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1. **Title:** Urinary neopterin: a novel biomarker of disease progression in amyotrophic lateral sclerosis

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Author contributions:

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B Benyamin: statistical analysis, reviewed manuscript

D W Schultz: patient recruitment, reviewed manuscript

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T Chataway: contributed to the conception and design of the study, reviewed manuscript

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Conflicts of Interest:

Research Assoc. Professor J. Wu reports grants from the National Institutes of Health and Target ALS during the conduct of the study

Professor M. Benatar reports grants from National Institutes of Health, the ALS Association, the Muscular Dystrophy Association, the Centers for Disease Control and Prevention, the Department of Defense, and Target ALS during the conduct of the study; personal fees from Roche, Biogen, Jazz Pharmaceuticals, and AveXis outside the submitted work. In addition, Dr. Benatar has a provisional patent entitled ‘Determining Onset of Amyotrophic Lateral Sclerosis’. Dr. Benatar also serves as a site investigator on clinical trials funded by Biogen and Orphazyme, and as the global coordinating investigator for Orphazyme’s trial of Arimoclomol in ALS.

Professor A. Malaspina reports grants from the ALS Association, MND Association UK, Wellcome Trust UK, NIHR UK, Barts and The London Charities UK. He has acted as consultant and received personal fees from Roche and Pfizer. He has a joint provisional patent entitled ‘Determining Onset of Amyotrophic Lateral Sclerosis’ with Dr Benatar.

Assoc Professor M-L Rogers reports grants from MND Research Australia, FightMND, the ALS Association, Target ALS and National Institutes of Health. In addition, Assoc Prof Rogers, Dr Shepherd and Assoc Prof Chataway have a provisional patent entitled “Antibody against ALS Biomarker”.

ABSTRACT

Background: To evaluate urinary neopterin, a marker of pro-inflammatory state, as a potential biomarker of disease prognosis and progression in amyotrophic lateral sclerosis (ALS); and to compare its utility to urinary neurotrophin receptor p75 extracellular domain (p75^{ECD}).

Methods: Observational study including 21 healthy controls and 46 people with ALS, 29 of whom were sampled longitudinally. Neopterin and p75^{ECD} were measured using enzyme-linked immunoassays. Baseline and longitudinal changes in clinical measures, neopterin and urinary p75^{ECD} were examined, and prognostic utility explored by survival analysis.

Results: At baseline, urinary neopterin was higher in ALS compared to controls (181.7 ± 78.9 $\mu\text{mol/mol}$ creatinine vs 120.4 ± 60.8 $\mu\text{mol/mol}$ creatinine, $p=0.002$, Welch's t-test) and correlated with ALSFRS-R ($r=-0.36$, $p=0.01$). Combining previously published urinary p75^{ECD} results from 22 ALS patients with a further 24 ALS patients, baseline urinary p75^{ECD} was also higher compared to healthy controls (6.0 ± 2.7 vs 3.2 ± 1.0 ng/mg creatinine $p<0.0001$) and correlated with ALSFRS-R ($r=-0.36$, $p=0.01$). Urinary neopterin and p75^{ECD} correlated with each other at baseline ($r=0.38$, $p=0.009$). In longitudinal analysis, urinary neopterin increased on average ($\pm\text{SE}$) by 6.8 ± 1.1 $\mu\text{mol/mol}$ creatinine per month ($p<0.0001$) and p75^{ECD} by 0.19 ± 0.02 ng/mg creatinine per month ($p<0.0001$) from diagnosis in 29 ALS patients.

Conclusion: Urinary neopterin holds promise as marker of disease progression in ALS and is worthy of future evaluation for its potential to predict response to anti-inflammatory therapies.

