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BIOFLUID AND NEUROPHYSIOLOGICAL BIOMARKERS IN AMYOTROPHIC LATERAL SCLEROSIS

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ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is a heterogeneous neurodegenerative disease, characterized by the degeneration of both upper and lower motor neurons. There is an undiscussed and urgent need of biomarkers in ALS for a deeper understanding of the pathogenic mechanisms of the disease and a better stratification of patients in clinical trials.

In this Ph.D. dissertation we aimed to confirm the role of an already recognized biomarker in ALS, the neurofilament light chains (NfL), and to explore other new candidate biomarkers, i.e. plasma p-tau phosphorylated at residue 181 (p-tau181) and glial fibrillary acidic protein (GFAP). Among neurophysiological biomarkers we investigated the prognostic role of conventional electromyography (EMG) in the bulbar region and the diagnostic value of a new method which estimates the number of motor units (MScanFit MUNE).

We confirmed that both cerebrospinal fluid (CSF) and plasma NfL showed a high accuracy in discriminating ALS patients from ALS mimics, and displayed an excellent prognostic value in detecting ALS patients with a faster disease progression and a shorter survival.

Then we focused on plasma p-tau181 values of patients with ALS compared to neurological controls and other neurodegenerative diseases. We found that plasma p-tau181 is increased in ALS patients compared to controls, and resulted highly correlated with clinical and EMG lower motor neuron dysfunction. These findings indicate that p-tau181 of putative peripheral origin might represent a confounding factor in using plasma p-tau181 for Alzheimer's disease (AD) pathology screening.

Subsequently, we focused on plasma GFAP, a biomarker of astrocytopathy, demonstrating that its increase in ALS is merely driven by amyloid-beta co-pathology and resulted well correlated with the cognitive profile of patients.

Exploring neurophysiological biomarkers, we demonstrated an excellent prognostic value of the genioglossus involvement as assessed by conventional quantitative EMG analysis, since it resulted associated to a worse prognosis even in patients without evidence of clinical bulbar involvement.

Finally, we performed a comparison between conventional quantitative EMG analysis and the novel MScanFit MUNE method, which allows to estimate the motor units lost in ALS. We concluded that the second method did not show a better sensitivity in detecting abnormalities in affected muscles, but it is probably useful for following the progression of the disease over time.

To conclude, we confirmed the undiscussed utility of some biomarkers and explored the potential of others in revealing some other pathogenic aspects of the disease in the living human brain. With these studies we produced several pieces of evidence which significantly contribute to this field of research in amyotrophic lateral sclerosis.

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1. Introduction

1.1 Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is a heterogeneous neurodegenerative disease, characterized by the degeneration of motor neurons in the motor cortex (upper) and in brainstem and spinal cord (lower motor neurons). Despite a predominant involvement of the motor system, over the last decade, several clinical, imaging and neuropathological studies have shown a more extensive involvement of the central nervous system (CNS), defining ALS as a multidomain neurodegenerative syndrome of motor and extra-motor systems (Swinnen et al., 2014).

Globally ALS occurs with an incidence ranging from 2 to 3 cases per 100,000 individuals and a prevalence of 6 to 9 per 100,000 persons (Longinetti et al., 2019; Brown et al., 2021). ALS is more common in men than in women, with a male-female ratio of 1.2–1.5:1 (Logroscino et al, 2010). The risk of developing ALS peaks at 50–75 years of age and decreases thereafter. The rate of disease progression is highly variable, but the neuromuscular respiratory failure usually leads to death about 2–4 years after onset (Chiò et al, 2013; Masrori et al., 2020).

Although the primary symptoms of ALS are related to the motor dysfunction, population-based phenotyping studies and the use of systematic detailed neuropsychological evaluations showed that subtle cognitive and behavioural disorders are present in up to 50% of patients affected by ALS, and around 5-10% develop a full-blown frontotemporal-dementia (FTD) (Phukan et al, 2012; Elamin et al, 2013).

Pathologically, the key features of ALS are the neuronal loss and gliosis affecting the primary motor cortex and the anterior horn of the spinal cord, associated to neurogenic changes in muscles (Neumann et al., 2006). The neuropathological hallmark of the disease is the aggregation and accumulation of ubiquitylated protein inclusions in motor neurons. In 97% of ALS, the main constituent of these inclusions is TDP-43, which is depleted in the nucleus and accumulated in cytosol of residual motor neurons, but some rarer forms not directly related to the TDP-43 aggregates are widely recognized, such as ALS caused by pathogenic variants in the *SOD1* (Cu–Zn superoxide dismutase) and *FUS* (fused in sarcoma) genes (Mackenzie et al., 2007; Vance et al., 2009). These aggregates might mediate cell-to-cell propagation of disease, suggesting a prion-like mechanism. However, multiple mechanisms have been progressively recognized, such as mitochondrial dysfunction, axonal transport, excitotoxicity, inflammation, and RNA toxicity (Hardiman et al, 2017).

ALS is an archetypal complex disease, with a monogenic cause in 5–10% of ALS patients, usually with a Mendelian autosomal dominant pattern of inheritance. Four genes account for up to 70% of all cases of familial ALS, namely *C9orf72*, *TARDBP* (encoding TAR DNA-binding protein 43), *SOD1* (encoding superoxide dismutase) and *FUS* (encoding RNA-binding protein FUS). However, even in

the case of these known Mendelian-inherited genes, familial forms of ALS are often characterized by less than 50% penetrance and genetic pleiotropy, with evidence of oligogenic and polygenic inheritance in individuals with seemingly sporadic disease (Al-Chalabi et al, 2011).

In 90-95% of cases ALS is sporadic, but genetic factors are considered important even in the absence of a family history. Overall, the disease is considered the result of both genetic and environmental risk factors (Al-Chalabi et al, 2014). Indeed, the late age at onset suggests a multistep process in which genetic factors are penetrant only when combined with lifestyle or environmental factors (Al-Chalabi et al, 2014). To date, the only confirmed epidemiological risk factors associated with the development of ALS are age and male gender (Longinetti et al., 2019). Recent Mendelian randomization studies have highlighted the robust causal link between strenuous physical exercise (Julian et al., 2022) and hyperlipidaemia (Bandres-Ciga et al., 2019), and the risk of developing ALS. Other potential environmental risk factors proposed include smoking (Armon et al., 2009), military service (McKay et al., 2021) and specific sporting activities, comprising soccer and American football (Lacorte et al., 2016).

1.2 The need for biomarkers in Amyotrophic Lateral Sclerosis

ALS can be defined as an "etiologically and biologically heterogeneous disease" (Taylor et al., 2016), associated with an extreme phenotypic variability, which have probably significantly contributed to the failure of the experimental drug trials conducted to date (van den Berg et al., 2019).

Indeed, expanded knowledge of the genotypic and phenotypic variability of the disease suggests the possibility of different pathogenic trajectories, which would explain, for example, the existence of certain extremes within the motor neuron disease (MND) spectrum with selective upper (UMN) or lower motor neuron (LMN) involvement and slower progression, such as primary lateral sclerosis (PLS) or progressive muscular atrophy (PMA). However, the findings of the peripheral involvement in post-mortem PLS patients (Le Forestier et al., 2001), such as the presence of ubiquitin-inclusions in the cortico-spinal tracts of patients clinically diagnosed as PMA (Ince et al., 2003) have confirmed that these entities are part of the larger spectrum of MND. This is the most evident example of the broad phenotypic variability of the disease, which sometimes makes the clinical counselling extremely changeling, together with the design of experimental drug trials (Mitsumoto et al., 2014). Furthermore, although clinical studies in ALS have well-defined end points such as the decline of the functional status measured by the ALSFRS-R score and survival, the lack of a robust surrogate marker of disease progression is a significant issue for the field. Finally, the diagnostic delay in ALS may imply that the disease process has progressed to a stage where compensatory mechanisms has failed and preserving motor neuron health can be particularly arduous, being the neurodegenerative process

already advanced and probably irreversible. At this stage, strategies to allow earlier intervention with potential neuroprotective agents would be valuable.

In this scenario, the research for biomarkers has multiple aims and applications, from a deeper understanding of the pathogenic mechanisms and pathological basis of the disease to implications in the clinical practice. Not surprisingly, the search of biomarkers has been incorporated into Airlie House consensus guidelines for trial implementation (van den Berg et al., 2019). In these consensus criteria, particular attention was focused on the importance to include prognostic and predictive biomarkers as eligibility criteria, and pharmacodynamics biomarkers as evidence of the adequacy of drug delivery, target engagement, or biological activity of the experimental therapy. The inclusion of validated prognostic biomarkers may help in the identification of subsets of patients with a higher likelihood of demonstrating the effect of the experimental drug, and also in reducing the necessity of broad recruitments and in shortening the duration of follow-ups. Furthermore, the emergence of promising biomarkers may be directly applied in routine clinical practice, shortening the diagnostic delay and therefore encouraging earlier referral to specialist ALS clinics, allowing patient recruitment into clinical trials at the earliest possible disease stage.

2. Plasma and CSF Neurofilament Light Chain in ALS: a cross-sectional and longitudinal study

2.1 Background and aims

In the last decade, neurofilaments (NFs) have emerged as an unspecific but extremely sensitive biomarker of neurodegeneration across many neurological diseases (Khalil et al., 2018; Gaetani et al., 2019).

NFs are a neuron-specific cytoskeletal structures belonging to the class of intermediate filaments, composed of 10 nm large filaments, with a diameter intermediate between actin (6.5 nm) and microtubules (25 nm). Three NF isoforms are recognized according to their molecular weight: neurofilament light chain, NfL; neurofilament medium chain, NfM; neurofilament heavy chain, NfH (Gaetani et al., 2019). NFs functions include a purely structural role in the axonal cytoskeleton, the transport of organelles such as mitochondria and endoplasmic reticulum, and the participation in intracellular signalling and transcription. NfM and NfH require post-translational modifications such as O-glycosylation or phosphorylation for proper stabilization and, consequently, to perform their correct function. Interestingly, NFs form a liquid crystal gel network in different neurodegenerative diseases, including ALS (Beck et al., 2012; Didonna et al., 2019). In particular, the aggregation of bundled NFs in axonal spheroids in post-mortem ALS studies is considered a histopathological hallmark, together with hyperphosphorylation of NfH and NfM and the presence of NF proteins in perikaryal inclusions (Sobue et al., 1990; Itoh et al., 1992; Mizusawa et al., 1989). Although the

mechanism of this misfolding process is still unclear, it seems to be mediated by the hyperphosphorylation, which can modify the stoichiometry of NF composition, leading these structures more prone to aggregate (Goldstein et al., 1987; Sihag et al., 2007).

Already in 1996, Rosengren had the intuition that neuronal proteins such as NFs might be increased in extracellular fluids as a result of the release from apoptotic processes in neurodegenerative diseases, demonstrating that cerebrospinal fluid (CSF) NfL levels were elevated in patients with ALS and other neurological disease (Rosengren et al., 1996). Since then, methodological advances have significantly improved the detection and measurement of NFs, moving from the semi-quantitative Western blots to the more sophisticated single-molecule array (SiMoA) technology, a fully automated procedure, which have dramatically reduced the sources of error and the inter-operator and intra-assay variability, reaching a high reproducibility.

Initial efforts to identify fluid biomarkers for neurological disorders focused on the cerebrospinal fluid (CSF), since it is close to the brain extracellular space and contains higher concentrations of CNS-derived proteins. Then, the development of fourth-generation immune assays brought the possibility of obtaining rapid and robust protein biomarker measurements from blood samples, opening up new perspectives in this field.

To date, Neurofilament light chain (NfL) can be reliably measured in both CSF (cNfL) and plasma (pNfL) (Gray et al., 2020), and showed the best performance in distinguishing patients with ALS from patients with diseases mimicking ALS (Steinacker et al., 2016; Poesen et al., 2017; Feneberg et al., 2018; Gille et al., 2019; Abu-Rumeileh et al., 2020; Ashton et al., 2021). Moreover, several authors highlighted the potential role of cNfL and pNfL as robust prognostic biomarkers, given the significant associations between the disease progression rate (DPR) and survival and the basal biomarkers values (Lu et al., 2015; Gaiani et al., 2017; Poesen et al., 2017; Steinacker et al., 2016; Feneberg et al., 2018; Benatar et al., 2020; Thouvenot et al., 2020). Finally, a few preliminary longitudinal studies suggested that pNfL levels remain stable in the disease course (Lu et al., 2015; Skillbäck et al., 2017; Verde et al., 2019; Benatar et al., 2020), making this novel biomarker a potential candidate for the monitoring of future therapeutic approaches in ALS.

In this first study (Vacchiano et al., 2021), we aimed to further explore the value of cNfL versus pNfL in distinguishing patients with ALS and ALS mimics in a large and deeply phenotyped cohort. Furthermore, we assessed the association of both biomarkers with clinical variables and with survival. Finally, we sought to describe the longitudinal behavior of pNfL, analyzing the biomarker values at different disease stages in a significant group of patients.

2.2 Material and Methods

2.2.1 Inclusion criteria and Clinical assessment

We included 171 ALS patients and 60 patients with an alternative clinical diagnosis (ALS mimics group) evaluated at the Institute of Neurological Sciences of Bologna between September 2014 and June 2021. We also analyzed blood and CSF samples from 57 non-neurodegenerative controls, namely 30 blood samples from healthy subjects and 27 CSF samples from patients lacking any clinical or neuroradiological evidence of CNS disease.

Patients with suspected ALS were prospectively enrolled, and underwent a standardized protocol including neurological examination, electromyography (EMG), lumbar puncture and ancillary exams to exclude any alternative clinical diagnosis. We included in the ALS group patients who received a diagnosis of ALS according to the Revised El Escorial criteria at baseline or during follow-up (Brooks et al., 2000), with available clinical data and at least one between CSF and plasma samples at baseline. Patients evaluated for ALS who received an alternative clinical diagnosis during the diagnostic work-up and/or follow-up and with at least one biofluid available were included in the ALS mimics group. For ALS patients the following clinical data were collected at the time of diagnosis (baseline visit): age at onset, sex, disease duration (time elapsed between the first referred symptom and sampling), type of onset (bulbar, spinal, pseudopolyneuritic or pyramidal according to Swinnen et al., 2014), clinical phenotype (classical, bulbar, predominant upper motor neuron [PUMN], predominant lower motor neuron [PLMN], [Chiò et al., 2011; Al-Chalabi et al., 2016]), ALS Functional Rating Scale-revised (ALSFRS-R) score, forced vital capacity (FVC) expressed as a percentage of predicted volume, and body mass index (BMI). Patients were classified according to the Revised El Escorial criteria in 31 definite ALS, 69 probable ALS, 31 probable laboratory-supported ALS and 40 possible ALS (Brooks et al., 2000), and staged in agreement with King's clinical staging system (Roche et al., 2012). All patients underwent genetic screening for the most frequent ALS genes (i.e., *SOD1*, *FUS*, *TARDBP*, and the repeats expansion of the *C9Orf72* gene) (Bartoletti-Stella et al., 2021). The degree of the UMN involvement was defined as the number of regions (bulbar, cervical and lumbosacral region) showing UMN signs at clinical examination, while for the extent of the LMN involvement both clinical and EMG assessment were considered, as stated by the Awaji criteria (de Carvalho et al., 2008). The DPR at the baseline visit was calculated as follows: (48-ALSFRS-R score at the time of sampling)/months elapsed between disease onset and sampling (Lu et al., 2015), and patients were accordingly divided into slow (DPR < 0.5), intermediate (DPR 0–5–1) and fast progressors (DPR > 1), as previously described (Lu et al., 2015). Moreover, the Medical Research Council (MRC) scale of 0–5 (calculated as the sum of 10 muscles for each side score/20; score 0–5 points) was provided for each patient at the time of clinical evaluation.

A subgroup of ALS patients underwent the Edinburgh Cognitive and Behavioral ALS Screen (ECAS) (Abrahams et al., 2014; Siciliano et al., 2017) to investigate the presence of cognitive impairment up to a full-blown frontotemporal dementia (FTD).

Baseline CSF and plasma samples were used for a cross-sectional study of NfL levels.

Fifty-seven of the 171 ALS patients had plasma samples available from two or more visits. Longitudinal plasma samples were obtained during multidisciplinary follow-up visits from ALS patients who accepted to donate further blood samples after baseline sampling. No selection criteria were applied to identify these patients. In details, 24 patients were sampled twice, 20 patients had three plasma samples, 11 patients were sampled four times and for two subjects we had five samples available. Patients were repeatedly sampled at non-standardized time points, with a median follow-up period of 12 months (IQR 8–26). We observed 55 patients for more than 3 months, 50 subjects for at least 6 months, 32 and 17 patients for at least 12 and 24 months, respectively. The most extended follow-up duration was 55 months (two patients). For patients with more than one sampling, we calculated the longitudinal disease progression rate (l-DPR), as the change in the ALSFRS-R between the last and the baseline visits divided by the number of months between the visits (Vu et al., 2020). Accordingly, ALS patients were further classified into fast progressors (l-DPR > 1), intermediate progressors (l-DPR 0.5–1), and slow progressors (l-DPR < 0.5).

The study was conducted according to the revised Declaration of Helsinki and Good Clinical Practice guidelines. Written informed consent was given by study participants. The study was approved by the ethics committee of "Area Vasta Emilia Centro."

2.2.2 CSF and plasma analyses

EDTA plasma samples were collected, aliquoted, and stored at −80°C according to standard procedures. CSF samples were obtained by LP following a standard procedure, centrifuged in case of blood contamination, divided into aliquots, and stored in polypropylene tubes at −80°C until analysis.

Both cNfL and pNfL concentrations, in the entire sample cohort, were determined with the Single molecule array (Simoa) technology on a Simoa SR-X instrument (Quanterix, Billerica, MA, United States) using the commercially available NF-light advantage kit (Quanterix). The mean intra- and inter-assay coefficients of variation (CVs) were below 15% for both cNfL and pNfL.

2.2.3 Genetic Analyses

Molecular genetic analyses were performed as previously described (Bartoletti-Stella et al., 2021). Briefly, genomic DNA (gDNA) was extracted from peripheral blood by standard procedures (Bartoletti-Stella et al., 2021). gDNA was quantified using the Quantus Fluorometer (Promega) with

QuantiFluor double stranded DNA system (Promega). Patients were screened for mutations in ALS major genes: *SOD1* (all exons), *FUS* (exons 6 and 15), *TARDBP* (exons 2, 3, and 5) genes and for pathogenic repeat expansion (RE) in the *C9orf72* gene as previously reported (Bartoletti-Stella et al., 2021).

2.2.4 Statistical Analyses

Statistical analyses were performed using IBM SPSS Statistics version 21 (IBM, Armonk, NY, United States), Stata SE version 14.2 (StataCorp LLC, College Station, TX, United States) and GraphPad Prism 7 (GraphPad Software, La Jolla, CA, United States) software.

