS participants (n=99) is not balanced. To better evaluate the specificity of urinary $p75^{ECD}$ as a biomarker for ALS, research with a larger cohort including neurological controls is needed. A previous study in a Chinese cohort of patients had 108 patients with other neurological conditions as a comparison.[27] They found that urinary $p75^{ECD}$ in ALS participants (n=101) was significantly higher than other neurological conditions (p=<0.001). They found that it had a sensitivity of 86.1%, specificity of 89.8% and area under the curve of 0.923 (95% CI 0.888 to 0.959) for detecting ALS.[27] Additionally, a recent meta-analysis showed that urinary $p75^{ECD}$ levels were significantly higher in participants with ALS (n=211) compared with both non-neurological controls (n=177) (weighted mean difference (WMD)=4.18, 95% CI (2.525 to 6.990), p<0.001)) and participants with other neurological conditions (n=127), with significant WMD in both comparisons (WMD=6.005, 95% CI (1.596 to 10.414), p=0.008).[7] Previous reports have therefore highlighted a significant difference between neurological controls and urinary $p75^{ECD}$ values in ALS. In this current study, despite a lack of disease controls, we provide new data on a large longitudinal UK cohort. These results highlight a significant difference between healthy controls and ALS participants with blinding to the sample origin, decreasing the potential for bias. There was also no observed difference between subgroups of ALS in our cohort, although this is likely to be due to the small number of participants in the less common subgroups. This is due to the blinding of the sample selection, and these results may have been obscured by phenotypic-specific variations in urinary $p75^{ECD}$ levels. It would be beneficial to test larger cohorts of each subgroup to see if there is a significant difference.

The urinary $p75^{ECD}$ levels were compared against urine creatinine levels, as has been done previously.[8] This study found significant differences in blood creatinine levels between ALS patients and healthy controls. Plasma creatinine has previously been suggested as a biomarker itself in ALS-COSMOS,[31] where baseline levels of plasma creatinine predicted survival and correlated with ALSFRS-R scores over time.[31 32] Plasma and urinary creatinine both reflect muscle health and broader metabolic processes. Therefore, to avoid false positive results due to the creatinine levels rather than $p75^{ECD}$ levels, we also normalised $p75^{ECD}$ measurements to urinary osmolality.

The importance of developing a biomarker for ALS is paramount, due to its devastating nature and current lack of disease-modifying treatments.[1] The absence of validated, efficient biomarkers of disease progression affects both clinical practice and clinical trial research.[33] A biomarker that has been developed and emerged in the last few years for ALS is neurofilament light chain (NfL).[34] It has been shown to be a good prognostic biomarker when measured early in

the disease course and may be useful in highlighting susceptibility or risk in populations with an elevated risk. NfL has been criticised for having a lack of specificity, despite being the most robust marker of disease 'aggressivity' in ALS.[34 35] Therefore, a potential panel of biomarkers for ALS progression, severity and therapeutic efficacy in clinical trials could be used. The data shown in this current study highlight that $p75^{ECD}$ could be used within this proposed panel, as an easily accessible biomarker to assess the disease stage and the extent of disability. Therefore, it could prove useful in trial responses as an indicator of treatment efficacy, with easily accessible repeated measurements. To enhance the validation of biomarkers and urinary $p75^{ECD}$ as a biomarker, future studies should include pathological controls, ensure extended follow-up periods as the disease allows and research further into a potential biomarker panel.

CONCLUSION

Urinary $p75^{ECD}$ is a urinary biomarker that is significantly raised in ALS patients in comparison to healthy controls. It increases during disease progression, with the speed of disease progression and is higher with greater levels of disability measured by the ALSFRS-R. $p75^{ECD}$ represents a promising potential biomarker that can be taken forward in further investigations and evaluated as a biomarker of therapeutic efficacy in clinical trials of neuroprotective agents in the future.

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Contributors Dr LRC contributed to the planning, conduct and reporting of this work. She conducted the statistical analysis, interpreted the data, created the figures, drafted the manuscript and submitted the final version. Dr SS performed the p75 assays and experimental work at Flinders University, contributing to the acquisition and analysis of data. Professor PJS and Dr M-LR conceived and designed the study, supervised the research throughout and oversaw the interpretation and reporting of results. NV contributed to the conceptualisation of the study and assisted with data interpretation. Professor MRT and Professor AM provided access to patient samples through their leadership in the AMBROSIA study and contributed to the planning and execution of this research. They also critically reviewed and approved the final manuscript. All listed authors were involved in the conception and design of the work, data analysis or interpretation and critical revision of the manuscript. All authors meet the ICMJE authorship criteria and take collective responsibility for the integrity and accuracy of the work. Professor PJS is the guarantor of this work and accepts full responsibility for the integrity of the data and the accuracy of the analysis and reporting. Non-author contributions: we thank the participants of the AMBROSIA study and the research teams involved in patient care and sample collection. Their contributions were vital to the conduct of this research. Members of the public who participated in the study contributed as research volunteers by providing biosamples, without which this work would not have been possible. While not meeting authorship criteria, their contribution is gratefully acknowledged.

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