INTRODUCTION

Biomarkers are widely believed to hold great potential for accelerating efforts to develop effective therapies for patients with amyotrophic lateral sclerosis (ALS). A critical issue is that potential biomarkers should be fit for purpose^{1,2}. Prognostic markers, which aid in predicting the future course of disease, could be used to reduce patient heterogeneity and potentially to yield sample size savings in mid-phase clinical trials. Pharmacodynamic markers, a subset of which may also be markers of disease progression, have the potential to show that a biological response has occurred following administration of an experimental therapeutic; and thereby provide a rationale for advancing experimental compounds from phase 2 to phase 3 clinical trials. Significant progress has been made in developing generic biomarkers of neuronal degeneration that have utility as prognostic, disease progression and potential pharmacodynamic markers, with two notable examples being the urinary concentration of the extracellular domain of the common neurotrophin receptor p75 (p75^{ECD})^{3,4} and blood-based measurement of neurofilament light⁵. However, with the exception of genetic markers, which identify patients most likely to benefit from therapies targeting the underlying genetic cause of disease⁶, predictive biomarkers are lacking in other forms of ALS. This is not to say that biomarkers indicative of a particular biological mechanism (e.g CSF chitotriosidase-1 (Chit-1) as a marker of microglial activation^{7 8, 9 10 11}) have not been identified, but rather that none of these biomarkers predict response to a particular experimental (or approved) treatment. The development of such predictive biomarkers could transform how we identify subsets of ALS patients most likely to benefit from an experimental therapeutic with a particular mechanism of action.

Immune dysregulation has been identified as an important component of the underlying disease process in ALS, comprising both anti-inflammatory and pro-inflammatory phases¹². It is hypothesised that the initial protective anti-inflammatory phase involves microglia in the central nervous system (CNS)¹³, as well as T-regulatory and T-helper type 2 (Th-2) cells in the periphery. Pre-clinical data suggest that this

protective response emerges as motor neurons and neuromuscular junctions begin to degenerate¹⁴. Evidence from animal models¹⁵ suggest that this response shifts from anti- to pro-inflammatory as motor neurons continue to degenerate and accumulate ALS-associated protein aggregates. Microglia become pro-inflammatory^{15 14} and induce release of neurotoxic factors from astrocytes that can kill motor neurons^{16,17}. In the periphery, the pro-inflammatory state is evidenced by a switch to T-helper cells types 1 and 17 (Th-1 and Th-17), as well as induction of cytotoxic CD8 cells, inflammatory monocytes and natural killer (NK)¹⁸. This cascade also results in Th-1 cell release of pro-inflammatory cytokines such as interleukins and interferon- γ (IFN- γ)¹².

While neuroinflammation has been the target of several ALS clinical trials, including NP001, Tocilizumab, and Masitinib, results have been disappointing. This may, at least in part, reflect the limitations of existing markers of neuroinflammation, the extent to which they reflect a pro- vs. anti-inflammatory state, and the extent to which blood and CSF concentrations of these markers reflect inflammation in the CNS vs. the periphery. For example in the phase II placebo controlled trial of NP001 that aimed to inhibit the pro-inflammatory response¹⁹, baseline levels of plasma monocyte chemoattractant protein-1 (MCP-1) failed to identify the small group of responders in post-hoc analysis²⁰; but the rationale for this analysis is unclear since MCP-1 has been defined as a Th-2 anti-inflammatory chemokine. Moreover, it is CSF levels of MCP-1 that are elevated in ALS^{21, 22}, but this trial relied on plasma levels of MCP-1²³. A number of Th-1 cytokines (e.g. IL-18, IL-6) in CSF are also being investigated as markers of the pro-inflammatory state in the CNS, but there is sparse information on peripheral or systemic pro-inflammatory signals. The small molecule neopterin, released from macrophages and dendritic cells²⁴ in the periphery in response to IFN- γ , and from microglia²⁵ and neurons²⁶ in the CNS, is a candidate marker of the pro-inflammatory state. Urinary neopterin has been used to assess the Th-1-type immune response in inflammatory conditions^{27,28} and previously suggested as a biomarker in ALS^{29,30}, but has not been extensively studied. Here we have sought to understand the

potential utility of urinary neopterin as a disease progression biomarker, with the knowledge that it might also serve as a biomarker of an underlying pro-inflammatory state. Secondly, we compared urinary neopterin and p75^{ECD} with respect to their potential utility as markers of disease progression.

Materials and Methods

Participant cohort and urine samples

Patients with ALS and healthy controls without underlying autoimmune disorder or immunosuppressive therapies, neurological disorders or untreated illness that affects kidney function were recruited between September 2011 and May 2021. All willing ALS patients were recruited without bias through the South Australian MND clinic at Flinders Medical Centre, and or the ALS Center at the University of Miami, and diagnosed with ALS according to the revised El Escorial criteria³¹. Written informed consent was obtained from all study participants following study approval from the Southern Adelaide Clinical Human Research Ethics Committee or the University of Miami IRB as appropriate. Study results were reported using the STROBE cohort reporting guidelines³².