Continuous variables were presented as mean and Standard Deviation (SD) or median and interquartile range (IQR), while categorical variables were presented as absolute number (n) and relative frequency (%). For continuous variables, based on the data distribution, the Mann-Whitney U test or the student t-test were adopted to evaluate the differences between the groups, while the Kruskal-Wallis test (followed by Dunn-Bonferroni post hoc test) or the one-way analysis of variance (ANOVA) (followed by Tukey's post hoc test) were used for multiple group comparisons. Chi-Square test was applied for categorical variables. Biomarker values were transformed into a logarithmic scale to obtain a normal data distribution.

For the analysis of diagnostic value, receiver operating characteristic (ROC) analyses were performed to establish the accuracy in the distinction between ALS and ALS mimics, as well as the sensitivity and specificity of biomarkers. The optimal cut-off value for each biomarker was calculated using the maximed Youden Index. A subgroup analysis was also carried out according to patients' median age (≤55 *vs.* > 55 years) and sex (female *vs.* male). De Long test was used to compare the areas under the curve of pNfL and cNfL in the whole groups and between subgroups.

For the cross-sectional analysis, Spearman's rho coefficient was used to test the correlation between cNfL and pNfL levels and clinical variables. Moreover, the association between biofluids NfL and the degree of UMN and/or LMN involvement was analyzed using univariate and multivariate linear regression models with the log-transformed biomarker values (cNfL and pNfL) as dependent variables and the extent of: (1) UMN involvement, (2) LMN involvement, (3) UMN and LMN involvement as independent variables. In the multivariable models we adjusted for age at sampling, sex, genetic status, basal ALSFRS-R score, DPR, MRC and King's scores. The results are presented as β coefficients and 95% confidence intervals (95% CI).

For the prognostic analysis the cumulative time-dependent probability of death was calculated by the Kaplan-Meier estimate. The time of entry into the analysis was the date of the first sampling (at baseline), and the time of the endpoint was the date of death/tracheostomy or the date of the last follow-up information, whichever came first. We performed univariate and multivariate Cox

regression models to study the association between time to death/tracheostomy and prognostic factors in ALS. The multivariate Cox regression analysis was adjusted for age at baseline, sex, baseline ALSFRS-R score, genetic status, DPR, MRC and King' scores. The results are presented as Hazard Ratios (HR) and 95% CI. The assumption of proportional hazard was assessed by Schoenfeld residuals. Differences were considered significant at $p < 0.05$.

For the longitudinal analysis, a linear mixed effect modeling analysis with random slope and random intercept was performed to evaluate the rate of change over the time of both cNfL and pNfL in the ALS patients stratified into fast, intermediate and slow progressors, as previously described (Vu et al., 2020). The results are presented as β coefficients and 95% CI.

2.3 Results

2.3.1 Demographic features, distribution and diagnostic performance of Plasma Neurofilament Light Chain and Cerebrospinal Fluid Neurofilament Light Chain

Demographic and clinical features of the study population are detailed in Tables 1, 2.

Age at baseline and sex distribution were not significantly different among the three diagnostic groups (age, $p = 0.575$; sex, $p = 0.728$). No effect of sex and age on cNfL and pNfL values was detected in the ALS group, while there was a moderate effect of age on pNfL and cNfL levels in both the ALS mimics (age vs. pNfL: rho = 0.546, $p < 0.001$; age vs. cNfL: rho = 0.536, $p < 0.001$) and the control groups (age vs. pNfL: rho = 0.691, $p < 0.001$; age vs. cNfL: 0.451, $p = 0.018$).

Table 1. Demographic and clinical features of the study population.

ALS patients - clinical characteristics	**N (tot. 171)**	**%**
Gender	68 (F)	39.8
Type of onset		
Bulbar	42	24.6
Spinal	113	66.1
Pseudopolyneuritic	9	5.3
Pyramidal	7	4.1
Deceased/with tracheostomy	72	42.1
Genetic screening	(N tot. 167)	
C9Orf72 RE carriers	18	10.8
SOD1 mutation carriers	7	4.2
FUS mutation carriers	1	0.6
TARDBP mutation carriers	2	1.2
FTD status	21	12.3
		Median (IQR)
Age at first sampling (y)		65 (56-74)
DD from first symptom to sampling (m)		16 (9-27)
ALSFRS-R score		41 (34.5-44)
MRC score		4.6 (4.1-4.8)
FVC		90 (70-106)
Biomarker values		Median (IQR)
cNfL	114	6543 (3697-12719)
pNfL	170	73.0 (45.9-114.2)
ALS mimics group	**N (tot. 60)**	**%**
Gender	24 (F)	40
Age at first sampling (y)		
Median (IQR)		65 (56.3-71.8)
Biomarker values		Median (IQR)
cNfL	53	1140 (589.5-1937)
pNfL	30	22.5 (11.4-28)
Clinical and healthy controls	**N (tot. 57)**	**%**
Gender	26 (F)	45.6
Age at sampling (y)		
Median (IQR)		63 (56.5-69)
Biomarker values		Median (IQR)
cNfL	30	682.3 (498.7-934.3)
pNfL	27	9.4 (6.8-15.5)

Biomarker values are expressed in pg/ml. Key: ALS, amyotrophic lateral sclerosis; ALSFRS-R, Revised Amyotrophic Lateral Sclerosis Functional Rating; cNfL, cerebrospinal fluid neurofilament light chain; DD, disease duration; FVC, forced vital capacity; FTD, frontotemporal dementia; IQR, interquartile range; m, months; MRC, Medical Research Council; PLMN, predominant lower motor neuron; pNfL, plasma neurofilament light chain PUMN, predominant upper motor neuron; RE, repeats expansion; y, years.

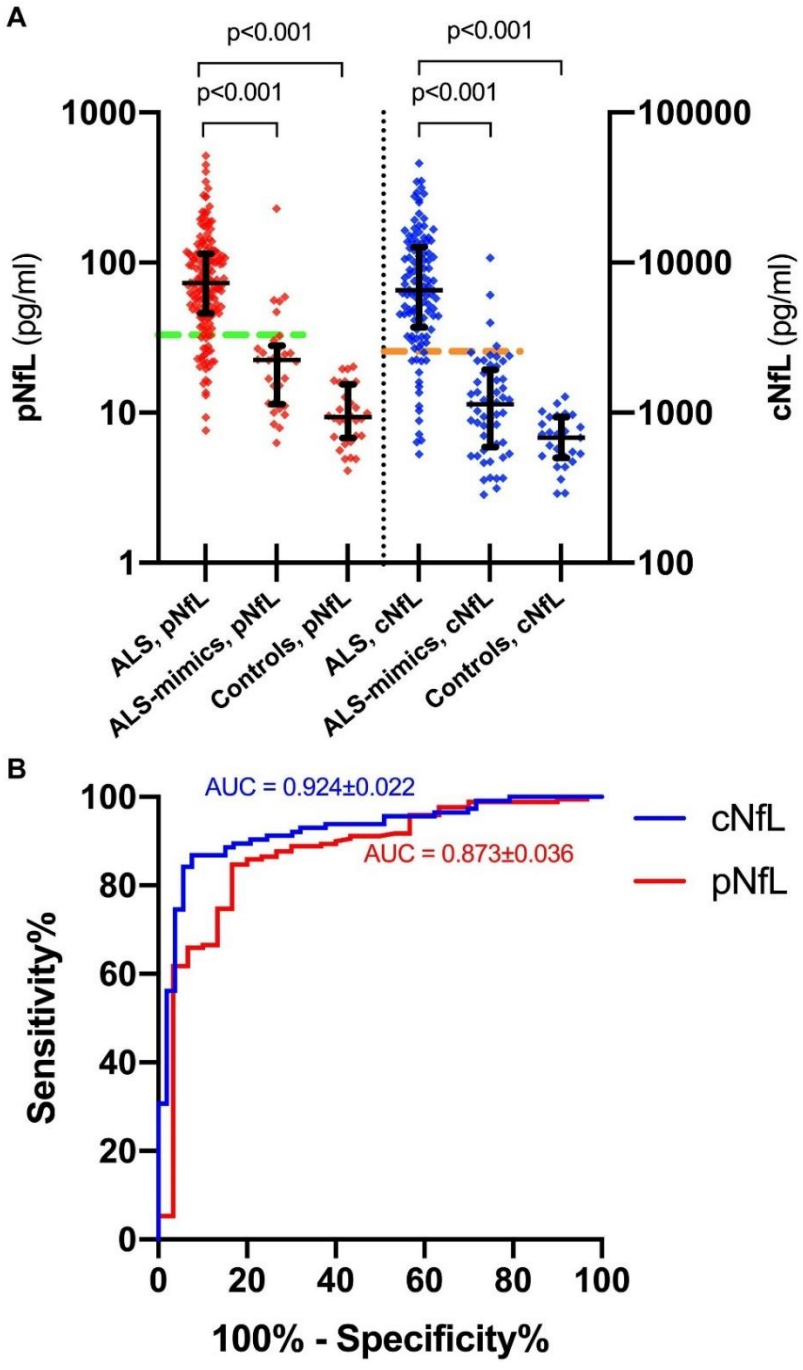
Table 2. Diagnostic categories in the ALS mimics group.

ALS mimic diagnosis	**60**
Hereditary or idiopathic spastic paraplegia	12
Chronic inflammatory demyelinating polyneuropathy	5
Polyneuropathy	6
Myelopathy/myelitis	3
Multineuropathy	3
Spinal muscular atrophy 3	2
Myopathy/myositis	4
Cramp-fasciculation syndrome	1
Spinocerebellar ataxia	1
Focal amyotrophy	2
Amyloidosis	1
Myasthenia gravis	3
Post-polio syndrome	1
Caspr2 antibody-associated disease	1
Anti-IgLON5 disease	1
Meningioma	1
Hydrocephalus	1
PSP-PLS	1
Atypical parkinsonism	2
Alexander's Disease	1
Lumbar spinal stenosis	1
Unclassified	7

Key: ALS, amyotrophic lateral sclerosis; PLS, primary lateral sclerosis; PSP, progressive supranuclear palsy.

ALS patients showed significantly higher pNfL ($p < 0.0001$) and cNfL ($p < 0.0001$) values compared to subjects belonging to the ALS mimics and control groups (Figure 1A). When evaluating the ROC curves, cNfL yielded a higher diagnostic value than pNfL ($p = 0.043$) in discriminating patients with ALS and subjects with an alternative ALS-mimicking disease (cNfL: AUC 0.924 ± 0.022, sensitivity 86.8%, specificity 92.4, cut-off 2,517 pg/ml; pNfL: AUC 0.873 ± 0.036, sensitivity 84.7%, specificity 83.3%. cut-off 32.7 pg/ml) (Figure 1B). After patient stratification, we found no significant influence of age ($p = 0.149$) and sex ($p = 0.644$) on the diagnostic performance of cNfL. Age but not sex ($p = 0.981$) slightly influenced the diagnostic accuracy of pNfL, although the effect did not reach statistical significance (≤ 55 years: AUC 0.939 ± 0.026 vs. > 55 years: AUC 0.804 ± 0.067; $p = 0.062$). Finally, the diagnostic accuracy of pNfL almost reached that of cNfL (AUC 0.906 ± 0.026, sensitivity 84.7%, specificity 86.4%, cut-off 32.7 pg/ml), when we limited the analysis to the subjects with alternative diseases only involving the CNS.

Figure 1. pNfL and cNfL levels in the diagnostic groups and ROC curves for pNfL and cNfL. Both cNfL and pNfL demonstrate high diagnostic value in the distinction between ALS and ALS mimics. (A) pNfL and cNfL levels in ALS patients, ALS-mimics and control groups. Thick lines represent medians and interquartile ranges. Biomarker values are expressed in the logarithmic scale. Dotted horizontal lines indicate the optimal cut-off values for pNfL (green) and cNfL (orange) in the distinction between ALS and ALS-mimics patients, as calculated through the maximized Youden Index. Only p-values of significative comparisons are shown (Kruskal-Wallis followed by Dunn-Bonferroni post hoc test). (B) ROC curves for pNfL (red) and cNfL (blue) in the comparison between ALS patients and ALS-mimics. Key: cNfL, cerebrospinal fluid neurofilament light chain; pNfL, plasma neurofilament light chain.



2.3.2 Association between CSF and plasma Neurofilament light chain and clinical variables

NfL and pNfL values strongly correlated at baseline (Spearman's rho = 0.836, $p < 0.0001$).

When evaluating the associations between biofluid biomarkers and measures of ALS severity, we found a marked association between both cNfL and pNfL concentrations and DPR (rho = 0.493, $p < 0.0001$; rho = 0.525, $p < 0.0001$, respectively), and a weaker association of NfL values in both biofluids with the MRC score (rho = 0.231, p = 0.014; rho = 0.248, p = 0.002), FVC (rho = 0.363, p = 0.003; rho = 0.276, p = 0.001), and ALSFRS-R (rho = 0.206, p = 0.023; rho = 0.217, p = 0.006) values. cNfL levels were also weakly correlated with the King's stage (rho = 0.249, p = 0.008).

Moreover, fast progressors (i.e., ALS patients with DPR > 1) showed higher cNfL and pNfL compared to intermediate (p = 0.026 and p = 0.001) and slow progressors ($p < 0.001$). In contrast, there was no significant association between pNfL/cNfL and ECAS (total, ALS-specific and ALS non- specific scores) and BMI, and between pNfL and King's stage. cNfL levels significantly differed across onset types (p = 0.011), and post hoc analysis revealed significantly higher levels in patients with bulbar than in those with spinal onset (p = 0.038). We found no significant differences across ALS variants, FTD, or genetic status, although cNfL resulted higher in ALS-FTD patients than in pure ALS (8637.2, IQR 6331.9-13979.9 vs. 6155.7, IQR 3231.4-12011, p = 0.093) and in *C9Orf72* RE carriers (p = 0.14) (Table 3). pNfL levels did not significantly differ among ALS phenotypes and type of onset but were slightly increased in FTD-ALS patients compared to those with ALS alone, with a trend of significance (110.8, IQR 55.5-165 vs. 70.7, IQR 43.4-109.5, p = 0.054).

Moreover, pNfL values were significantly higher in *C9Orf72* RE expansion carriers than in the other patients (p = 0.010) (Table 3). Finally, both pNfL and cNfL levels increased according to the accuracy level of the categories of the Revised El Escorial diagnostic criteria (Brooks et al., 2000) (for pNfL: probable laboratory-supported vs. definite ALS, p = 0.001; probable laboratory-supported vs. probable ALS, p = 0.002; for cNfL: possible ALS vs. probable ALS, p = 0.005; probable laboratory supported ALS vs. definite ALS, p = 0.043; possible ALS vs. definite ALS, p = 0.004, Table 3), likely reflecting the effect of the progressive spreading of the neurodegeneration and the increase of body regions involved during the disease course.

Table 3. pNfL and cNfL levels according to the accuracy level of the categories of the Revised El Escorial diagnostic criteria and to genetic status (i.e., wild type vs. ALS gene mutations).

Revised El Escorial Criteria	**N**	**pNfL** Median (IQR)	**N**	**cNfL** Median (IQR)
Possible ALS	38	64.9 (27.6-101.3)	25	4536 (2232-8853)
Probable laboratory-supported ALS	31	45.8 (31.4-70.7)	24	5100 (3145.2-7760)
Probable ALS	68	86.1 (57.7-127.2)	45	7572 (4770-15569)
Definite ALS	31	100.5 (58.8-135.5)	19	10892.4 (6156-14629)
Genetic status	**N**	**PNfL** Median (IQR)	**N**	**cNfL** Median (IQR)
Wild-type	137	73.5 (43.9-113.7)	98	6317 (3574-13476)
SOD1	6	36.0 (14.0-59.4)	2	2252; 4536
TARDBP	2	32.8; 51.8	1	3018.5
FUS	1	37.2	0	NA
C9Orf72	18	107.1 (64.5-125.3)	11	10796 (7950-12031)

Biomarker values are expressed in pg/ml. Key: ALS, amyotrophic lateral sclerosis; cNfL, cerebrospinal neurofilament light chain; IQR, interquartile range; N, number; NA, not available; pNfL, plasma neurofilament light chain.

Both cNfL and pNfL were associated with the number of body regions displaying UMN signs (rho = 0.325, $p < 0.0001$; rho = 0.308, $p = 0.001$). Accordingly, both cNfL and pNfL levels significantly raised with increasing number of regions affected by UMN signs only ($p = 0.008$ and $p = 0.001$) or displaying both UMN and LMN signs ($p = 0.001$ and $p = 0.002$). Both results remained statistically significant after adjusting for covariates (i.e., age at sampling, sex, genetic status, basal ALSFRS-R, DPR, MRC, and King's scores) (cNfL vs. UMN, three regions vs. zero or one region: b = 0.834, CI

0.316–1.636, p = 0.042; pNfL vs. UMN, three regions vs. zero or one region: b = 0.609, CI 0.348–1.185, p = 0.038; cNfL vs. UMN C LMN, three regions vs. zero or one region: b = 1.003, CI 0.265–1.741, p = 0.008; pNfL vs. UMN C LMN, three regions vs. zero or one region: b = 0.529, CI 0.206–1.038, p = 0.042). In contrast, there was no association with the number of LMN affected regions (p = 0.467 and p = 0.537) (Table 4).

Table 4. pNfL and cNfL levels according to the extent of UMN and/or LMN degeneration.

		N	**pNfL** **Median (IQR)**	**N**	**cNfL** **Median (IQR)**
UMN and LMN degeneration	Zero region	15	51.8 (32.8-103.9)	10	4161 (2165-8816)
	One region	52	58.2 (32.7-95)	35	4938 (2926-7964)
	Two regions	65	76.8 (48-120.8)	45	7187 (4209-14901)
	Three regions	36	104.0 (64.5-139.1)	23	11052 (6970-15995)
UMN degeneration	Zero region	10	40.9 (24.3-108.7)	7	3574 (1103-8778)
	One region	29	49.7 (35.8-72)	21	4938 (3814-6590)
	Two regions	60	67.8 (43.4-112.3)	40	5943 (3211-13758)
	Three regions	69	97.2 (58.83-136.9)	45	9440 (5624-14653)
LMN degeneration	Zero region	6	59.0 (46.9-76.6)	3	4747 (-)
	One region	20	80.4 (22.8-116.0)	11	6263 (881.6-13732)
	Two regions	65	63.4 (38.8-117.3)	41	5784 (3392-13805)
	Three regions	77	79.2 (51.2-112.4)	58	7378 (4496-12147)

Biomarker values are expressed in pg/ml. Key: ALS, amyotrophic lateral sclerosis; cNfL, cerebrospinal neurofilament light chain; LMN, lower motor neuron; IQR, interquartile range; N, number; pNfL, plasma neurofilament light chain; UMN, upper motor neuron.