Clinical information was collected by investigators blinded to urinary neopterin and p75^{ECD} results and included ALSFRS-R scores; medications; and latencies between symptom onset, diagnosis, sample collection; use of non-invasive ventilation, tracheostomy, and death. Baseline Δ FRS, the estimated monthly change in ALSFRS-R prior to enrolment, was calculated as $(48 - \text{ALSFRS-R at baseline}) / \text{number of months between symptom onset and baseline}$ ³³. For the prognostic analysis we analysed the association between baseline information and time to death or censorship.

Urine samples were collected and stored as per Urine & Kidney Proteome Project Standards³⁴ and were coded to ensure participant anonymity. Samples were tested by urinalysis (Siemens Multistix) and samples with signs of infection (blood and high pH) or diabetes (e.g high glucose) were excluded.

Samples collected in Miami were shipped on dry ice. All samples were stored in 200 µl aliquots at -80°C until analysis.

Urinary neopterin measurement

Urinary neopterin was quantified using the IBL International/Tecan competitive enzyme-linked immunoassay (RE59321) as per manufacturer's instructions²⁴. Briefly, urine samples were diluted 1:100 in buffer, and samples, standards and controls were added to plates, followed by enzyme conjugate and neopterin antiserum. Following incubation, plates were washed, and neopterin quantity visualised using a tetramethylbenzidine (TMB) reaction, with optical density measured at 450nm. Neopterin concentration was determined as per the manufacturer's instructions using a four-parameter nonlinear inhibitor vs response standard curve using GraphPad Prism 9. Storage of urine at -80C was sufficient to prevent degradation of neopterin post storage, when measured by this ELISA³⁵. The average coefficient of variation over 3 separate assays per sample in this study was 12.23 ± 5.61 % (range 0.6-19.34).

Urinary p75^{ECD} measurement

Urinary p75^{ECD} was quantified using a sandwich ELISA modified from that previously described³. Our previously published ELISA used a monoclonal capture antibody (MLR1) and a polyclonal detection antibody³. The modified ELISA used the MLR1 capture antibody, and a monoclonal detection antibody (NGFR5³⁶, 2.0 µg/mL) that was biotinylated as per manufacturer's instructions (Thermo Fisher Scientific Australia, #UG283022). The blocking and sample buffers were also changed from 2% bovine serum albumin in PBS to BlockAce (BioRad, BUF-029). The enzyme reaction was achieved as previously described³ using streptavidin horse radish peroxidase (Jackson ImmunoResearch Laboratories, #JIO16030084) diluted to 1.0 µg/mL and colour developed using TMB (A:B; BioRad Australia, #1721067). This assay was transferred to a Hamilton Starlet, integrated with an MD reader and Biotek

405 washer, to increase testing capacity. The average coefficient of variation over 3 separate assays per sample was $11.10 \pm 5.3 \%$ (range 1.6-18.21). We verified the new method by quantifying p75^{ECD} in 69 urine samples using the modified assay, and comparing to previously published results³. There was a 97% association between the new method and our published results in quantified p75^{ECD} levels (n=69 samples, CI: 0.96 to 0.98; see Passing-Bablok equation and correlation in Supplementary **figure e-1**). This also indicates there was no degradation of p75^{ECD} measured post long-term (up to 10 years) storage of urine at -80C.

Urinary creatinine measurement

Urinary neopterin and p75^{ECD} measurements were corrected for urine dilution using urinary creatinine³, expressing values as $\mu\text{mol neopterin/mol creatinine}$ or $\text{ng p75}^{\text{ECD}}/\text{mg creatinine}$. Urinary creatinine was measured using Enzo Life Sciences Creatinine kits (ADI-907-030A) as per manufacturer's instructions³. Samples with urinary creatinine below $0.3 \pm 0.03 \text{ mg/ml}$, or above $3.0 \pm 0.3 \text{ mg/ml}$ were rejected as per WHO guidelines³⁷. These indicate that 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.