2.3.3 Prognostic value of CSF and plasma Neurofilament Light Chain and longitudinal trajectories of plasma Neurofilament light chain during the follow-up

Based on univariate Cox regression analysis (171 ALS patients; 72 dead), age at sampling ($p = 0.034$), basal ALSFRS-R ($p < 0.001$), DPR ($p < 0.001$), C9orf72 status ($p = 0.031$), MRC score ($p = 0.001$), King's score ($p < 0.001$), FVC ($p < 0.001$), cNfL ($p < 0.001$) and pNfL ($p < 0.001$) were identified as predictors of the mortality in ALS patients.

Multivariate Cox regression confirmed the value of both cNfL (HR 2.44, CI 1.52–3.90, $p < 0.001$) and pNfL (HR 2.06, CI 1.31–3.22, $p = 0.002$) as independent predictors of the mortality in ALS. Accordingly, ALS patients with higher baseline cNfL and pNfL levels were associated with shorter survival (highest tertile of cNfL vs. lowest tertile of NfL, HR 4.58, CI 1.57–13.41, $p = 0.005$; highest tertile of pNfL vs. lowest tertile of NfL, HR 2.59, CI 1.20–5.58,
$p = 0.015$) (Figure 2).

When stratifying ALS patients according to the l-DPR, baseline levels of both cNfL and pNfL were significantly higher in ALS fast progressors than the slow progressors ($p = 0.002$ and $p = 0.001$, respectively, Table 5). In contrast, there was no significant rise or decline in the slopes of pNfL levels during follow-up in the three ALS groups (slow b = –0.001, CI –0.009 to 0.007, $p = 0.773$; intermediate b = 0.006, CI –0.002 to 0.013, $p = 0.126$; fast b = –0.0001, CI –0.009 to 0.009, $p = 0.974$, Figure 3), highlighting the overall stability of the biomarker during the disease course.

Figure 2. Prognostic value of pNfL and cNfL. Survival curves in ALS patients according to the values of pNfL (A) and cNfL (B). A greater increase in baseline cNfL and pNfL levels is associated with shorter survival in patients with ALS. Key: c-NfL, cerebrospinal fluid neurofilament light chain; pNfL, plasma neurofilament light chain.

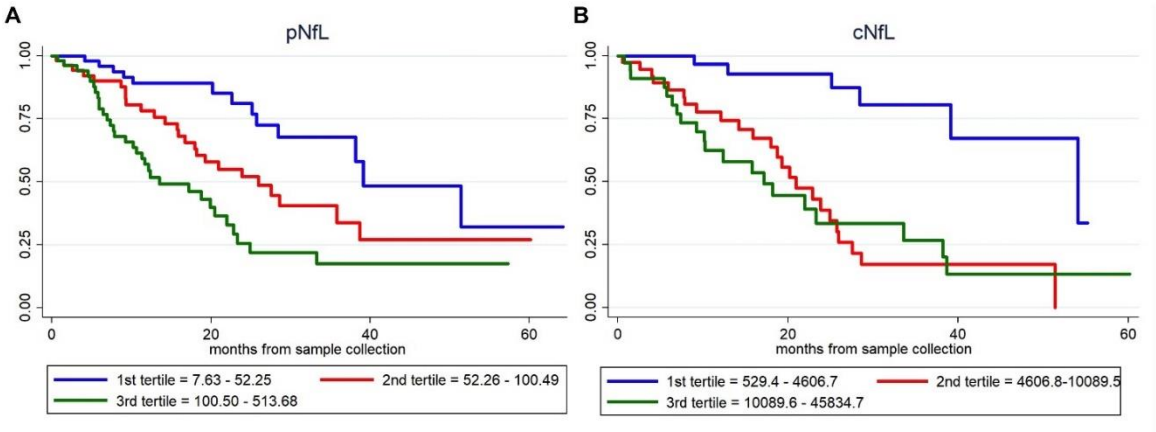


Figure 3. Longitudinal trajectories of pNfL during the follow-up. Overall and single-patient longitudinal pNfL behavior in the slow (A), intermediate (B) and fast (C) progressors showing a stable longitudinal biomarker trajectory. Thick lines represent the overall biomarker trend. Analyses were conducted through a linear mixed effects model. Biomarker values are expressed in the logarithmic scale. Key: pNfL, plasma neurofilament light chain.

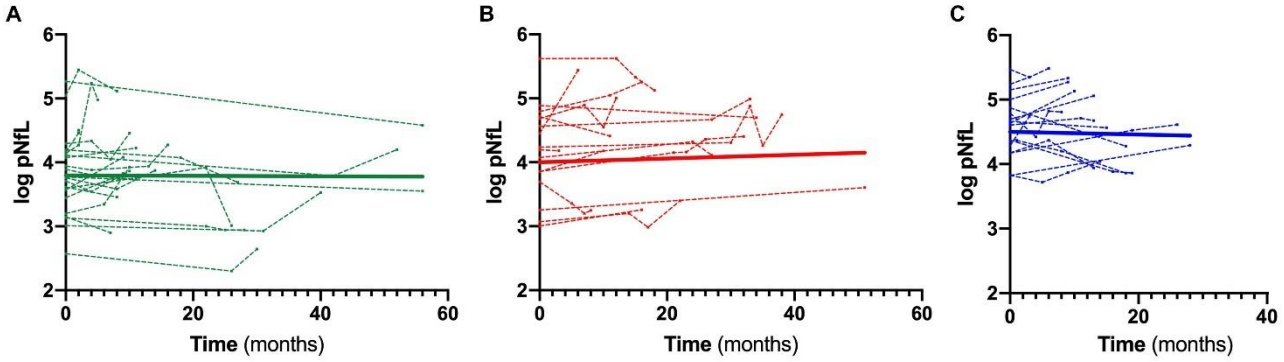


Table 5. Longitudinal ALS cohort: patients' characteristics and biomarkers stratification according to the l-DPR.

Groups (l-DPR)	N	Age at sample (mean ± SD)	Time from onset to sample (m, mean ± SD)	Sex F/M	Type of onset, SPI/BUL/ PSE/PYR	cNfL median (IQR)	PNfL median (IQR)
ALS Fast	17	55.6 (13.5)	14.6 (15.6)	8/9	9/6/1/1	9175 (6021-14887)	101.4 (68.7-134.7)
ALS Intermediate	16	67.4 (11.9)	20.8 (10.7)	10/6	11/3/0/2	5520 (3738-8345)	67.8 (43.7-109.9)
ALS Slow	24	64.8 (12.4)	31.7 (24.9)	13/11	17/3/2/2	3250 (2365-5193)	43.4 (31.4-64.4)
p-value		0.021*	0.021**	0.71	0.461	0.002°	0.002°°

*Post-hoc analysis revealed a significant difference between Fast and Intermediate ALS patients (p=0.028)
**Post-hoc analysis showed a significant difference between Fast and Slow ALS patients (p=0.021)
° Post-hoc analysis revealed a significant difference between Fast and Slow ALS patients (p=0.002)
°° Post-hoc analysis revealed a significant difference between Fast and Slow ALS patients (p=0.001)
The p-values reported directly in the table refer to the multiple-groups comparison analyses. Only the p-values of the comparisons showing a statistically significant difference at the post-hoc analysis are further detailed in the Table legend.
Biomarker values are expressed in pg/ml. Key: ALS, amyotrophic lateral sclerosis; BUL, bulbar; cNfL, cerebrospinal neurofilament light chain; F, females, l-DPR, longitudinal disease progression rate; IQR, interquartile range; m, months; M, Males; N, number; pNfL, plasma neurofilament light chain; PSE, pseudopolyneuritic; PYR, pyramidal; SPI, spinal; SD, standard deviation.

2.4 Discussion

In the context of motor neuron diseases, biofluid markers may aid in the diagnosis of clinically subtle or atypical ALS variants, in the prognostic evaluation of patients and their stratification for clinical trials. Here we confirmed the value of cNfL in distinguishing between patients with ALS and ALS mimics in a large clinical cohort. Additionally, in line with previous studies (Gaiottino et al., 2013; Lu et al., 2015; Benatar et al., 2018; Feneberg et al., 2018; Verde et al., 2019; Ashton et al., 2021), we demonstrated a strong association between cNfL and pNfL, and showed that pNfL also provides a robust diagnostic marker for ALS, especially after excluding patients with peripheral neuropathy, a condition associated with a higher increase of NfL values in plasma than in CSF (Bischof et al., 2018; Mariotto et al., 2018; Sandelius et al., 2018). Given that an extensive clinical and electrophysiological evaluation can reliably identify a PNS involvement, the diagnostic value of pNfL may be considered almost comparable to that of cNfL in the clinical routine. Furthermore, after stratification for age, we found a slight decrease of diagnostic accuracy of pNfL in elderly patients, likely reflecting the physiological increase of the biomarker levels with age, which did not involve the ALS patients, given the marked abnormal concentrations, but that was evident in the ALS mimics cohort.

To address the still debated issue of the pathophysiology of NfL release according to the involvement of upper and lower motor neurons (Zucchi et al., 2020), we investigated the association between biomarker levels and the extent of UMN and LMN degeneration. We found that both pNfL and cNfL levels increased with the number of UMN regions, which is in line with several studies showing a significant correlation between serum (Gille et al., 2019) or CSF (Menke et al., 2015) NfL levels and clinical signs of UMN damage or the extent of corticospinal tract involvement assessed by diffusion tensor MRI (Menke et al., 2015). However, other studies, including our previous evaluation limited to CSF NfL in a smaller cohort, did not confirm this association (Steinacker et al., 2016; Gaiani et al., 2017; Abu-Rumeileh et al., 2020). Beside the possible effects of patient selection and cohort size and the type of assay chosen for the analysis, one likely explanation for these conflicting results relies on the well-known high inter-rater variability in the clinical evaluation of UMN and LMN signs. Indeed, there is still disagreement among neurologists on how to define the presence of UMN-signs given that some consider a preserved reflex in an otherwise atrophic muscle to be a sign of upper motor neuron involvement, while others require the reflex to be hyperactive to reach the same conclusion (Swinnen et al., 2014). Likewise, given that both clinical and neurophysiological assessment help evaluate LMN involvement, a between-center standardization of neurophysiological techniques is also needed. In our cohort, both cNfL and pNfL showed higher values in *C9Orf72*-expanded ALS patients than in those with sporadic ALS, likely reflecting the more severe disease course in this patient subgroup. Notably, the current literature does not show full agreement also on

this issue with three previous studies supporting our findings (Gendron et al., 2017, Benatar et al., 2020; Huang et al., 2020), and two others not detecting any difference in CSF or serum NFL levels between patients with mutations in *SOD1, TARDBP, FUS* or the RE of *C9orf72* and sporadic cases (Weydt et al., 2016; Verde et al., 2019). Another debated issue concerns the potential effect of cognitive impairment on neurofilament levels in ALS. FTD-ALS patients in our cohort presented with higher levels of both pNfL and cNfL than ALS alone, reaching a trend of significance only for the plasma biomarker. Similarly, one study demonstrated higher, although not significant, plasma neurofilament heavy chain levels in ALS-FTD than in ALS patients (Falzone et al., 2020). However, other studies failed to find a correlation between cognitive functions decline and NfL levels (Gaiani et al., 2017; Feneberg et al., 2018), suggesting that the increase of biomarker levels in ALS is probably relatively independent of the brain regions involved compared to the effect of progression rate. These discordances in the current literature may also be attributable to the small number of ALS-FTD patients enrolled in the available studies. Further studies are, therefore, needed to establish whether the abnormal accumulation of neurofilaments might contribute to the definition of the pathologic ALS-FTD continuum.

On another critical issue, our results confirmed the predictive value on disease progression of cNfL and pNfL assessment (De Schaepdryver et al., 2020). Indeed, our data showed a strong correlation between the biofluid levels of the biomarker and the DPR. Accordingly, when stratifying patients in fast, intermediate, and slow progressors by tertiles score, biofluid NfL levels were significantly higher in fast progressors compared to the other two groups, in line with previous results (Poesen et al., 2017; Feneberg et al., 2018; Verde et al., 2019; Abu-Rumeileh et al., 2020; Dreger et al., 2021). In the present study, we also confirmed that both CSF and plasma NfL levels are independent prognostic factors in ALS, even after adjusting for potential clinical prognostic predictors, such as basal ALSFRS-R, genetic status, DPR, MRC, and King's scores (Benatar et al., 2020). This implies that NfL assessment in both plasma and CSF allows an early diagnosis of ALS in clinical trials, considering the high clinical variability of this devastating disease. Accordingly, a recent study (Benatar et al., 2020) showed that using the baseline serum NfL level as a pharmacodynamic biomarker instead of the ALSFRS-R slope would yield a significant patient sample size saving in a clinical trial. While the absolute pNfL values varied between patients in our cohort, they remained largely stable in individual patients over time, consistent with previous observations (Lu et al., 2015; Verde et al., 2019). This finding further confirms the potential clinical utility of plasma NfL as a marker of drug effect, provides that the tested novel therapeutics will result in a significant reduction of NfL levels, as recently proved for nusinersen in pediatric spinal muscular atrophy (Darras et al., 2019; Johannsen et al., 2021).

The present study has some limitations. Although we enrolled a significant number of ALS patients, the well-known high variability of the disease did not allow us to draw definitive conclusions about the effect of ALS clinical variants, FTD status, and ALS gene mutations on plasma and CSF NfL levels.

Moreover, our demonstration of NfL concentration stability during the disease course was based on the analysis of a relatively small cohort and on longitudinal blood samples collected at nonstandardized time points, suggesting caution in interpreting these results. Another partial limitation concerns the small number of ALS patients with a recent onset of symptoms and the absence of pre-symptomatic subjects carrying mutations in ALS genes. The inclusion of such patients could provide additional information about the behavior of biofluids NfL during the presymptomatic and early symptomatic phases of the disease, as already pointed out in recent studies (Benatar et al., 2018).

Interestingly, a more recent study (Thompson et al., 2022) confirmed our results on the diagnostic value of both CSF and plasma NfL, on their independent prognostic value and also on their longitudinal behavior, validating our findings in a multicentre longitudinal cohort.

In conclusion, the results of the present study support the use of pNfL as a pharmacodynamic marker in clinical trials. However, despite the positive results, to fully understand the diagnostic potential of biofluid NfL in ALS, it would be important to perform more detailed comparisons between ALS patients and homogeneous larger cohorts of single categories of mimic diseases. Finally, a better understanding of how NfL is released in response to pathology, especially in the early disease stages, would also facilitate the use of NfL in the diagnostic work-up and therapeutic trials in ALS.

3. Elevated plasma p-tau181 levels unrelated to Alzheimer's disease pathology in amyotrophic lateral sclerosis

3.1 Background and aims

As mentioned above, in recent years innovative biofluid biomarkers have contributed to remarkable progress in neurodegenerative diseases, allowing earlier and more accurate diagnostic and prognostic evaluations and a deeper understanding of pathophysiological mechanisms.

Biomarkers of Alzheimer's disease (AD) pathophysiology, i.e. amyloid-beta, phospho-tau (p-tau) and total tau (t-tau), and those detecting neuroaxonal degeneration, as NfL, have provided the most substantial impact (Hansson et al., 2021; Bridel et al., 2019; Vacchiano et al., 2021). Moreover, reliable assays that can detect p-tau and NfL in blood have become available, paving the way for more widespread use of these biomarkers in clinical practice (Palmqvist et al., 2021; Sturmey et al., 2022). In particular, the measurement of blood p-tau is increasingly considered a realistic, cost-effective and non-invasive assay that will help the diagnostic process for patients with cognitive decline (Palmqvist et al., 2021; Thijssen et al., 2021; Baiardi et al., 2022). Nevertheless, whether plasma measures of these biomarkers exclusively reflect their CSF concentration or are also influenced by peripheral sources remains to be fully explored. As previously confirmed also by our results (Vacchiano et al., 2021), NfL levels in CSF and plasma have been shown to accurately distinguish patients with ALS from their mimics (Lu et al., 2015; Steinacker et al., 2016; Verde et al., 2019; Abu-Rumeileh et al., 2020), correlate with disease severity and predict survival (Sturmey et al., 2022; Lu et al., 2015; Steinacker et al., 2016; Verde et al., 2019; Abu-Rumeileh et al., 2020).

Tau protein isoforms in biofluids have also been investigated in ALS, either as a marker of neurodegeneration (t-tau) or as a follow-up of studies reporting a small amount of p-tau deposition in the brain and the spinal cord of patients with ALS with cognitive dysfunction (Strong et al., 2006; Yang et al., 2012; Behrouzi et al., 2016; Moszczynski et al., 2018) and, to a lesser extent, in those with pure motor ALS (Stevens et al., 2019). ALS subjects showed significantly higher CSF t-tau levels than controls, probably reflecting unspecific massive neurodegeneration, whereas inconclusive results were obtained for CSF p-tau (Abu-Rumeileh et al., 2020; Grossman et al., 2014; Wilke et al., 2015; Agnello et al., 2021). Unexpectedly, a recent study (Cousins et al., 2022) showed that patients with ALS exhibit significantly increased levels of plasma p-tau phosphorylated at residue 181 (p-tau181) compared with controls. Intriguingly, the authors found that plasma p-tau181 levels do not correlate with CSF p-tau181 levels and AD postmortem neuropathological changes. Moreover, they demonstrated a significant association between plasma p-tau181 levels and the degree of LMN loss in the cervical, thoracic and lumbosacral districts, supporting a peripheral origin of the plasma p-tau181 elevation. Given the potential relevance of the finding also for the AD fields, given that plasma

p-tau isoforms, including p-tau181, are being increasingly proposed as a screening marker of AD pathology (Karikari et al., 2020; Palmqvist et al., 2021), we aimed to expand the current data on the plasma p-tau181 levels in patients with ALS (Vacchiano et al., 2023). Furthermore, we explored for the first time the association of the biomarker with electrophysiological variables and survival and studied the longitudinal trajectory of the biomarker during the disease course. Finally, we extended the analysis of plasma p-tau181 in patients with a different form of motor neuron disease, namely, spinal muscular atrophy (SMA).