Statistical analysis

Statistical analysis of correlation, logistic regression, Cox proportional-hazard models and longitudinal change (linear mixed model analysis, taking into account repeated measures) in urinary neopterin, p75^{ECD} and ALSFRS-R, were examined using SPSS, R, and GraphPad Prism 9. Sensitivity and specificity were examined using receiver operating characteristic (ROC) analysis using the area under the curve (AUC) in GraphPad Prism 9. The cut-off levels for sensitivity and specificity of the ROC curves was determined using the Youden Index³⁸. All statistical analysis was done by B.B and M.L.R.

Results

Study population

The study population included 21 healthy controls and 46 people with ALS, with longitudinal data available from n=29 (total of 103 person-visits). Most ALS (n=44) was sporadic, with no documented family history; genetic mutations were identified in two patients – one with a *C9orf72* repeat expansion and one with an E101G (E100G) mutation in *SOD1*. Disease characteristics of the ALS population are similar to previously reported studies (Table-1)^{3 5}.

Cross Sectional Analysis

Baseline urinary neopterin levels were elevated in people with ALS (n=46, 181.7 ± 78.5 $\mu\text{mol/mol}$ creatinine, $p=0.002$, Welch's t-test) compared to controls (n=21, 120.4 ± 60.8 $\mu\text{mol/mol}$ creatinine; **Fig 1A**). Combining previously published urinary $p75^{\text{ECD}}$ results from 22 ALS patients (in red symbols)³ with data from an additional 24 ALS patients, urinary $p75^{\text{ECD}}$ was higher among the ALS patients compared to healthy individuals (6.0 ± 2.7 ng/mg creatinine vs. 3.2 ± 1.0 ng/mg creatinine, $p<0.0001$ (red symbols from 10 previously published data³), Welch's t-test, **Fig 1B**). The type of $p75^{\text{ECD}}$ assay (new or old) did not contribute to the significance (logistic regression; $p=0.729$ for assay type and $p75^{\text{ECD}*}$ assay type $p=0.437$).

Interestingly, among the n=46 ALS patients, there was a correlation between baseline levels of urinary neopterin and urinary $p75^{\text{ECD}}$ (Pearson's correlation, $r=0.38$, $p=0.009$; **Fig 1C**). Receiver operating characteristic analysis using the area under the curve (AUC) indicated neopterin distinguished ALS from controls 74% of time ($p=0.002$; CI: 0.61-0.87) with 56% sensitivity whereas $p75^{\text{ECD}}$ distinguished ALS from controls 87% of time ($p<0.0001$; CI: 0.78-0.96), with 76% sensitivity (**Fig 1D**). Both neopterin and $p75^{\text{ECD}}$ were inversely correlated with ALSFRS-R at baseline: $r=-0.36$, $p=0.01$ (**Fig 2A**) and $r=-0.36$,

$p= 0.01$ (**Fig 2B**) respectively. Among controls ($n=21$, median age 61.3; range 48-72), there was no correlation between age and neopterin, or between age and $p75^{ECD}$ (**Fig 3A and 3C**). Among the 46 ALS patients (median age 66.7; range 42-85), baseline age did correlate with neopterin ($r = 0.5$, $p= 0.001$) and $p75^{ECD}$ ($r= 0.37$, $p= 0.01$). After excluding those over 72 and below 48 years of age, such that both control ($n= 21$) and ALS ($n= 26$) had a similar age range (median 63.5, range: 48.6.-71.5 years **Fig 3B** and **Fig 3D**), there was no correlation. Baseline urinary neopterin levels in these 26 with ALS, however, remains elevated ($167.1 \pm 66.36 \mu\text{mol/mol creatinine}$), compared to 21 controls ($120.4 \pm 60.8 \mu\text{mol/mol creatinine}$ $p= 0.01$, Welch's t-test). Urinary $p75^{ECD}$ was also higher in $n= 26$ people with ALS in the 48-72 years old age-range at baseline compared to healthy controls ($5.49 \pm 2.52 \text{ ng/mg creatinine}$ vs. $3.2 \pm 1.0 \text{ ng/mg creatinine}$, $p<0.0001$).

Prognostic Biomarkers

To investigate the potential utility of baseline urinary neopterin and $p75^{ECD}$ as predictors of prognosis, we performed survival analyses on 46 ALS patients, 44 of which who have died during follow-up and 2 that were alive. In univariate analysis, age at diagnosis, sex, and site of onset were not associated with survival, but consistent with previously published data³ higher $p75^{ECD}$ (HR= 1.13, 95% CI: 1.02-1.25, $p= 0.019$) and faster estimated progression rate (ΔFRS) predicted shorter survival (HR= 3.66, 95% CI: 1.9-6.85, $p<0.0001$); with $p75^{ECD}$ levels having only modest predictive value. Higher neopterin levels at baseline, while statistically significant, did not meaningfully predict survival (HR = 1.01, 95% CI: 1.001-1.01, $p= 0.01$). Urinary neopterin and $p75^{ECD}$ were dichotomized above or below their median baseline values, and their Kaplan Meier survival plots are shown in **Fig 4A and 4B**. ALS patients with lower than the median neopterin ($161 \mu\text{mol/mol creatinine}$) survived 19.8 months versus 10.7 months for those with neopterin above the median value ($p= 0.046$). Longer median survival from baseline was also observed

for ALS with urinary p75^{ECD} levels below versus above the median value of 5.33 (20.5 vs. 8.7 months p= 0.005 (Wilcoxon test, SPSS and Prism 9).

In multivariate Cox regression analysis, only Δ FRS (HR= 3.61, CI: 1.62-8.05, p=0.002) predicted survival; but not urinary neopterin, urinary p75^{ECD}, sex, site of onset or baseline age. This indicates that neither neopterin nor p75^{ECD} add prognostic value beyond what can be determined based on Δ FRS.

Urinary biomarkers of disease progression

The potential utility of urinary neopterin as a biomarker of disease progression was investigated in 29 patients with ALS in whom longitudinal clinical data and urine samples were available. All 29 had more than 1 sample post baseline (median of 3 time points, 3.0 (range 2- 6); Table 1). The median duration between first and second sample was 3.9 months (range: 2- 14.7 months). Fourteen of these patients were included in our prior publication focused on p75^{ECD}³, but eight of these had additional samples collected at later time points. To illustrate that the utility of urinary neopterin and p75^{ECD} as markers of disease progression is not dependent on timing of initial measurement, we have examined changes in these biomarkers from, symptom onset, diagnosis and baseline. Since the dates of diagnosis and baseline are reliably obtained from clinical notes³, we have chosen to use time from diagnosis and baseline to each sample collection for our primary analyses (**Fig 5B, 5C, 5E and 5F**). The urinary concentration of neopterin increased from diagnosis of 6.8 μ mol/mol creatinine each month (95% CI: 4.6-9.1; p<0.0001; **Fig 5B**) and baseline by an average of 6.8 μ mol (CI: 4.1 to 9.5, p<0.0001; **Fig 5C**), with a similar magnitude increase from symptom onset (**Fig 5A**). Urinary p75^{ECD} increased at an average of 0.19 ng/mg creatinine per month from diagnosis (**Fig 5E** 95% CI: 0.14-0.23, p<0.0001), which is the same as our previously published study³ and 0.19 ng/mg creatinine from baseline (CI 0.14–0.24, p<0.0001 **Fig 5F**) and similarly from onset (**Fig 5D**).

Among the 29 patients with longitudinal data, the slope of the ALSFRS-R declined by an average of -0.61 points/month from diagnosis (CI -0.49 to -0.73, $p < 0.0001$) and by -0.66 points/month (CI -0.54 to -0.79, $p < 0.0001$) from baseline, similar to that reported in a recent study examining NfL in a larger cohort⁵. Comparing the rate of change in ALSFRS-R and the urinary biomarkers, the monthly increase in neopterin (-0.015; CI: -0.02 to -0.004; $p = 0.009$) and p75^{ECD} (-1.475; CI: -1.95 to -0.995; $p < 0.0001$) are significantly associated with a monthly decrease in ALSFRS-R.

To illustrate faster and slower progressors, we split the data around the median Δ FRS at baseline (-0.49 ALSFRS points/month) into slower (< 0.49 points/month; $n = 13$) and faster (> 0.49 ; $n = 16$), progressors. In the slower progressors, neopterin increased by 4.7 $\mu\text{mol/mol}$ creatinine per month (95% CI: 1.4-8.0; $p < 0.0001$) from diagnosis and in the faster progressing group neopterin increased by 16.6 $\mu\text{mol/mol}$ creatinine per month (95% CI: 13-20; $p < 0.0001$; red lines in Fig 5B). The difference between groups was significant ($p = 0.0005$). p75^{ECD} increased by 0.17 ng/mg creatinine per month from diagnosis (CI 0.12-0.22, $p < 0.0001$) in the slower and 0.36 ng/mg creatinine per month in the faster progressors (CI 0.26-0.45, $p < 0.0001$; red lines in Fig 5E). This difference was significant ($p = 0.002$).