3.2 Methods

3.2.1 Inclusion criteria and clinical assessment

Our cohort comprised 148 patients with a clinical diagnosis of ALS according to the Revised El Escorial criteria (Brooks et al., 2000) evaluated at the Institute of Neurological Sciences of Bologna between September 2014 and July 2022. Among them, 130 had samples of both CSF and plasma available and a negative amyloid status according to the A/T/N classification (Jack et al., 2016). We also included 18 patients with ALS with only plasma samples available because their age at sampling (less than 60, median 54.5, IQR 47.25– 57) made a concomitant AD pathology unlikely (doi: 10.1002/alz.12068). Finally, we included 20 ALS patients with CSF evidence of underlying amyloid co-pathology (A+), 12 SMA patients, 88 patients with AD and 60 healthy controls. All SMA patients (7 SMA type 2 and 5 SMA type 3) had a genetically confirmed diagnosis and were treatment-naïve. Patients with AD fulfilled the criteria for ‘probable AD dementia with evidence of the AD pathophysiological process’ according to the 2011 NIAAA criteria (McKhann et al., 2011). For ALS patients, the following clinical data were collected at baseline: age at onset, sex, disease duration (time elapsed between the first referred symptom and sampling), type of onset (Swinnen et al., 2014), clinical phenotype (Chiò et al., 2020), ALS Functional Rating Scale-Revised (ALSFRS-R) score, Medical Research Council (MRC) scale of 0–5 (calculated as the sum of 10 muscles for each side score/20; score 0–5 points), forced vital capacity (FVC), body mass index (BMI), creatinine levels, King’s (Roche et al., 2012), Milan-Torino (MiToS) (Fang et al., 2017), and Fine'til 9 (FT9) clinical stages (Thakore et al., 2018). Patients were stratified according to the validated clinical classification (Chiò et al., 2020) in classic, bulbar, respiratory, UMN-predominant (PUMN), primary lateral sclerosis (PLS), flail arm syndrome, flail leg syndrome and progressive muscular atrophy (PMA). However, to allow comparisons with sufficient statistical power, we grouped them in main categories: classic (including respiratory), bulbar, PUMN (ie, PUMN and PLS) and LMN-predominant (PLMN, including flail arm/leg and PMA). Details on cognitive function assessment in patients with ALS are

provided in online supplemental materials. One hundred and forty-two (96%) patients underwent genetic screening for the most frequent ALS-associated genes (ie, *SOD1*, *FUS*, *TARDBP* and the repeats-expansion of *C9Orf72*) (Bartoletti-Stella et al., 2021). UMN involvement was evaluated by the number of regions (bulbar, cervical and lumbosacral region) showing UMN signs at clinical examination. In contrast, we used clinical and electromyographic (EMG) assessments according to the Awaji criteria to define the extent of LMN involvement (de Carvalho et al., 2008). To further investigate the correlation between plasma p-tau181 levels and LMN dysfunction, we assigned to each patient with available EMG data (n=119) a denervation score (DS), as reported (Abu-Rumeileh et al., 2020). Briefly, in the affected muscle with the highest denervation activity (DP, sharp waves or fibrillation) in each region (bulbar, cervical or lumbosacral), we derived a numerical score (0–10) based on the number of sites per muscle showing DP, with each muscle explored in 10 sites. The disease progression rate at the baseline visit (b-DPR) was calculated as follows: (48−ALSFRS-R score at the time of sampling)/months elapsed between disease onset and sampling) (Lu et al., 2015). Accordingly, patients were divided into slow (b-DPR< 0.5), intermediate (b-DPR 0.5–1) and fast progressors (b-DPR> 1). Thirty-nine of the 148 patients with ALS had plasma samples from two or more follow-up visits. Repeated sampling was performed at non-standardised time points after the diagnostic assessment. In detail, 15 patients were sampled twice, 12 three times, and nine and three patients had samples from 4 and 5 visits, respectively. The median follow-up was 13 months (IQR 7–22). For these patients, we calculated the longitudinal disease progression rate (l-DPR) as the change in the ALSFRS-R between the last and the baseline visits divided by the number of months between the visits. Accordingly, patients with ALS were further classified into fast progressors (l-DPR>1), intermediate progressors (l-DPR 0.5–1) and slow progressors (l-DPR<0.5).

3.2.2 CSF and plasma analyses

EDTA plasma samples were collected, aliquoted and stored at −80°C according to standard procedures. CSF samples were obtained by lumbar puncture (LP), centrifuged in case of blood contamination, divided into aliquots and stored in polypropylene tubes at −80°C until analysis. Plasma p-tau181 and NfL levels in all participants, and CSF NfL values in patients with ALS, were determined with the Single molecule array (Simoa) technology on a SR-X instrument using commercially available kits (Quanterix, Billerica, Massachusetts, USA). The mean intra-assay and interassay coefficients of variation (CVs) were below 15% for both biomarkers. CSF NfL in patients with AD was quantified by a validated commercial ELISA assay (NfL ELISA kit, IBL, Hamburg, Germany). CSF t-tau, p-tau181, Aβ42 and Aβ40 were measured by automated chemiluminescent enzyme immunoassay on the Lumipulse G600II platform (Fujirebio, Gent, Belgium). The mean

intraassay and interassay CVs for these markers were <8%. The Aβ42/Aβ40 was calculated as described (Baiardi et al., 2019). Pathological values for the AD core markers were determined according to in-house validated cutoffs. Specifically, a CSF Aβ42/Aβ40×10 ratio < 0.68 was considered supportive of amyloid deposition (i.e. A+ according to the ATN classification).

3.2.3 Statistical analyses

Statistical analysis was performed using Stata SE V.14.2 (StataCorp) and GraphPad Prism V.7 (GraphPad Software, La Jolla, California, USA) software. Biomarker values were transformed into a logarithmic scale to obtain a normal data distribution. For continuous variables, the Kruskal-Wallis test (followed by Dunn-Bonferroni post hoc test) or the one-way analysis of variance (followed by Tukey's post hoc test) were used for multiple group comparisons. The $\chi 2$ test was applied for categorical variables. For the cross-sectional analysis, Spearman's r coefficient was used to test the correlation between plasma p-tau181 and clinical/neurophysiological variables. Furthermore, the association between plasma p-tau181 and the degree of UMN and/or LMN involvement was assessed using univariate and multivariate linear regression models with the log-transformed plasma p-tau181 values as dependent variables and the extent of (1) UMN involvement, (2) LMN involvement, (3) UMN and LMN involvement as independent variables. In the multivariable models, we adjusted for age at sampling, sex, genetic status, presence of FTD, ALSFRS-R scale, ALS phenotype, type of onset, MRC and King's scores. The results are presented as ß coefficients and 95% CI. For the prognostic analysis, the cumulative time-dependent probability of death was calculated by the Kaplan-Meier estimate. The time of entry into the analysis was the date of the first sampling, and the time of the endpoint was the date of death/ tracheostomy or the date of the last follow-up information, whichever came first. We performed univariate and multivariate Cox regression models to study prognostic factors in ALS. The multivariate Cox regression analysis was adjusted for age at onset, type of onset, ALSFRS-R score, presence of FTD, b-DPR and King's score. The results are presented as hazard ratios (HRs) and 95% CI. The assumption of proportional hazard was assessed by Schoenfeld residuals. Differences were considered significant at $p < 0.05$.

For the longitudinal analysis, a linear mixed effect modelling analysis with a random slope and random intercept was performed to evaluate the rate of change over the time of plasma p-tau181 in the patients with ALS stratified into fast, intermediate and slow progressors, according to both basal and longitudinal DPR. The results are presented as ß coefficients and 95% CI.

3.3 Results

3.3.1. Demographic data and distribution of plasma p-tau181 values across the diagnostic groups

Demographic and clinical data of the studied population are shown in Tables 1 and 2. Post hoc analysis showed no significant difference in the age at sampling between ALS patients and controls ($p>0.99$). Plasma p-tau181 levels significantly differed across diagnostic categories (Table 1 and Figure 1). Post hoc analysis showed that patients with ALS and AD had substantially higher p-tau181 levels than controls ($p <0.001$), with significantly lower levels in ALS compared with AD ($p=0.02$). SMA patients also showed significantly higher p-tau181 levels than controls ($p=0.03$), in line with ALS participants ($p=0.42$). Of note, patients with ALS (main group) had significantly lower plasma p-tau181 values than A+ALS patients ($p=0.005$). In contrast, patients with ALS showed significantly lower CSF p-tau values than AD participants ($p <0.001$).

Table 1. Demographic variables and biomarker values in the study population across the different diagnostic categories

	ALS n=148	**ALS A+ n=20**	**AD n=88**	**SMA n=12**	**Controls n=60**	**p values**
Female, N (%)	54 (36.5)	9 (45.0)	53 (60.2)	6 (50)	26 (43.3)	**0.07**
Age at plasma sampling, years	62 (51-69)	74.5 (70.0-81.5)	67 (61-73.5)	35.5 (25.5-48.5)	60.5 (58.2-63.0)	**<0.0001[g]**
Plasma p-tau 181[a]	2.47 (1.40-4.29)	4.39 (2.68-6.31)	3.26 (2.46-4.30)	1.62 (0.95-2.69)	1.04 (0.78-1.26)	**<0.0001[h]**
Plasma NfL[a]	73.5 (42.8-116.1)[b]	58.8 (38.5-103.0)[e]	21.1 (16.8-26.4)	-	10.1 (8.5-14.6)	**<0.0001[i]**
CSF p-tau181[a]	33 (26.2-42.6)[c]	58.1 (43.7-80-1)	109 (82-159)	-	-	**<0.0001[j]**
CSF NfL[a]	6307 (3250-12011)[d]	4324 (2329-6390)[f]	1076 (862.5-1488)	-	-	**<0.0001[k]**

a: Data are expressed as median (interquartile range); b: Data are available only in 144 patients; c: Data are available only in 130 patients; d: Data are available only in 117 patients, e: Data are available only in 19 patients; f: Data are available only in 18 patients; g: the p value shown in the table was calculated through the Kruskal-Wallis test, significant post-hoc comparisons (Dunn-Bonferroni test) ALS vs. ALS A+, ALS A+ vs. SMA, ALS A+ vs. controls and AD vs. SMA $p<0.0001$, ALS vs. AD and ALS vs. SMA $p=0.002$, ALS A+ vs. AD and AD vs. controls $p=0.01$, SMA vs. controls $p=0.008$; h-k: p values shown in the table were calculated through the ANOVA test (biomarker values were transformed into a logarithmic scale to obtain a normal data distribution; p values of statistically significant post-hoc comparisons (Tukey's test) are detailed in the table legends; h: ALS vs. ALS A+ $p=0.005$, ALS vs. AD $p=0.02$, ALS vs. controls, ALS A+ vs. controls and AD vs. controls $p<0.0001$, ALS A+ vs. SMA $p=0.002$, AD vs. SMA $p=0.02$, SMA vs. controls $p=0.03$; i: ALS vs. AD, ALS vs. controls, ALS A+ vs. controls, ALS A+ vs. AD, AD vs. controls $p<0.0001$; j: ALS vs. ALS A+, ALS vs. AD and ALS A+ vs. AD, $p<0.0001$; k: ALS vs. AD and ALS A+ vs. AD $p<0.0001$.

Keys: ALS, Amyotrophic Lateral Sclerosis; AD, Alzheimer's Disease; A+, amyloid positive; CSF, cerebrospinal fluid; NfL, Neurofilament light chain; p-tau 181, phosphorylated tau 181; SMA, spinal muscular atrophy.

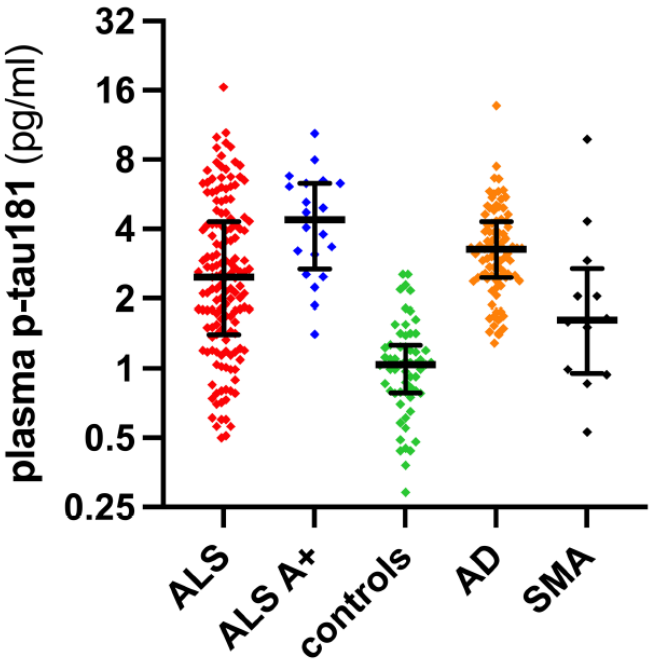
Table 2. Clinical features of patients with ALS

ALS patients (n=148)			
	N (%)		**Median (IQR)**
Type of onset		DD from first symptom to sampling (m)	13.5 (8-24)
Bulbar	33 (22.3)	ALSFRS-R scale *(n=144)*	42 (38.2-44.0)
Spinal	96 (64.9)	MRC score *(n=147)*	4.6 (4.2-4.8)
Pseudopolyneuritic	11 (7.4)	FVC[a] *(n=134)*	92.5 (76.7-106.3)
Pyramidal	8 (5.4)	BMI *(n=141)*	24.6 (22-27.55)
Clinical phenotype		Creatinine *(n=139)*	0.78 (0.69-1.05)
Classic			N (%)
Bulbar		**King's staging**	
Respiratory		1	7 (4.7)
PUMN		2	47 (31.7)
PLS		3	79 (53.4)
Flail arm syndrome		4	15 (10.1)
Flail leg syndrome		**MiToS staging *(n=140)***	
PMA		0	118 (84.3)
Deceased/with tracheostomy	67 (45.3)	1	18 (12.8)
Genetic status *(n=142)*		2	3 (2.1)
C9Orf72 RE carriers	15 (10.6)	3	1 (0.7)
SOD1 mutation carriers	3 (2.1)		
TARDBP mutation carriers	2 (1.4)	**FT9 staging *(n=140)***	
Definite ALS	24 (16.2)	0	27 (19.3)
Probable ALS	54 (36.5)	1	69 (49.3)
Probable laboratory-supported ALS	33 (22.3)	2	32 (22.8)
Possible ALS	28 (18.9)	3	8 (5.7)
Unclassified (PMA)	9 (6.1)	4	4 (2.8)
Patients with FTD	17 (11.5)		

a: FVC is expressed as a percentage of the predicted volume.

Key: ALS, amyotrophic lateral sclerosis; ALSFRS-R, Revised Amyotrophic Lateral Sclerosis Functional Rating; BMI, body mass index; DD, disease duration; FVC, forced vital capacity; FTD, frontotemporal dementia; FT9, Fine'til staging; IQR, interquartile range; m, months; MiToS, Milan-Torino staging; MRC, Medical Research Council; PLMN, predominant lower motor neuron; PLS, primary lateral sclerosis; PMA, progressive muscular atrophy; PUMN, predominant upper motor neuron; RE, repeats expansion; y, years.

Figure 1. Plasma p-tau181 values in the different diagnostic categories included. Thick lines represent median and IQR. p-tau, phosphorylated tau 181.



3.3.2 Association between biomarkers and clinical variables in ALS patients

CSF p-tau181 and plasma p-tau181 did not correlate at baseline (r=0.08, p=0.37). In contrast, cNfL and pNfL values strongly correlated at first LP (r=0.79, p<0.001). Plasma p-tau181 was not associated with pNfL (r=0.03, p=0.69) or cNfL (r=−0.03, p=0.72). The lack of correlation between plasma p-tau181 and NfL levels extended to the ALS A- (r=0.01, p=0.87) and ALS A+ (r=0.28, p=0.25) subgroups.

Plasma p-tau181 levels were weakly correlated with age at sample collection (r=0.25, p=0.02) and showed significantly higher values in males than females (median 2.77, IQR (1.55– 4.82) vs 1.89 (1.18–2.92), p=0.009).

We also found a weak correlation between plasma p-tau181 and ALSFRS-R (r=−0.21, p=0.01) and MRC (r=−0.37, p<0.0001), while there were no associations with BMI (p=0.098), King's stage (p=0.06), MiToS (p=0.33), FT9 (r=0.16, p=0.052), creatinine values (p=0.46), CVF (p=0.22) or b-DPR (p=0.78). The disease duration correlated weakly with only a trend of significance (r=0.16, p=0.05). Plasma p-tau181 levels significantly differed across clinical onset types (p=0.005), and post hoc analysis revealed significantly higher levels in spinal than in bulbar onset (p=0.005).

Accordingly, plasma p-tau181 levels significantly differed across ALS phenotypes (p=0.004), with post hoc analysis revealing considerably higher levels in classic than bulbar ALS (p=0.004) and in PLMN compared with bulbar ALS (p=0.006, Table 3).

Table 3. Plasma p-tau181 across ALS type of onset, ALS phenotypes and genetic status

Onset type (N)	**Plasma p-tau[a]**	**ALS phenotypes (N)**	**Plasma p-tau[a]**	**Genetic Status (N)**	**Plasma p-tau[a]**
Bulbar (33)	1.59 (1.01-2.6)	**Bulbar (18)**	1.28 (0.8-1.89)	**Wild-type patients (121)**	2.55 (1.55-4.22)
Spinal (96)	2.76 (1.69-4.59)	**Classic (89)**	2.72 (1.55-4.44)	***SOD1* patients (3)**	6.05 (2.92-6.67)
Pseudopolyneuritic (11)	3.02 (1.79-5.36)	**PLMN (26)**	2.76 (1.8-4.68)	***TARDBP* patients (2)**	0.955 (0.71-1.2)
Pyramidal (7)	1.8 (0.74-2.47)	**PUMN (13)**	1.69 (1.02-2.73)	***C9Orf72* patients (15)**	1.4 (0.61-2.11)

a: values are expressed as median (interquartile range). Key: p-tau, phosphorylated tau; N, number; PLMN, predominant lower motor neuron; PUMN, predominant upper motor neuron

Plasma p-tau181 levels were significantly lower in FTD-ALS than in pure motor ALS patients (2.7 (1.73–4.68) vs 1.33 (1.04–1.59), p=0.0001). Finally, plasma p-tau181 levels were significantly influenced by genetic status (p=0.007), with increased levels in patients carrying mutations in *SOD1* compared with *C9ORF72* (p=0.04) and *TARDBP*-mutated patients (p=0.04) (table 3).

3.3.3 Association between CSF and plasma p-tau181 and the extent of UMN and/or LMN degeneration in ALS patients

Plasma p-tau181 levels were not associated with either the number of body regions displaying UMN signs (p=0.10) or the number of districts showing both UMN and LMN signs (p=0.98). Conversely, there was a weak association with the number of body regions displaying LMN signs (Rho=0.28, p=0.0008). Accordingly, p-tau181 levels significantly increased with the increasing number of regions affected by isolated LMN signs (p=0.007), but not with the number of areas displaying isolated UMN signs or UMN and LMN signs (p=0.20 and 0.92 respectively), Table 4.