Discussion

Our results show that urinary neopterin is elevated in ALS patients and increases as disease progresses. Previous work has shown neopterin levels in healthy individuals are not impacted by age between the ages of 18 and 75 years³⁹. p75 expression is also known to be increased in older individuals⁴⁰. Consistent with these published results, we found an association between age and neopterin only amongst those older than 72 years of age. Neopterin and p75^{ECD} remain elevated in ALS compared to control in those aged 48-72. Age, therefore, does not appear to confound our observation that neopterin increases

measurably over time as ALS advances – i.e. that it is a marker of disease progression. Neopterin, therefore, together with p75^{ECD}³, might have potential utility as pharmacodynamic biomarkers in clinical trials of ALS treatments, where a decrease in rate of change of these markers post treatment might indicate a biological effect of an experimental therapeutic.

Urinary neopterin and urinary p75^{ECD} correlate with the ALSFRS-R at baseline. While univariate analysis suggests that that p75^{ECD} has modest prognostic value, multivariate analysis suggests that neither neopterin nor p75^{ECD} add prognostic value to what can be discerned from the Δ FRS³. We found no relationship between sex or site of onset with ALS prognosis, even though some other studies have reported such an association⁵. By contrast, serum NfL has been verified in large cohorts as prognostic of future ALSFRS-R decline and survival duration, providing information that is not captured by readily available clinical predictions such as Δ FRS and site of onset⁵.

There have been many clinical trials of experimental therapeutics that target the inflammatory system, (e.g. COX2 inhibitor; glatiramer acetate)²⁰ and their negative results have highlighted the need to better understand the immune response in ALS, and to identify biomarkers that might identify potential responders at trial enrolment. Previous work on immune dysfunction in ALS¹² has led us to investigate neopterin as a potential biomarker. Low levels of urinary neopterin in healthy individuals indicate that Th-1 lymphocytes are suppressed in the absence of an inflammatory response. However, progressively increasing neopterin in ALS patients suggests the emergence of a pro-inflammatory state in which Th-1 and Th-17 lymphocytes release IFN- γ and tumour necrosis factor (TNF)- α , which in turn induce neopterin release from monocytic cells such as proinflammatory macrophages, dendritic cells, microglia and astrocytes^{18 41 42}, with eventual removal from the circulation by the kidney²⁴. It will be valuable, in future studies, to determine if high baseline neopterin levels might identify the subset of ALS patients most likely to benefit from anti-inflammatory drug in trials. Neopterin might also be used as a

pharmacodynamic biomarker in clinical trials that target the immune system, where a decrease in the monthly change in neopterin levels with an experimental treatment might suggest a beneficial reduction in the pro-inflammatory response.

Chitotriosidase-1 (CHIT-1), and monocyte chemoattractant protein-1 (MCP-1) have also been investigated⁴³ as inflammatory biomarkers of ALS. CHIT-1 is released from microglia, and in five studies^{7 8, 9 10 11} found to be significantly elevated in the cerebrospinal fluid (CSF) of patients with ALS compared to controls, but in those that examined progression, CHIT-1 was relatively stable as disease progressed^{9 11} and did not always correlate with the ALSFRS-R⁸. In serum and plasma, there are conflicting reports as to the significance of CHIT-1^{44 9 11}, with only one study reporting a significant amount of CHIT-1 in serum⁴⁴ and there was no correlation between serum and CSF CHIT-1. MCP-1 levels in CSF appear to be related to faster progression^{22 21} but are fairly stable over progression^{45 22}. A meta-analysis of 8 studies⁴⁶, found serum MCP-1 is not significantly increased in ALS compared to controls. MCP-1 is a Th-2 anti-inflammatory chemokine, however, other proinflammatory cytokines including IL-6, IL-18, and TNF- α that are associated with Th-1 phenotypes are also upregulated in the CSF in ALS¹². For example, serum IL-6 was not prognostic for ALS, but was found to be robustly increased, especially at later stages of disease⁴⁷. However, it has not been clearly demonstrated that the serum/plasma levels of these markers increase (or decrease) from baseline at a measurable rate as ALS progresses^{9 11 48}. By contrast, we have found urinary neopterin is correlated to ALSFRS-R and changes over time as disease progresses, suggesting measurement of immune markers may be useful in the less complex proteome of human urine, compared to serum. The levels of neopterin are understood to reflect the Th-1 pro-inflammatory IFN- γ , and found at similar levels in serum and urine⁴⁹. This suggests urinary neopterin may be a marker that reflects pro-inflammation and disease progression. Urinary neopterin measurement does not require stringent collection protocols⁴⁹ whereas for reliable IFN- γ measurement, fresh serum or plasma sample (within 1 hour) must be used, or measured from samples that were