Table 4. Plasma p-tau181 levels according to the extent of UMN and/or LMN degeneration

		N	Plasma p-tau[a]
UMN and LMN degeneration	Zero region	16	2.39 (1.72-5.68)
	One region	46	2.11 (1.45-4.14)
	Two regions	57	2.6 (1.2-4.22)
	Three regions	28	2.29 (1.31-4.99)
UMN degeneration	Zero region	10	3.76 (2.3-6.74)
	One region	27	2.7 (1.79-4.18)
	Two regions	52	2.49 (1.19-4.06)
	Three regions	57	2.01 (1.33-4.02)
LMN degeneration	Zero region	5	1.64 (1.22-1.8)
	One region	19	1.51 (1.14-2.84)
	Two regions	58	2.21 (1.37-3.96)
	Three regions	65	3.1 (1.89-5.36)

a: values are expressed as median (interquartile range). Key: p-tau, phosphorylated tau; LMN, lower motor neuron; UMN, upper motor neuron; N, number.

After adjustment for covariates (i.e., age, sex, genetic status, presence of FTD, ALSFRS-R scale, ALS phenotype, type of onset, MRC score, and King's stage), the association between plasma p-tau181 levels and number of regions displaying LMN signs remained statistically significant (three areas *vs.* one region: ß=-0.46, 95% CI -0.89-0.03, p=0.036).

Regarding the extent of denervation, we found a significant correlation with the denervation degree in the lumbosacral region (Rho=0.51, p<0.0001) but not in the bulbar or cervical area (p=0.89 and p=0.77, respectively).

3.3.4 Prognostic value of plasma p-tau181 in ALS patients

Based on univariate Cox regression analysis (134 ALS patients; 67 dead), ALSFRS-R (p < 0.0001), DPR (p < 0.0001), FTD status (p=0.042), King's score (p < 0.0001), FVC (p < 0.0001), bulbar onset (p=0.004) and plasma p-tau181 (p=0.027) were identified as predictors of the mortality in ALS patients. Multivariate Cox regression confirmed the value of plasma p-tau181 (HR 1.90, CI 1.24-2.90, p=0.003) as independent predictors of mortality in ALS (Table 5).

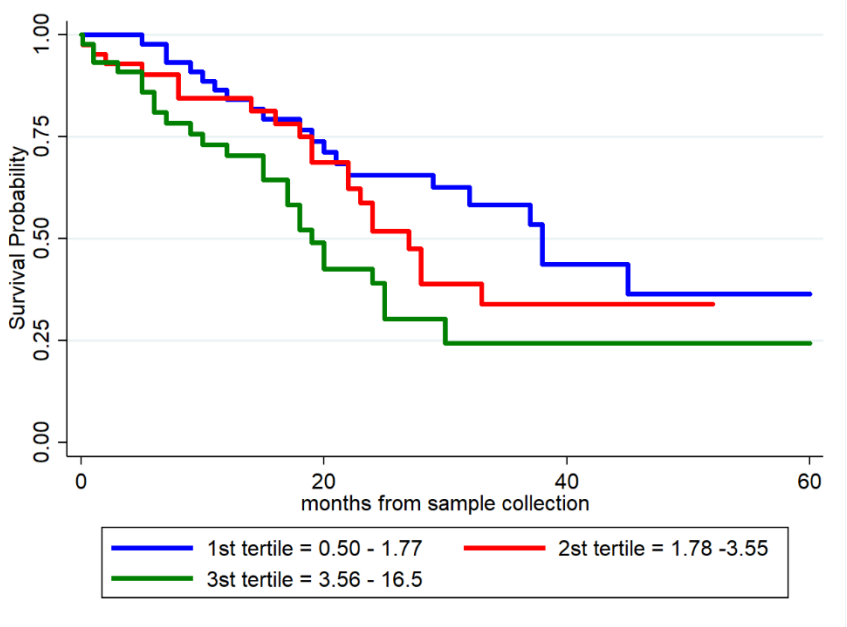
Accordingly, ALS patients with higher baseline plasma p-tau181 levels showed shorter survival (highest tertile of plasma p-tau181 vs. lowest tertile, HR 3.57, 95% CI=1.51-8.41, p=0.004) (Figure 2).

Table 5. Multivariate Cox Regression analysis for plasma p-tau181 and clinical prognostic factors in ALS

Variable		**HR (95% CI)**	**P-value**
Plasma p-tau181		1.90 (1.25-2.90)	**0.003**
Age at onset disease		1.02 (0.99-1.04)	0.08
Onset type	**Spinal**	Ref	Ref
	Bulbar	2.22 (1.11-4.42)	**0.024**
	Pyramidal	0.33 (0.92-1.19)	0.09
	Pseudopolyneuritic	0.35 (0.09-1.49)	0.15
ALSFRS-R scale		0.96 (0.92-1.001)	0.06
FTD status		1.95 (0.86-4.46)	0.11
King's score		1.72 (0.99-2.97)	0.053
b-DRP	**Slow progressors**	**Ref**	**Ref**
	Intermediate progressors	2.49 (1.35-4.61)	**0.004**
	Fast progressors	5.16 (2.40-11.07)	**<0.001**

Key: ALSFRS-R, Revised Amyotrophic Lateral Sclerosis Functional Rating; b-DPR, basal disease progression rate; CI, confidence interval; FTD, frontotemporal dementia; HR, hazard ratio; p-tau181, phosphorylated tau 181; Ref, reference

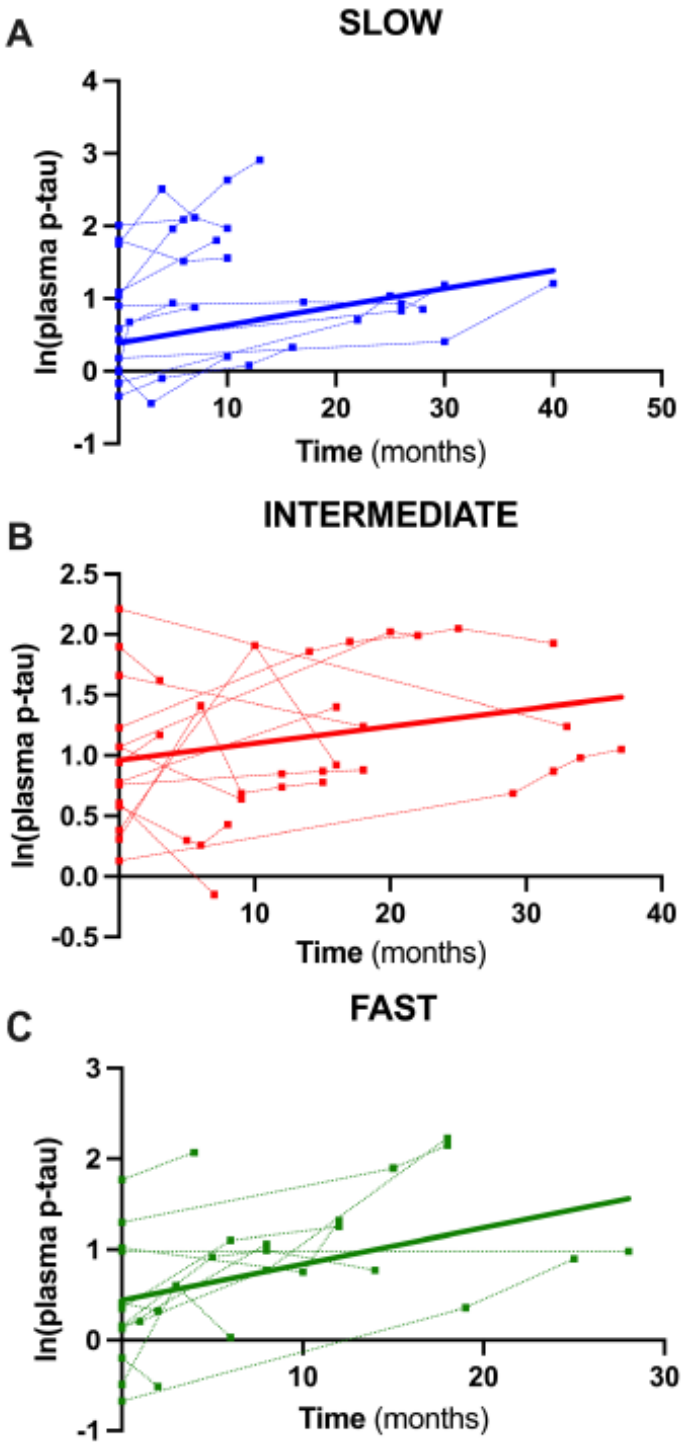
Figure 2. **Prognostic value of plasma p-tau181**. Survival curves in ALS patients according to the values of plasma p-tau181. Biomarker levels were stratified into low, mid and high tertiles and are expressed in pg/ml.



3.3.5. Longitudinal trajectories of plasma p-tau181 in ALS patients

No significant differences in the basal plasma p-tau181 values were detected among patients in the three disease progression groups (as calculated by both b- and l-DPR, Supplementary Table 5). After stratifying ALS patients according to the l-DPR, we observed a significant rise in the slopes of p-tau181 values over time (months) in all groups, with the fastest progressing group showing the most consistent increase in the biomarker levels (slow: β=0.025, CI 0.013-0.037, p<0.001; intermediate β=0.014, CI 0.002-0.026, p=0.02, fast β=0.044, CI 0.025-0.063, p<0.001, Figure 3). A similar rising trend in the biomarker values in all groups was also noted when stratifying patients according to the b-DPR (slow: β=0.021, CI 0.011-0.030, p<0.001; intermediate: β=0.026, CI 0.006-0.047, p=0.01; fast: β=0.033, CI 0.006-0.060, p=0.01).

Figure 3. **Longitudinal trajectories of plasma p-tau181**: Overall and single-patient longitudinal plasma p-tau behavior in the slow (A), intermediate (B) and fast (C) progressors, as defined through the l-DPR, showing an increasing trend over time. Thick lines represent the overall biomarker trend. Analyses were conducted through a linear mixed effects model. Key: p-tau, phosphorylated tau 181.



3.4 Discussion

In this study, we confirmed in a large cohort that patients with ALS show significantly elevated plasma p-tau181 levels that in most cases is unrelated to AD pathology. The biomarker change is likely also unrelated to the overall neuroaxonal damage, given the lack of association between plasma p-tau181 and both CSF and plasma NfL. Moreover, the lack of correlation between CSF and plasma p-tau181 strongly suggests a peripheral origin of the biomarker elevation. Our finding of an association between plasma p-tau181 levels and LMN dysfunction supports this interpretation. In contrast to Cousins et al (Cousins et al., 2022) we quantified the number of regions affected by clinical and/or EMG LMN signs rather than determining the presence or absence of LMN involvement in each region. Using both clinical and EMG assessments rather than the sole clinical evaluation, we could detect a subclinical LMN pathophysiological involvement (Krarup et al., 2011), adding strength to our results. Moreover, we showed that the association remained significant after covarying for potentially confounding clinical factors. Finally, we tested, for the first time, the association of plasma p-tau181 and quantitative EMG correlates of denervation. We found a moderate correlation between plasma p-tau181 levels and the denervation score in the lumbosacral region but not in the bulbar and cervical areas. Given the potential peripheral axonal derivation of plasma p-tau181, we speculate that the higher length of the nerve fibres arising from the lumbosacral region, compared with those of bulbar and cervical areas, implying a wider exchange surface with the vascular bed, might explain these results. Another explanation could be that plasma p-tau181 levels reflect the amount of denervated muscular fibres. We also found that patients with a spinal onset and with PLMN or classic phenotypes had significantly higher p-tau181 levels than those presenting with a bulbar onset and a bulbar phenotype, respectively. Notably, unlike Cousins et al (Cousins et al., 2022) we used a standardised phenotype classification based on the clinical longitudinal assessment of patients by expert neurologists besides the UMN-onset or LMN-onset anamnestic distinction. We also confirmed that patients with ALS with concomitant FTD display lower levels of plasma p-tau181 compared with those with pure motor ALS, making the sporadic reports on a limited tauopathy in ALS-FTD patients unrelated (Strong et al., 2006; Strong et al., 2020) to plasma p-tau181 concentrations and further indicating a peripheral contribution. Additionally, in our cohort, plasma p-tau181 levels were elevated in *SOD1* mutated patients compared with *C9Orf72* and *TARDBP* patients. With the necessary caution related to the low sample size, these data, in line with those previously reported (Cousins et al., 2022) also support a peripheral contribution to plasma p-tau 181 levels, given that the *SOD1* ALS phenotype is classically associated with a prevalent LMN degeneration (Chiò et al., 2020). Given the association of plasma p-tau181 with LMN dysfunction, we measured the biomarker in patients with SMA. Our

finding of significantly higher plasma p-tau181 values in SMA patients than in controls, with no significant difference with the ALS group, supports the association between LMN involvement and increased plasma p-tau181 levels. Considering the relatively small number of SMA patients, all classified as adult SMA2 and SMA3 patients, a more extensive study, including all SMA types, should confirm these results. Further studies are also needed to investigate plasma p-tau181 levels in other diseases affecting LMN, such as motor axonal neuropathies. These findings have significant implications for current proposed biomarker strategies to detect early AD pathology in the general population. Evidence indicates that blood-based biomarkers, especially p-tau181 and other p-tau isoforms, can discriminate patients with AD pathology even at a preclinical or prodromal stage (Palmqvist et al., 2021; Karikari et al., 2020). However, determining if confounding factors affect the blood levels of the biomarker, and maybe even their clinical utility, is necessary before widespread implementation. Our results combined with those of a previous study (Cousins et al., 2022) suggest that tau isoforms, likely of peripheral origin rather than brain derived, might represent a significant confounding factor for these assays. Future studies comparing assays targeting different p-tau and tau isoforms should validate p-tau assays for their specificity for brain-derived p-tau. In this study, we also showed that plasma p-tau181 levels predict survival in ALS, regardless of other clinical variables already associated with ALS prognosis. Furthermore, we explored the longitudinal behaviour of this biomarker in a subset of patients with ALS, showing a consistent increase in its levels in the disease course, especially in patients with a faster disease progression, as stratified at both basal visits and during the disease course. This is divergent from the longitudinal behaviour of blood NfL, which is stable during the disease course, as shown by several studies including our previous findings (Lu et al., 2015; Verde et al., 2019). The longitudinal behaviour of p-tau181 in patients with ALS could reflect the ongoing denervation until the final phase of the disease, with an addictive effect of damage of the peripheral fibres, initially insulted but still undergoing axonal rearrangement (Marshall et al., 2021). CSF and blood NfL probably have a more robust predictive value on survival (Benatar et al., 2020) than plasma p-tau181 in patients with ALS. Similarly, plasma NfL might be a more promising treatment-monitoring candidate for its stability during the disease course. Nevertheless, the discovery of other reliable biomarkers is valuable due to the recent advances in ALS clinical trials and the highly variable pharmacodynamic targets implied in ALS research (Suzuki et al., 2022). Including a significant number of well-characterised patients with ALS with available quantitative electromyographic data is the major strength of our study. The association with survival data and the availability of longitudinally repeatedly sampled patients with ALS constitute a significant added value to our work. Our study is not free of limitations. First, we could not exclude a

concomitant AD pathology neuropathologically, the current gold-standard approach. However, CSF analyses with automated platforms, including the determination of the Aβ42/ Aβ40 ratio, have demonstrated high accuracy in predicting AD pathology in vivo. A second limitation is the lack of standardised time points for the longitudinal sampling of patients with ALS. Finally, the limited number of SMA patients included did not allow us to draw a definitive conclusion on the significance of the trend of increased plasma p-tau181 in these patients. In conclusion, our study provides evidence that plasma p-tau181 is elevated in patients with ALS and is related to LMN dysfunction, especially at the lumbosacral level. Moreover, plasma p-tau181 levels, likely from a peripheral source, increase progressively in the disease course and predict survival in patients with ALS. Finally, the study further demonstrates that plasma p-tau181 is a less specific AD biomarker than CSF p-tau, making the peripheral source of p-tau a possible confounding factor in the use of this marker for the screening of the general population with cognitive decline.

4. Amyloid-beta co-pathology is a major determinant of the elevated plasma GFAP values in amyotrophic lateral sclerosis

4.1 Background and aims

As previously mentioned, the clinical spectrum of ALS is not limited to motor abnormalities, with up to half of patients displaying cognitive and/or behavioral impairment at different stages of severity and around 10-15% of subjects fulfilling the criteria for full-blown frontotemporal dementia (FTD). Moreover, the severity of cognitive decline seems to worsen with the progression of the disease, similar to motor impairment, further contributing to the disability of patients (Chiò et al., 2019). Neuropathologically, ALS is a TDP-43 proteinopathy characterized by TDP-43 enriched inclusions in affected neurons. However, due to the high disease prevalence, a significant proportion of ALS patients develop secondary Alzheimer's disease (AD) pathological changes of various severity, which may also contribute to cognitive impairment in these patients (Behrouzi et al., 2016; Hamilton et al., 2004).

As stated before, biofluid biomarkers are urgently needed in the ALS field to improve the diagnostic accuracy in vitam, predict and track the disease progression and monitor the response to potential disease-modifying agents. Besides neurofilament light chain protein, glial activation and neuroinflammation markers are also increasingly exploited in neurodegenerative diseases, given their relevance to the pathogenesis of many neurological disorders (Lee et al., 2022; Qian et al., 2023). Glial fibrillary acidic protein (GFAP) is the main component of the intermediate filaments, and is expressed in mature astrocytes in the grey and white matter, the cerebellum, the subventricular and subgranular zones, and Mueller cells in the retina (Abdelhak et al., 2022). GFAP levels have been recently explored in both CSF and blood of patients with different central nervous system (CNS) disorders. The mechanisms underlying drainage of GFAP and its breakdown products into the blood under pathological conditions is still not completely understood and matter of continuing debate. Evidence indicates that GFAP drainage is likely result from a continuous bidirectional fluid exchange at the barriers of the CNS (that is, the blood-brain and blood–CSF barrier) (Tumani et al., 2017), and its spillover in the extracellular space increases following astrocyte damage (Abdelhak et al., 2022; Yang et al., 2015). Most significantly, plasma GFAP levels have shown to be considerably higher in patients with Alzheimer's disease than in other diseases associated with dementia, even in a prodromal or asymptomatic phase (Pereira et al., 2021; Benedet et al., 2021; Baiardi et al., 2022; Bellaver et al., 2023).

As for ALS, data on CSF and blood GFAP levels are fewer and less concordant, with a preliminary study showing elevated CSF GFAP values in ALS patients compared to controls (Benninger et al., 2016), and others reporting no difference, in blood or CSF, between ALS and healthy subjects (Oeckl

et al., 2019; Falzone et al., 2022). Recently, a single study showed higher blood GFAP values in ALS than in controls, also reporting a correlation with parameters of cognitive and behavioral impairment (Verde et al., 2023).

In the present study (Mastrangelo and Vacchiano et al., 2023), we compared plasma GFAP levels in the most extensive ALS cohort examined to date with those of patients with fronto-temporal dementia (FTD) and neurological controls. Furthermore, we evaluated the association of plasma GFAP values with clinical variables and plasmatic and CSF levels of other biofluid biomarkers, including those reflecting AD pathology. Finally, we studied the value of plasma GFAP in predicting survival in ALS patients.