processed and frozen no more than 1h post collection^{50 51}. Since it is difficult to ensure such stringent collection protocols, and ALS patients are willing to provide urine, urinary neopterin is thus an easily accessible marker of systemic proinflammation and ALS progression.

In ALS, prior small cross-sectional studies of neopterin have been inconclusive, variably showing an increase in serum and CSF concentrations^{29,52}. Our results are consistent with a recent study by Lunetta et al, that examined urinary neopterin in ALS, using a non-specific high-performance liquid chromatography (HPLC) with neopterin measured at 355 nm³⁰; this study reported a significantly higher level of urinary neopterin in 81 individuals with ALS (mean= 263.9 μ mol neopterin/mol creatinine) compared to 68 healthy controls (169.6 μ mol neopterin/mol creatinine). While these levels were higher than we observed (mean 181.7 μ mol versus control at 120.4 μ mol neopterin/mol creatinine), the apparent discordance may reflect the fact that we analysed baseline levels of neopterin at an average of 16.9 \pm 8.8 months following onset, compared to an average of 26.4 months in the prior study³⁰ i.e., that patients were later in their disease course may account for the higher neopterin levels. In addition, the non-specific UV detection at 355 nm used in the prior study³⁰ may yield apparently higher levels of neopterin than the ELISA used in our study, which has higher specificity²⁴. A more recent alternative to HPLC/UV detection is to use mass spectrometry to accurately detect and quantify neopterin and a HPLC-mass spectrometry method. This technique has been shown to be robust in measuring urinary neopterin⁵³ and will be pursued in the future.

The limitations of the study include the relatively small number of samples. Future directions would be to analyse larger cohorts over progression, refining neopterin quantification using the HPLC-Mass spectrometry method. We will also determine the usefulness of neopterin in ALS clinical trials that target neuroinflammation.

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The data that support the findings of this study are available from the corresponding author(s) upon reasonable request.

Figure Legends

Figure 1: Urinary neopterin (**A**, $p=0.0017$) and $p75^{\text{ECD}}$ (**B**, $p<0.0001$) levels are higher in patients with ALS ($n=46$) than in controls ($n=21$) at baseline (2-sample t test), with previously published $p75^{\text{ECD}}$ data in red. Neopterin levels correlate with $p75^{\text{ECD}}$ (**C**) sampled in the same patients ($n=46$) at baseline (Pearson's $r=0.38$; $p=0.009$). ROC analysis of urinary neopterin and $p75^{\text{ECD}}$, illustrating discrimination between ALS patients and controls, with an AUC of 0.74 ($p=0.0024$) and 0.87 ($p<0.0001$) respectively (**D**) (Wilson-Brown method). Analysis: SPSS and Prism 9.

Figure 2: Urinary neopterin (**A**) is correlated with ALSFRS-R at baseline for each of the 46 patients with ALS in the study ($r=-0.36$, $p=0.013$). As previously reported, urinary $p75^{\text{ECD}}$ also correlates with ALSFRS-R ($r=-0.36$, $p=0.013$) at baseline (**B**). Pearson's Tests in SPSS/ Prism 9.

Figure 3: There is no correlation between urinary neopterin and age (**A**, $r=-0.2$, $p=0.40$) or between urinary $p75^{\text{ECD}}$ and age (**C**; $r=-0.2$, $p=0.4$) among 21 healthy individuals aged 49-72 years. Similarly, there was no correlation between neopterin levels and age (**B**; $r=0.02$, $p=0.93$) or between urinary $p75^{\text{ECD}}$ and age (**D**; $r=0.002$, $p=0.99$) among the $n=26$ ALS patients aged 49-72 years. Pearson's Tests in SPSS/ Prism 9.