4.2 Methods

4.2.1 Inclusion criteria and clinical assessment

We included 156 patients diagnosed with ALS according to the Revised El Escorial criteria (Brooks et al., 2000), evaluated at the Institute of Neurological Sciences of Bologna between September 2014 and December 2022. All patients had baseline CSF and plasma samples available. We also separately studied 50 patients with a clinical diagnosis of FTD according to international criteria (Rascovsky et al., 2011; Gorno-Tempini et al., 2011), without any signs of UMN or LMN impairment (pure FTD) and a negative CSF amyloid profile. Finally, 48 subjects without clinical evidence of neurological disease were also included as controls. For ALS patients, we collected the following clinical variables at baseline: age at onset; sex; disease duration (time elapsed between the disease onset and CSF/plasma sampling); type of onset; ALSFRS-R; MRC scale of 0 to 5; FVC; BMI; and King's clinical stage. Patients were subdivided according to a validated classification (Chiò et al., 2011) into the following phenotype categories: classic, bulbar, respiratory, UMN-predominant (PUMN), primary lateral sclerosis (PLS), flail arm syndrome, flail leg syndrome, and progressive muscular atrophy (PMA). However, to reach sufficient statistical power for comparisons, we also grouped patients into main categories, i.e., classic (including respiratory), bulbar, PUMN (i.e., PUMN and PLS), and LMN-predominant (PLMN, including flail arm/leg and PMA). One hundred and fifty-three ALS patients performed genetic analysis, including the screening for mutations in the most frequent ALS-related genes (i.e., *SOD1*, *FUS*, *TARDBP*, and the *C9Orf72* repeats expansion) (Bartoletti-Stella et al., 2021). Furthermore, the apolipoprotein E (APOE) genotype was analyzed, and APOE ε4 carriers were defined as individuals with at least one APOE ε4 allele. Cognitive status was evaluated through a neuropsychological assessment encompassing executive function, memory, visuospatial function, language, and social cognition domains. The battery included the MMSE, the Frontal assessment battery (FAB) (Dubois et al., 2000), the Letter Fluency Test (FAS); the Category Fluency

Test; the BMDB (Gallassi et al., 1986), and the ECAS (Poletti et al., 2016). For this latter, we computed the five cognitive domains of executive functions, verbal fluency, language, memory, and visuospatial functions, composite ALS-specific (i.e., executive + verbal fluency + language) and ALS-nonspecific (i.e., memory + visuospatial) subscores. ECAS scores were adjusted for age and education, as previously reported (Siciliano et al., 2017). Patients were classified accordingly into five categories (purely motor ALS (ALS-CN), ALS with cognitive impairment (ALSci), ALS with behavioral impairment (ALSbi), and ALS with cognitive and behavioral impairment (ALScbi), FTD) (Strong et al., 2017). To enable statistical analysis with sufficient power, we grouped ALSbi and ALSci categories. We also used a binary classification (ALS-FTD or pure motor ALS patients), according to the presence of FTD only, as clinically assessed (Rascovsky et al., 2011). UMN involvement was scored by the number of regions (bulbar, cervical, and lumbosacral region) showing UMN signs at clinical assessment. In contrast, we used clinical and electromyography (EMG) assessments to establish the extent of LMN involvement according to the Awaji criteria (de Carvalho et al., 2008). The DPR was calculated using the following formula: (48−ALSFRS-R score at the time of sampling)/months elapsed between disease onset and sampling) and patients were accordingly divided into slow (DPR < 0.5), intermediate (DPR 0–5–1), and fast progressors (DPR > 1). Patients performed routine laboratory blood examinations, among which we collected serum creatinine, CPK, and serum albumin. None of the ALS patients were under Riluzole treatment at the time of sampling.

4.2.2. CSF and Plasma analyses

EDTA plasma samples were collected, aliquoted, and stored at −80 ◦C, according to standard procedures. CSF samples were obtained by lumbar puncture following a routine procedure, centrifuged in case of blood contamination (even minimal), divided into aliquots, and stored in polypropylene tubes at −80 ◦C until analysis. From CSF routine analysis, we extrapolated CSF albumin to calculate the albumin index.

Plasma GFAP, Plasma NfL, and CSF NfL levels were determined with the Single molecule array (Simoa) technology (Wang et al., 2018) on a Simoa SR-X instrument using the commercially available GFAP Discovery and NF-light Advantage Kits (Quanterix, Billerica, MA, USA). The mean intra- and inter-assay coefficients of variation (CVs) were below 15% for all analyses.

CSF Aβ42, Aβ40, p-tau, and t-tau were measured by automated chemiluminescent enzyme immunoassay on the Lumipulse G600II platform (Fujirebio, Gent, Belgium). The inter-assay CVs were <8% for all biomarkers. The Aβ42/Aβ40 was calculated as described (Baiardi et al., 2019). We used in-house validated cutoffs to determine pathological values for the AD core markers. In particular, a CSF Aβ42/Aβ40 ratio < 0.68 was considered supportive of amyloid deposition (i.e., A+

according to the ATN classification (Jack et al., 2016)), while a CSF phosphorylated tau at site 181 (p-tau181) > 62 pg/mL was considered indicative of p-tau deposition (i.e., T+)

4.2.3 Statistical Analyses

Statistical analyses were performed using Stata SE V.14.2 (StataCorp, College Station, TX, USA) and GraphPad Prism V.7 (GraphPad Software, La Jolla, CA, USA) software. For continuous variables, the Mann–Whitney or the Kruskal–Wallis test (followed by the Dunn–Bonferroni post hoc test) was used for comparisons between groups. Fisher's test was applied for categorical variables. We used Spearman's Rho coefficient to test the correlation between plasma GFAP levels and other CSF/plasma biomarkers (i.e., plasma p-Tau181, CSF/plasma NfL) and age- and education-adjusted scores from neuropsychological tests.

The association between plasma GFAP levels and clinical variables was assessed using univariable and multivariable models with the log-transformed plasma GFAP values as dependent variables and the clinical variables as independent variables. In the multivariable models, we adjusted for age at sampling, ALSFRS-R scale, A status, DPR, FVC, and creatinine. The results are presented as ß coefficients and 95% Confidence Interval (95% CI).

Receiver operating characteristic (ROC) analyses were performed to establish the accuracy of different plasma biomarkers in the discrimination of ALS patients according to their A and T status. ROC curves were compared through the DeLong test. The optimal cut-off value for each biomarker was defined using the maximized Youden Index.

For the prognostic analysis, the Kaplan–Meier estimate calculated the cumulative time-dependent probability of death. The time of entry into the analysis was the date of the first sampling, and the endpoint was the date of death/tracheostomy or the date of the last follow-up information, whichever came first. Univariable and multivariable Cox regression models were performed to study prognostic factors in ALS. In detail, we performed two separate multivariable analyses: one including plasma GFAP and clinical variables (age at onset, type of onset, ALSFRS-R score, presence of FTD, DPR) and the other one with other plasma biomarkers with known prognostic value (i.e., plasma GFAP, NfL, and p-tau181). The results are presented as Hazard Ratios (HR) and 95% CI.

The assumption of proportional hazard was assessed by Schoenfeld residuals.

Differences were considered significant at $p < 0.05$.

4.3 Results

4.3.1 Distribution of Plasma GFAP Level Values across the Diagnostic Categories and Clinical Correlates of Plasma GFAP in ALS Patients

The demographic variables of ALS patients and controls and clinical features of the ALS cohort are detailed in Tables 1 and 2.

Table 1. Demographic variables and biomarkers values in ALS patients and controls.

	ALS patients (n=156)	**Controls (n=48)**	**p values**
Age at sampling, years*	66.0 (56.0-72.0)	61.0 (60.0-64.0)	0.07
Female, n (%)	58 (37.2)	16 (33.3)	0.73
Plasma GFAP, pg/ml*	159.70 (117.30-236.70)	125.9 (93.52-154.70)	**0.0004**
Plasma NfL, pg/ml*	70.50 (41.25-113.70)	10.76 (9.41-15.71)	**<0.0001**
Plasma p-tau181, pg/ml*	2.71 (1.74-4.96)	0.99 (0.76-1.36)	**<0.0001**

*: data are expressed as median (IQR). Significant p-values are reported in bold. Abbreviations: ALS, amyotrophic lateral sclerosis; GFAP, glial fibrillary acidic protein; NfL, neurofilament light chain; p-tau181, plasma phosphorylated tau 181.

Table 2. Clinical features of ALS patients

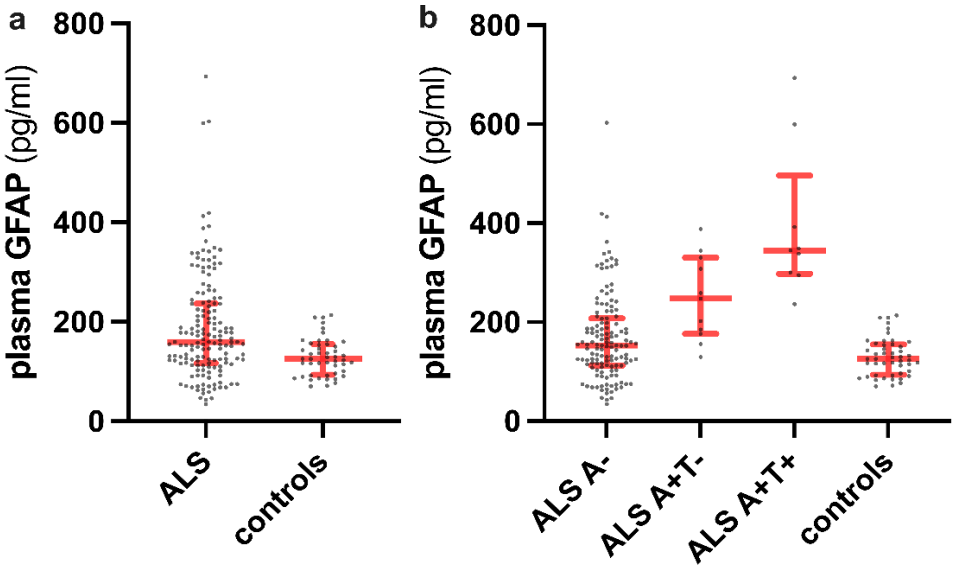
ALS patients (n=156)	
	N (%)
Type of onset	
Bulbar	34 (21.8)
Spinal	105 (67.3)
Pseudopolyneuritic	12 (7.7)
Pyramidal	5 (3.2)
Clinical phenotype	
Classic	92 (58.9)
Bulbar	22 (14.1)
Respiratory	1 (0.6)
PUMN	11 (7.0)
PLS	3 (1.9)
Flail arm syndrome	10 (6.4)
Flail leg syndrome	7 (4.5)
PMA	10 (6.4)
Diagnostic categories	
Definite ALS	27 (17.3)
Probable ALS	58 (37.2)

Probable laboratory-supported ALS	35 (22.4)
Possible ALS	26 (16.7)
Unclassified (PMA)	10 (6.4)
King's staging	
1	8 (5.1)
2	53 (34.0)
3	83 (53.2)
4	12 (7.7)
Strong's categories *(n=128)*	
ALS-CN	77 (60.1)
ALSbi	21 (16.4)
ALSci	7 (5.5)
ALScbi	9 (7.0)
ALS-FTD	14 (10.9)
Genetic status *(n=153)*	
C9Orf72 RE carriers	15 (9.8)
SOD1 mutation carriers	3 (2.0)
TARDBP mutation carriers	1 (0.6)
Wild-type	134 (87.6)
Deceased/with tracheostomy	77 (49.3)
	Median (IQR)
Disease duration (months)	13 (8-24)
ALSFRS-R scale *(n=154)*	41 (38.0-44.0)
MRC score *(n=155)*	4.6 (4.25-4.8)
FVC* *(n=140)*	140 (74.5-106.0)
BMI *(n=146)*	24.6 (22.1-27.7)
Creatinine	0.74 (0.65-0.85)
CPK *(n=155)*	197 (120-379)
Blood-brain barrier index *(n=153)*	7.0 (5.5-10.6)

*Expressed as a percentage of the predicted volume. If not otherwise specified, data are available for the whole ALS cohort. Abbreviations: ALS, amyotrophic lateral sclerosis; ALSbi, amyotrophic lateral sclerosis with behavioral impairment; ALScbi, amyotrophic lateral sclerosis with combined cognitive and behavioural impairment; ALSci, amyotrophic lateral sclerosis with cognitive impairment; ALS-CN, cognitively normal amyotrophic lateral sclerosis; ALSFRS-R, Revised Amyotrophic Lateral Sclerosis Functional rating scale; BMI, body mass index; CPK, creatine-phosphokinase; FVC, forced vital capacity; FTD, frontotemporal dementia; IQR, interquartile range; MRC, Medical Research Council; PLS, primary lateral sclerosis; PMA, progressive muscular atrophy; PUMN, prevalent upper motor neuron; RE, repeats expansion.

Age at sampling (p=0.07) and sex distribution (p=0.73) were not significantly different between ALS patients and controls. ALS patients showed higher plasma GFAP levels than controls (p=0.0004) (Figure 1a).

Figure 1. Plasma GFAP levels in the whole ALS cohort compared to controls **(a)** and in the ALS patients stratified by A and T status **(b)**.



Plasma GFAP levels were not significantly different across onset types (p=0.52), clinical phenotypes (p=0.65), King's stages (p=0.52), genetic status (p=0.59) and different numbers of regions with UMN (p=0.07) or LMN signs (p=0.57) or both (p=0.07). A slight increase in plasma GFAP levels in ALS females compared to males almost reached statistical significance (179.43 [126.4-238] *vs.* 152.19 [110.4-231.9], p=0.052).

Using regression analysis, we found that GFAP levels were significantly influenced by age at both onset (β=0.026, 95% CI 0.02 to 0.032, p<0.0001) and sampling (β=0.026, 95% CI 0.020 to 0.032, p<0.001), ALSFRS-R score (β=-0.031, 95% CI -0.046 to -0.017, p<0.001), DPR (β=0.119, 95% CI 0.023 to 0.217, p=0.016), FVC values (β=-0.004, 95% CI -0.008 to -0.001, p=0.02), and creatinine levels (β=0.699, 95% CI 0.310 to 1.088, p=0.0005).

In contrast, GFAP values were not related to disease duration (p=0.7), MRC score (p=0.36), CK levels (p=0.095), albumin index (p=0.2), and BMI (p=0.28). A multivariable linear regression model after adjusting for age at sampling, FVC, creatinine values, ALSFRS-R score, DPR, and A status confirmed that GFAP values were significantly influenced by age at sampling (β=0.019, 95% CI 0.013 to 0.026, p<0.001), creatinine (β=0.573, 95% CI 0.239 to 0.906, p=0.001), ALSFRS-R scale (β=-0.023, 95% CI -0.039 to -0.007, p=0.004) and A status (β=0.333, 95% CI 0.101 to 0.565, p=0.005).

4.3.2 Association of plasma GFAP with measures of cognitive impairment in ALS patients

Plasma GFAP did not statistically differ among ALS patients belonging to different Strong's Categories (p=0.16) but was higher in ALS patients with associated FTD (230.7 [154-317.9] *vs.* 157.8 [116.6-225.25], p=0.042) as compared to pure motor ALS.

Plasma GFAP levels significantly differed among ALS-FTD, pure FTD and pure motor ALS patients (namely without clinical signs of FTD) with a negative A status (p=0.001), with the post-hoc analysis revealing significantly higher levels in pure FTD (199.0 [132.3-293.9]) than in pure motor ALS subjects (n=125) (152.2 [111.3-197.3], p=0.001).

GFAP levels correlated with ALS-specific subscores of ECAS (Rho=-0.22, p=0.04), BMDB total score (Rho=-0.23, p=0.019), Category Fluency scores (Rho=-0.20, p=0.036) and Freehand copy of drawings (Rho=-0.26, p=0.01). A trend of significance was observed with ECAS total score (Rho=-0.20, p=0.06), ECAS executive functions (Rho=-0.19, p=0.07), ECAS memory (Rho=-0.2, p=0.06), Letter Fluency scores (Rho=-0.17, p=0.07). No correlations were found with other ECAS subscores and other neuropsychological tests.

The association of plasma GFAP with BMDB total score (Rho=-0.20, p=0.048) and Freehand copy of drawings (Rho=-0.24, p=0.02) was retained after excluding ALS patients with a positive CSF amyloid profile.

4.3.3 Association of plasma GFAP with other plasma and CSF biomarkers in ALS patients

In ALS patients, a moderate inverse correlation was found between plasma GFAP and CSF Aβ ratio (Rho=-0.34, p<0.001), which was consistent even after accounting for age at sampling (β=-0.84; p<0.001). Plasma GFAP was weakly associated with plasma NfL (Rho=0.30, p=0.0001), CSF t-tau (Rho=0.27, p=0.004), plasma p-tau181 (Rho=0.25, p=0.001) and CSF p-tau (Rho=0.23, p=0.004). There was no association between plasma GFAP and CSF NfL (Rho=0.09, p=0.25), even after accounting for plasma creatinine (p=0.23) or disease duration (p=0.11).

4.3.4 Plasma GFAP levels and clinical variables according to A and T status in ALS patients

Due to the moderate association between plasma GFAP and CSF Aβ ratio, we stratified ALS patients according to their A and T status.

20 ALS patients (12.8%) showed a positive amyloid status (A+), and 9 of them (5.8% of the whole ALS cohort) had a CSF profile also suggestive of tau deposition (A+T+ profile). At sampling, A+ ALS patients were significantly older than those A- (74.5 [70.2-81.5] vs. 64.0 [55.0-71.0], p<0.0001). Plasma GFAP significantly differed among A+T+, A+T-, A- ALS patients and controls (p<0.0001), with each A+ ALS subgroup showing higher values than controls (A+T+ *vs.* controls, p<0.0001; A+T- *vs.* controls, p=0.0003) and A- subjects (A+T+ *vs.* A-, p<0.0001; A+T- *vs.* A-, p=0.02). Plasma

GFAP did not significantly differ between A+T+ and A+T- ALS patients (p>0.99), while the comparison between A- ALS patients and controls reached a trend of significance (p=0.07) (Figure 1b). Biomarker values in ALS patients stratified by their A and T status are reported in Table 3.

Table 3. Biomarkers values and APOE status in ALS patients stratified by their A and T status

	ALS, A+T+ (n=9)	**ALS, A+T- (n=11)**	**ALS, A- (n=136)**	**p values**
Plasma GFAP, pg/ml*	345.0 (297.4-496.2)	247.4 (176.2-330.0)	153.0 (112.2-207.6)	**<0.0001** †
Plasma NfL, pg/ml*	66.21 (52.95-172.10)	55.20 (31.30-103.0)	73.50 (41.25-115.0)	0.57†
Plasma p-tau181, pg/ml*	4.72 (2.87-5.66)	3.79 (2.81-6.46)	2.55 (1.56-4.63)	**0.01**†
CSF p-tau, pg/ml*	82.00 (73.25-96.55)	45.90 (41.30-52.60)	32.65 (26.70-42.13)	**<0.0001** †
CSF t-tau, pg/ml*	585.0 (455.5-635.0)	286.0 (265.0-358.0)	255.5 (204.8-357.0)	**<0.0001** †
CSF NfL, pg/ml*	6135 (3737-10570)	2618 (1731-6390)	6102 (3165-10844)	0.21†
***APOE* ε4 carriers, positive (%)**	3 (33.3)	4 (36.4)	15 (11.0)	**0.01§**

* Expressed as median and interquartile range; †Kruskal-Wallis test; §Fisher's test. Significant p-values are reported in bold. Abbreviations: *APOE*, apolipoprotein E; ALS; amyotrophic lateral sclerosis; CSF, cerebrospinal fluid; GFAP, glial acidic fibrillary protein; NfL, neurofilament light chain; p-tau, phosphorylated tau protein; p-tau181, plasma phosphorylated tau 181; t-tau, total tau protein.

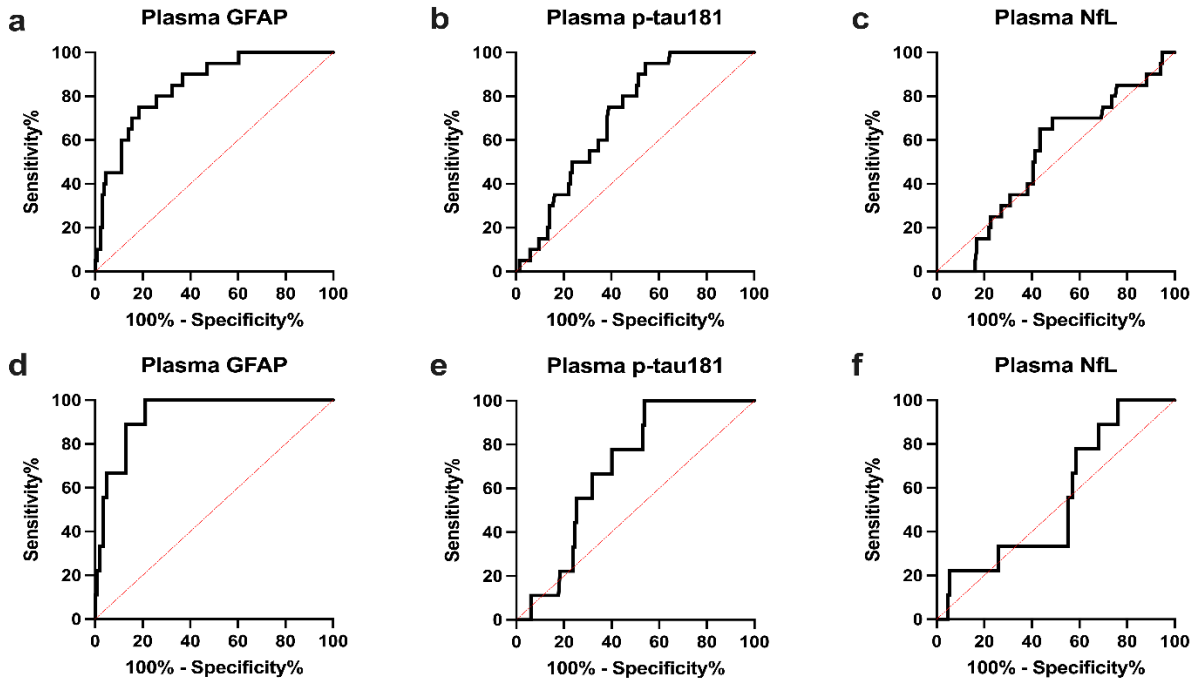
Accordingly, plasma GFAP yielded a high value in the discrimination between A+ and A- ALS patients (AUC 0.847±0.041), significantly higher than that of other plasma biomarkers (plasma p-tau181 AUC 0.706±0.048, plasma GFAP vs. plasma p-tau181, p=0.008; plasma NfL AUC 0.528±0.064, plasma GFAP vs. plasma NfL, p=0.0003) and comparable to that of CSF p-tau (CSF p-tau AUC 0.875±0.038, plasma GFAP vs. CSF p-tau p=0.52) (Table 4, Figure 2a-c).

Table 4. Value of different plasma and CSF biomarkers in the discrimination of ALS patients according to their A and T status.

ALS A+ vs. ALS A-					
	AUC (95% CI)	**p-value***	**Sensitivity, % (95% CI)**	**Specificity, % (95% CI)**	**Optimal cutoff value†**
Plasma GFAP	0.847 (0.766-0.929)	-	75.0 (53.1-88.8)	81.6 (74.3-87.2)	>236.3
Plasma p-tau181	0.706 (0.611-0.800)	**0.0008**	95.0 (76.4-99.7)	45.6 (37.4-54.0)	>2.22
Plasma NfL	0.528 (0.403-0.653)	**0.0003**	65.0 (43.3-81.9)	56.6 (48.2-64.5)	<67.0
CSF p-tau181	0.875 (0.801-0.949)	0.52	90.0 (69.9-98.2)	75 (67.1-81.5)	>41.15
AD/ALS vs. not-AD/ALS					
	AUC (95% CI)	**p-value***	**Sensitivity, % (95% CI)**	**Specificity, % (95% CI)**	**Optimal cutoff value†**
Plasma GFAP	0.932 (0.879-0.985)	-	100 (70.1-100)	78.9 (71.6-84.7)	>236.3
Plasma p-tau181	0.692 (0.578-0.807)	**0.0008**	100 (70.1-100)	46.2 (38.4-54.3)	>2.47
Plasma NfL	0.549 (0.376-0.721)	**<0.0001**	100 (70.1-100)	23.8 (17.6-31.3)	>38.1

*Comparison with the AUC of plasma GFAP (DeLong Test); †: expressed in pg/ml and calculated through the Youden Index. AD/ALS patients show a A+T+ CSF profile. Not-AD/ALS subjects do not show a CSF profile consistent with a full-blown AD pathology (namely A- and A+T-). Significant p-values are reported in bold. Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; AUC, area under the curve; CSF, cerebrospinal fluid; CI, confidence interval; GFAP, glial fibrillary acidic protein; NfL, neurofilament light chain; p-tau181, plasma phosphorylated tau protein 181.

Figure 2. ROC curves of plasma biomarkers in the discrimination of ALS patients with amyloid co-pathology (A+ status) (a-c) and ALS patients with concomitant full-blown AD pathology (A+T+ status) (d-f). AUC, area under the curve; GFAP, glial fibrillary acidic protein; NfL, neurofilament light chain; p-tau181, plasma phosphorylated tau protein 181; ROC, receiver operating characteristic.



Plasma GFAP showed very high accuracy (AUC 0.932±0.027) in discriminating A+T+ patients (AD/ALS) from those not displaying a CSF profile consistent with a full-blown AD pathology (not-AD/ALS, namely A- and A+T- ALS patients), which was significantly higher than that of any other plasma biomarker (plasma p-tau181 AUC 0.692±0.058, plasma GFAP vs. plasma p-tau181 p=0.0008; plasma NfL AUC 0.548±0.088, plasma GFAP vs. plasma NfL p<0.0001) (Table 4, Figure 2d-f).

In comparison to not-AD/ALS, AD/ALS patients showed significantly lower scores at the ECAS battery (ECAS total equivalent scores, p=0.04), at the MMSE test (albeit not age- and education-adjusted) (p=0.03), and at neuropsychological tests exploring short-term visual memory (p=0.01) (Table 5). A trend of significance was also found for ECAS ALS-specific equivalent scores (p=0.06).

Table 5. Clinical and neuropsychological features in AD/ALS and not-AD/ALS patients.

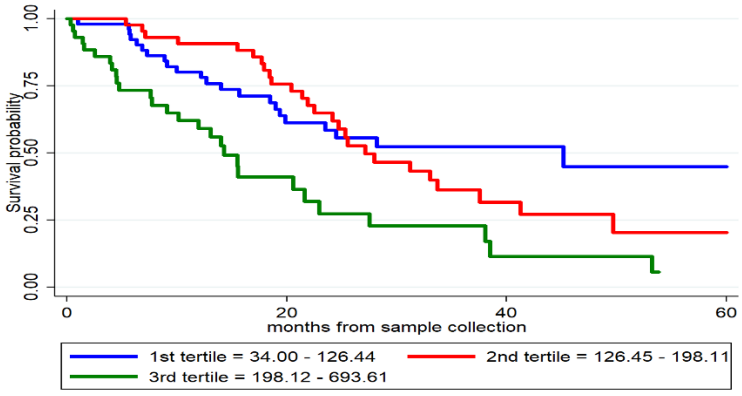
	AD-ALS (n=9)	**not-AD/ALS (n=147)**	**p values**
MMSE scores, n	28.0 (22.5-29.0), 5	29.0 (28.0-30.0)	**0.03***
MMSE scores (age- and education-adjusted), **n**	25.70 (22.90-29.36), 5	28.16 (26.70-28.99), 108	0.35*
ECAS total scores (age- and education-adjusted), **n**	89.19 (79.91-112.0), 4	108.4 (96.81-116.80), 84	0.16*
ECAS total scores (equivalent scores), **n**	2.0 (1.25-3.5), 4	4.0 (3.0-4.0), 84	**0.04***
ECAS ALS-specific scores (age- and education-adjusted), **n**	64.44 (61.25-84.42), 4	79.87 (72.56-86.08), 84	0.21*
ECAS ALS-specific scores (equivalent scores), **n**	2.0 (2.0-3.5), 4	4.0 (3.0-4.0), 84	0.057*
ECAS ALS-nonspecific scores (age- and education-adjusted), **n**	24.77 (18.42-27.82), 4	27.34 (24.10-30.65), 84	0.20*
ECAS ALS-nonspecific scores (equivalent scores), **n**	3.0 (1.25-.4.0), 4	4.0 (2.0-4.0), 84	0.63*
Visual short-memory test (age- and education-adjusted), **n**	15.80 (14.18-17.97), 5	19.70 (17.43-20.90), 109	**0.01***
Visual short-memory test (equivalent scores), **n**	1.0 (1.0-2.5), 5	3.0 (2.0-4.0), 109	**0.04***
ALS-CN, n	4/6 (66.7)	73/122 (59.8)	>0.99†
ALSci, n	0/6 (0)	7/122 (5.7)	>0.99†
ALSbi, n	0/6 (0)	21/122 (17.2)	0.58†
ALScbi, n	1/6 (16.7)	8/122 (6.5)	0.36†
ALS-FTD, n	1/6 (16.7)	13/122 (10.6)	0.50†

*: Mann-Whitney test; †: Fisher's test. Significant p-values are reported in bold. Abbreviations: AD, Alzheimer's Disease; ALS, amyotrophic lateral sclerosis; ALSbi, amyotrophic lateral sclerosis with behavioral impairment; ALScbi, amyotrophic lateral sclerosis with combined cognitive and behavioural impairment; ALSci, amyotrophic lateral sclerosis with cognitive impairment; ALS-CN, cognitively normal amyotrophic lateral sclerosis; ECAS, Edinburgh Cognitive and Behavioural ALS Screen; FTD, frontotemporal dementia; MMSE, Mini Mental State Examination.

4.3.5. Prognostic value of plasma GFAP in patients with ALS

Univariable Cox regression analysis (156 patients with ALS, 77 dead) identified as prognostic factors the following clinical variables: age at onset (p=0.005), ALSFRS-R (p<0.001), DPR (p<0.001), bulbar onset (p=0.001), FTD status (p=0.048). As for biomarkers, plasma GFAP (HR 2.46, p<0.001), plasma NfL (HR 1.01, p<0.001), and plasma p-tau181 (HR 1.11, p=0.02) were identified as predictors of survival (Figure 3).

Figure 3. Survival curves in patients with ALS according to the values of plasma GFAP. Biomarkers levels were stratified into low, mid and high tertiles and are expressed in pg/ml. GFAP, glial fibrillary acidic protein.



In the multivariable analysis including plasma biomarkers, plasma GFAP (p=0.032) and both plasma NfL (p<0.001) and p-tau181 (p=0.042) independently predicted survival in ALS patients (Table 6).

Table 6. Multivariable Cox Regression analysis for survival in ALS patients including plasma biomarkers.

Variable	HR (95% CI)	P-value
Plasma GFAP	1.73 (1.05-2.87)	**0.032**
Plasma p-Tau181	1.09 (1.00-1.19)	**0.042**
Plasma NfL	1.01 (1.00-1.01)	**<0.001**

Data are expressed as Hazard Ratios and 95% CI. Significant p-values are reported in bold. Abbreviations: CI, confidence interval; GFAP, glial fibrillary acidic protein; HR, hazard ratio; NfL, neurofilament light chain; p-tau181, plasma phosphorylated tau protein 181.

4.4 Discussion

In this work, we investigated the distribution of plasma GFAP levels in an extensive cohort of deeply phenotyped ALS patients and explored their clinical and neuropsychological correlates.

In ALS patients, plasma GFAP values were significantly higher than in controls, correlated with age at sampling, in line with previous reports (Falzone et al., 2022; Verde et al., 2023), and showed slightly increased values in females than in males. Plasma GFAP was moderately associated with the

ALSFRS-R scale and DPR but did not show any relationship with other parameters of disease severity or extent of motor impairment, such as King's stage, MRC score, or the number of regions displaying UMN or LMN signs. Similarly, we found no differences in plasma levels across onset types and clinical phenotypes. Taken together, these data, in agreement with previous reports in smaller ALS cohorts (Falzone et al., 2022; Verde et al., 2023), suggest that plasma GFAP elevation in ALS also reflects the astrocytic activation secondary to neurodegeneration at sites unrelated to motor neurons. The moderate association between plasma GFAP and parameters of cognitive impairment, including ALS-specific ECAS scores, the BMDB total score, and the scores in tests exploring semantic fluency and constructional praxis, suggest a link with extra-motor cortical areas. Accordingly, plasma GFAP was significantly elevated in ALS patients displaying a full-blown FTD despite the lack of a significant difference in the biomarker levels across the Strong classification categories, probably due to the subgroups' scarce numerosity. Furthermore, plasma GFAP levels were significantly higher in pure-FTD patients than those with pure motor ALS. Increasing evidence suggests an extra-motor involvement in ALS (Chiò et al., 2019), including neuropathological studies (Schiffer et al., 2004) showing astrocyte activation or degeneration in brain areas different from those harboring motor neurons. Notably, preliminary data (Yang et al., 2012), albeit not confirmed by other authors (Behrouzi et al., 2016), indicate a higher representation of reactive astrocytes in brain areas relevant for superior functions in ALS patients with cognitive impairment than in pure motor ALS. In this scenario, the unique association of the biomarker with the ALSFRS-R scale and DPR could reflect the correlation with the spreading process of the disease, possibly driven by the correlation with age, with older patients typically showing a more severe disease. The results of our survival analysis in ALS patients align with these observations. Indeed, plasma GFAP significantly predicted survival in the univariate analysis. Still, the significance was lost when covarying with well-known prognostic clinical factors in ALS, such as type of onset and the ALSFRS-R scale. These data reflect the lack of correlation of plasma GFAP with scores of motor impairment severity, which plays the most important role in determining the disease course. Notably, plasma GFAP retained its prognostic value when we only accounted for plasma NfL, and p-tau181, which were previously shown also by our studies to predict survival in ALS patients (Benatar et al., 2020). This confirms the specific prognostic contribution of this biomarker, possibly indicating cognitive impairment, and suggests that prognostic estimates based on different blood biomarkers, with p-tau181 mainly reflecting LMN degeneration and NfL expressing the overall disease severity, may have an added value in ALS patients. As an important finding, we showed for the first time that plasma GFAP levels in ALS patients are significantly influenced by AD co-pathology. In detail, plasma GFAP was moderately associated with the CSF Aβ ratio, even after correction for age. Moreover, when stratifying patients according to

amyloid status, A+ subjects, independently from their T status, showed significantly higher GFAP values than A−ALS patients and controls. Interestingly, in the multiple-group comparison, the biomarker's values were not significantly different between controls and A−ALS, probably reflecting the relatively low degree of astrogliosis found in ALS patients' brains compared to that of subjects with AD. Plasma GFAP levels, more accurately than those in CSF, have been shown to distinguish patients with underlying amyloid pathology independently from the severity of cognitive impairment and even in patients with a primary alternative neurodegenerative disorder, such as Lewy Body disease (Pereira et al., 2021; Baiardi et al., 2022; Cousins et al., 2023). This probably reflects the strong relationship between activated astrocytes and amyloid plaques in AD patients' brains (Bellaver et al., 2023; Medeiros et al., 2013). In this view, plasma GFAP could serve as a valid surrogate blood biomarker for the identification of AD co-pathology in ALS patients, given the suboptimal value of plasma p-tau181 in these subjects due to its likely peripheral source, as already demonstrated by our findings and others (Cousins et al., 2022) and confirmed in this work in a larger cohort. Plasma GFAP showed the highest accuracy among the examined plasma biomarkers in identifying ALS patients with positive amyloid status and full-blown AD pathology. Interestingly, plasma GFAP values were not significantly different between ALS A+T− and A+T+, further indicating that astrogliosis, so GFAP elevation in blood, is an initial event in the AD pathogenetic cascade, as already supported by biomarkers studies in autosomal dominant AD mutation carriers (Johansson et al., 2023). Extensive studies on the prevalence of AD co-pathology in ALS patients are lacking, with some authors reporting a 20% prevalence, likely age-related (Behrouzi et al., 2016), while others show a higher percentage of AD neuropathological changes, mainly in subjects with cognitive decline (Hamilton et al., 2004). In our cohort, although only through a biofluid-biomarker-based approach, we reported an amyloid co-pathology in approximately 13% of ALS patients (only 6% with both A and T positive status), which is in line with the estimates of amyloid deposition prevalence in the age-matched general population (Jansen et al., 2022) and therefore not supporting a causal connection between AD and ALS pathologies. Albeit only preliminary, our data seem to support a different cognitive profile in ALS patients with concomitant AD co-pathology, with these latter showing significantly lower scores in multi-domain scales (ECAS total equivalent scores, MMSE) and in specific cognitive domains, such as visual memory, typically impaired at early stages in the AD continuum (Seo et al., 2021). Similarly, the association of plasma GFAP with scores of semantic fluency and constructional praxis may be interpreted in this view. In summary, plasma GFAP, being strictly associated with amyloid co-pathology in ALS, could serve as a biomarker of cognitive impairment in ALS and aid in identifying patients with cognitive features atypical for ALS-FTD dementia. Further studies are needed to show more detailed differences in the cognitive profile of AD/ALS subjects. Regarding the

possible influence of gender on plasma GFAP values, we found that in our ALS population females showed higher biomarker levels than males, with the difference almost reaching statistical significance. Higher plasma GFAP values in females were previously reported in ALS patients, albeit potentially related to the older age (Verde et al., 2023), and in subjects with other neurodegenerative disorders as well (Pereira et al., 2021; Benedet et al., 2021). In our cohort, the slightly higher plasma GFAP values in females could be at least partially related to the higher prevalence of beta-amyloid co-pathology (A+, females 15.5%, males 11.2%). Given the overall inconclusive data, further studies are required to fully explore the influence of gender on the distribution of plasma GFAP values. The moderate association of GFAP levels with plasma creatinine deserves further comments. The relationship between renal function and levels of plasma biomarkers, including GFAP, has already been reported (Verde et al., 2023; Pichet Binette et al., 2023). However, in one of these studies, a significant overall effect of creatinine on the accuracy of using plasma biomarker levels to predict the risk of conversion to dementia in AD patients could not be demonstrated (Pichet Binette et al., 2023). Nonetheless, given the high prevalence of chronic kidney disease in the general population, especially in the elderly, further studies are required to clarify the influence of renal function on plasma GFAP levels and their clinicopathological correlates. The inclusion of a large sample of deeply characterized ALS patients and a high number of different CSF and plasma biomarkers available is the main strength of our work. Secondarily, the deep categorization of patients' cognitive impairment through an extensive battery of neuropsychological tests, including the specific battery validated for ALS, (i.e., ECAS), is another added value. On the contrary, the lack of a systematized evaluation of the impact of comorbidities and medication on GFAP values in ALS patients is one of the limitations, as the lack of neuropathological correlates of GFAP elevation in our cohort and the relatively low number of ALS patients with a full-blown AD co-pathology, partially due to the rarity of ALS itself. Further studies involving neuropathological cohorts are required to confirm our results and address the relationship between plasma GFAP levels and the burden of AD co-pathology in ALS patients.