Figure 4: Kaplan-Meier survival estimates of 46 amyotrophic lateral sclerosis patients, comparing 22 ALS patients who had baseline urinary neopterin (**A**) and $p75^{\text{ECD}}$ (**B**) above (dashed line) and 22 with values below (black line) the median (161.1 μmol neopterin/mol creatinine; 5.33 ng $p75^{\text{ECD}}$ /mg creatinine), illustrates the longer median survival in those with lower compared to higher urinary neopterin ($p=0.046$; 10.7 versus 19.8 months) and $p75^{\text{ECD}}$ levels ($p=0.005$; 8.7 versus 20.5 months). Kaplan-Meier analysis (Wilcoxon test) was in SPSS and Prism 9.

Figure 5: Individual longitudinal trajectories of neopterin (A-C) and p75^{ECD} (D-F) for 29 ALS patients. Neopterin increased from symptom onset at a rate of 4.5 μmol neopterin/mol creatinine per month (**A**; CI: 2.2-6.8, $p < 0.0001$); from time of diagnosis (**B**) at a rate of 6.8 μmol neopterin/ mol creatinine per month (95% confidence interval (CI) 4.6-9.1; $p < 0.0001$) and baseline (**C**) at a rate of 6.8 μmol neopterin/ mol creatinine per month (CI: 4.1-9.5, $p < 0.0001$). p75^{ECD} also increased from time of symptom onset (**D**, 0.15 ng p75^{ECD}/mg creatinine/ month, CI 0.1-0.2, $p < 0.0001$); diagnosis (**E**, 0.19 ng/mg creatinine/ month, CI: 0.14-0.23, $p < 0.0001$) and baseline (**F**, 0.19 ng/mg creatinine/ month, CI 0.14–0.24, $p < 0.0001$). Each patient was sampled at least twice. Faster progressors ($>0.49 \Delta\text{FRS}$) are in red.

Supplementary Figure

Figure e1: Correlation between published urinary p75^{ECD} from ALS patients (n=69) measured by published sandwich ELISA method⁴ and automated method ($r = 0.97$, $p < 0.0001$). The Passing-Bablok equation was $y = -0.05 + 1.06$ (*old values), correlation, $r = 0.97$. The automated method has the same capture antibody to p75^{ECD}, but different detection antibody. Analysis in Prism 9.

Table 1: Study participant characteristics

		Controls	ALS	
			Baseline	Longitudinal
N		21	46	29
Age at baseline, years	Mean \pm SD (range)	60.6 \pm 6.9 (49-71)	66.0 \pm 11.7 (42-85)	65.7 \pm 12.9 (42-84)
Age at diagnosis, years	Mean \pm SD (range)	--	66.1 \pm 11.7 (42-85)	64.4 \pm 11.4 (42-84)
Sex, male	N (%)	10 (48%)	33 (72%)	22 (76%)
Familial disease	N (%)	--	2 (4.3%)	0
Bulbar onset	N (%)	--	17 (36%)	11 (38%)
Months from onset to diagnosis	Mean \pm SD (range)	--	10.4 \pm 8.1 (0.4-41)	10.4 \pm 8.9 (0.4-41)
Months from diagnosis to baseline	Mean \pm SD (range)	--	5.2 \pm 6.8 (0.3-37)	5.27 \pm 5.6 (0.5-21)
Baseline ALSFRS-R	Mean \pm SD (range)	--	39.8 \pm 4.6 (27-47)	41.4 \pm 3.9 (31-47)
Baseline Δ FRS	Mean \pm SD (range)	--	0.68 \pm 0.5 (0.02-2.7)	0.54 \pm 0.3 (0.02-1.17)
Death	N (%)	--	44 (96%)	27 (93%)
Disease duration, months ^a	Median (IQR)	--	19.5 (11.34-38.6)	22.14 (18.3-41.9)
Sampling time points	Median (range)	--	--	3 (2-6)

Δ FRS = estimated rate of progression (change in ALSFRS-R per month from onset of weakness to baseline)

IQR = interquartile range (25th-75th percentile)

^a From diagnosis to death