In conclusion, our work provides evidence that plasma GFAP is elevated in ALS patients compared to controls, but this elevation is mainly affected by concomitant amyloid-beta pathology. Plasma GFAP shows the highest accuracy among the most common plasma biomarkers in identifying AD co-pathology in ALS and is related to measures of cognitive impairment in ALS patients. Finally, including plasma GFAP in survival multivariable analyses with other plasma biomarkers could add value to the prognosis estimation of ALS patients.

5. Neurophysiological biomarkers in Amyotrophic Lateral Sclerosis

5.1 Prognostic value of conventional EMG in Amyotrophic Lateral Sclerosis

5.1.1 State of art

As already highlighted before, development of reliable and clinically applicable prognostic biomarkers in ALS could potentially drive patient management, enable more equitable patient stratification into clinical trials, and serve as outcome measurement for prompt assessment of drug effectiveness (Goutman et al., 2022). Identifying ALS patients with aggressive disease would facilitate care planning, such as timing of alternative feeding strategies, noninvasive pressure ventilation (NIV), and prescription of communication devices. In addition, phenotypic heterogeneity may be reduced, thereby facilitating patient stratification for clinical trials and increasing the likelihood of positive outcomes. Neurophysiological measures of lower motor neuron dysfunction have been proposed as prognostic biomarkers in ALS, ranging from the application of conventional methods to the development of advanced technologies (Vucic et al., 2018).

Indeed, neurophysiological studies are essential for the diagnosis of ALS, since this latter is currently based on the Revised El Escorial (Brooks et al., 2000), the Awaji (de Carvalho et al., 2008), and the most recent Gold-Coast criteria (Shefner et al., 2020). In particular, the Awajii criteria stated for the first time that clinical and electrophysiological involvement have the same diagnostic significance in any body region for evaluating the presence of LMN signs. Indeed, to define a region as affected, both signs of denervation (i.e., fibrillations and/or positive sharp waves) and re-innervation must be found through needle electromyography (EMG), with fasciculation potentials having the same electrophysiological significance of fibrillations and positive sharp waves. Interestingly, this allows a prompt diagnosis, due to the earlier neurophysiological involvement compared to the clinical signs of disease (de Carvalho et al., 2008).

However, beside the essential diagnostic value, conventional EMG has been recently investigated in a few studies to determine its possible prognostic burden. Just as an example, a previous our study revealed an association between the severity of the denervation findings in the three body regions (bulbar, cervical and lumbosacral regions) at EMG at diagnosis and the survival in ALS patients (Fileccia et al., 2020). Notably, the degree of denervation findings in the bulbar region was associated with the time to respiratory failure and survival. However, one limitation was the variable assessment of two different bulbar muscles (genioglossus and masseter muscles) to evaluate the presence of LMN signs in the bulbar region (Fileccia et al., 2020).

This present study (Vacchiano et al., 2021) aimed to evaluate the prognostic value of the EMG genioglossus involvement in ALS patients at diagnosis. In addition, we performed a comparison

between the prognostic value of EMG genioglossus and masseter involvement at diagnosis in a subset of patients in whom both muscles were explored at diagnosis, assessing for the first time the relative prognostic burden of these bulbar muscles in ALS patients.

5.1.2 Material and Methods

We retrospectively reviewed clinical and EMG data of patients diagnosed with ALS at our centre from 2009 to 2018. We included only the patients with available EMG genioglossus assessment at diagnosis and available clinical follow-up which allowed the diagnosis of clinically definite ALS (Brooks et al., 2000). During the study period, 309 ALS patients came to our attention. Among these, 190 underwent genioglossus EMG assessment, with ten patients excluded due to an incomplete study for patients' discomfort. Patients without genioglossus assessment were investigated through EMG masseter examination, as we introduced the systematic genioglossus assessment only from 2013. Among 180 patients with neurophysiological genioglossus data, only 103 were systematically followed-up in our centre and therefore included in the study. In addition, in a subgroup of these patients the masseter muscle was also investigated (N = 55). The EMG protocol was fully described in previous studies (Fileccia et al., 2020; Vacchiano et al., 2021). Briefly, for each completely relaxed muscle at least 10 different sites were investigated for the presence of fibrillations, positive sharp waves and fasciculations. A minimum of 20 different motor unit action potential (MUAP) in each muscle were quantitatively examined to assess the presence of re-innervation. The tongue was examined by direct needle insertion into the right or left edge of the anterior third of the tongue, carefully instructing the patient to relax mouth and chin with the tongue inside the mouth. MUAP analysis was assessed as neurogenic if the MUAP duration was increased as compared to normal values collected from healthy subjects from our laboratory. The muscle was considered affected if signs of denervation (and/or fasciculations) and neurogenic changes were observed (de Carvalho et al., 2008). Clinical variables of all patients were collected at the time of EMG examination. They included: age, gender, time and type of onset (bulbar vs spinal), clinical phenotype (defined as classical, bulbar, predominantly lower and upper motor neuron disease [Al-Chalabi et al., 2016; Chiò et al., 2011]), Revised Amyotrophic Lateral Sclerosis Functional Rating (ALSFRS-R) scale, presence of clinical UMN (pseudobulbar affect, hyperreflexia, spasticity) and LMN signs (weakness and wasting with or without fasciculations) in the bulbar region. We considered as clinical milestones: 1) the indication for noninvasive ventilation (NIV) according to the EFNS guidelines recommendation (when at least one respiratory clinical symptom or one of the following criteria is present: FVC < 80%, SNIP < 40 cm H2O, significant nocturnal desaturation, or pCO2 > 45 mmHg); 2) the onset of moderate dysphagia, defined as a ALSFRS-R item 3 (swallowing) score < 3 (the score 3 corresponds to ‘‘early eating problems; occasional chocking”); 3) the indication for percutaneous endoscopic

gastrostomy (PEG)/parenteral nutrition, namely severe dysphagia, choking, and/or weight loss > 10%; and 4) loss of intelligible verbal communication, defined as an ALSFRS-R item 1 (speech) score < 2 (the score 2 corresponds to intelligible speech with repeating). Survival was defined as the time from EMG assessment (corresponding in all patients to the time of diagnosis) to death or tracheostomy. Since the expansion of repeats (RE) in *C9Orf72* gene has been associated with a worse prognosis in ALS (Miltenberger-Miltenyi et al., 2019), we included the presence of the *C9Orf72* RE as a covariate in the survival models. The study was approved by the local ethics committee AVEC and conducted in accordance with the Declaration of Helsinki and the principles of Good Clinical Practice (GCP).

Statistical analyses were performed using SPSS 24.0. Mean and standard deviation (SD) and Student's T-Test were used for data normally distributed. Median and range and the Mann-Whitney U test were used for data not normally distributed. Categorical variables were expressed as counts and percentages. The chi-square test was adopted for categorical variables. The agreement between neurophysiological assessment of genioglossus and masseter was tested using Cohen's k statistics. To investigate the prognostic value of both clinical and neurophysiological bulbar involvement, we first explored the association between the presence of clinical LMN signs in the bulbar region at diagnosis and the clinical milestones (time from EMG to indication for NIV, to onset of moderate dysphagia, to indication for PEG/parenteral nutrition, to loss of verbal communication and to death/tracheostomy) using Cox proportional hazard models adjusting for age at EMG, sex, diagnostic delay, presence of bulbar UMN signs at diagnosis, neurophysiological cervical and lumbosacral involvement, ALSFRS-R score and the presence of the *C9ORF72* RE. Therefore, we investigated the association of EMG genioglossus involvement with clinical milestones using Cox proportional hazard models after adjusting for the same covariates. Finally, we adopted the same statistical method to compare the prognostic role of the EMG masseter and genioglossus muscles involvement at the diagnosis in the subset of patients undergoing both muscles examination. P-values < 0.05 were considered significant for all analyses.

5.1.3 Results

Demographic and clinical features of patients are detailed in Table 1.

Only 28 patients (27.18%) presented with clinical bulbar LMN signs, while, variably considering the EMG involvement of the genioglossus or masseter muscle, 49 patients (47.57%) showed a neurophysiological involvement of the bulbar region. EMG genioglossus abnormalities consistent with motor neuron disease were found in 45 out of 103 (43.68%) patients as compared to 19 patients (19/55; 34.55%) displaying EMG masseter involvement. The concordance between evaluations of the two muscles was 70.9% (kappa = 0.415, p = 0.001).

Table 1. Demographic and clinical features of the study population.

Patients/clinical characteristics	**N (tot. 103)**	**%**
Gender		
Male	60	58.3
Female	43	41.7
Type of onset		
Bulbar	38	36.9
Spinal	65	63.1
ALS variant		
Classic	51	49.5
Bulbar	36	35
PLMN	11	10.7
PUMN	5	4.9
Deceased/with tracheostomy	68	66
C9Orf72 repeat expansion		
Carriers	10	9.7
Bulbar clinical signs		
Upper bulbar clinical signs	67	65
Lower bulbar clinical signs	28	27.2
Prognostic milestones		
NIV indication	61	59.2
PEG/parenteral nutrition indication	52	50.5
Onset of moderate dysphagia	70	68
Loss of intelligible verbal communication	49	47.6
Age at EMG (y)		
Median (IQR)	66 (54-74)	
DD from EMG to death/tracheostomy (m)		
Mean (SD)	19.91 (13.27)	
Diagnostic delay (m)		
Median (IQR)	12 (8-23)	
ALSFRS-R score (m)		
Median (IQR)	41 (36-44)	
ALSFRS-R bulbar score (m)		
Median (IQR)	10 (9-12)	
Time to NIV indication (m)		

Median (IQR)	10 (4 – 20.5)
Time to PEG indication (m)	
Mean (SD)	11.33 (8.48)
Time to moderate dysphagia (m)	
Median (IQR)	7 (1 – 16.5)
Time to loss of intelligible verbal communication (m)	
Median (IQR)	10 (5.5 – 19.5)

Key: ALSFRS-R, Revised Amyotrophic Lateral Sclerosis Functional Rating; DD, disease duration; EMG, electromyography; m, months; NIV, non-invasive ventilation; PEG, percutaneous endoscopic gastrostomy; SD, standard deviation; y, years.

Patients with clinical bulbar LMN signs at diagnosis showed a significantly shorter disease duration ($p = 0.006$), a shorter time to NIV and PEG/parenteral nutrition indication ($p = 0.038$ and $p = 0.0001$ respectively), an earlier onset of moderate dysphagia ($p = 0.0001$) and a shorter time to loss of intelligible verbal communication ($p = 0.007$) as compared to patients without (Table 2).

Table 2. Prognostic milestones in the whole study population: results from Student's T and Mann-Whitney U tests.

	Bulb LMN+	**Bulb LMN -**	**p-value**	**Gen +**	**Gen -**	**p-value**	**Mass +**	**Mass -**	**p-value**
Disease Duration (m) Mean (SD)	13.43 (8.94)	22.81 (13.92)	**0.006**	13.84 (10.07)	25 (13.50)	**0.0001**	13.58 (10.33)	22.23 (10.95)	**0.032**
Time to NIV (m) Median (IQR)	5 (3 – 13)	12.50 (5.50 – 23)	**0.038**	6.50 (3 – 13.25)	16 (9 - 27)	**0.017**	3 (2 - 14)	12 (6 – 27)	**0.047**
Time to PEG/parenteral nutrition (m) Mean (SD)	6.35 (7.02)	14.44 (7.9)	**0.0001**	9.10 (9.16)	14.13 (6.74)	**0.033**	9.11 (10.36)	11.77 (7.95)	0.527
Time to moderate dysphagia (m) Median (IQR)	1 (0 – 3)	12.50 (6 – 19.75)	**0.0001**	2 (0 – 5.50)	14 (7.05 - 24)	**0.0001**	3 (0.50 - 7)	12 (1 - 25)	**0.037**
Time to loss of verbal communication (m) Median (IQR)	8 (1.50 – 11)	14 (7 – 21)	**0.007**	9 (4 - 11)	19 (7.75 – 26.75)	**0.002**	8.50 (3 – 10.25)	9 (4 - 19)	0.238

Key: bulb, bulbar; Gen, genioglossus; LMN, lower motor neuron signs; mass, masseter; m, months; NIV, non-invasive ventilation; PEG, percutaneous endoscopic gastrostomy; SD, standard deviation; y, years; +, present; -, absent.

However, Cox proportional hazard models only revealed a significant association with the time to onset of moderate dysphagia (p = 0.0001, Fig. 1D and Table 3).

Figure 1. Cox proportional hazard models after adjusting for age at EMG, sex, diagnostic delay, presence of bulbar UMN signs, EMG cervical and lumbosacral involvement, ALSFRS-R score and the presence of C9Orf72 repeat expansion showed that EMG genioglossus involvement was associated with a shorter survival (Panel A), a shorter time to onset of moderate dysphagia (Panel B), and a shorter time to loss of intelligible verbal communication (Panel C). Cox proportional hazard models after adjusting for the same covariates showed that clinical bulbar LMN were only associated with a shorter time to moderate dysphagia (Panel D). Abbreviations: ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale; EMG, electromyography; LMN, lower motor neuron; UMN, upper motor neuron.

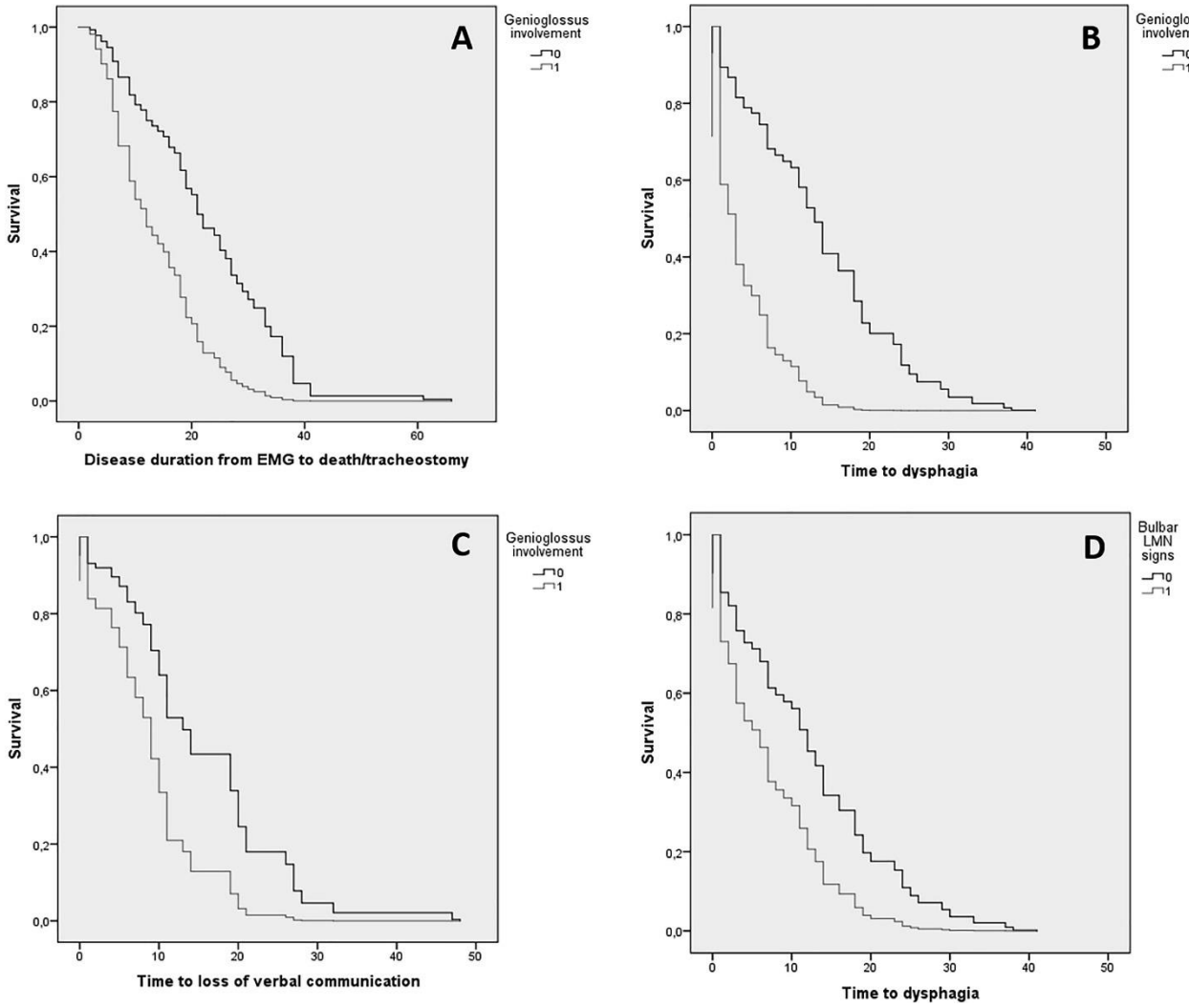


Table 3. Prognostic milestones in the whole study population: results from Cox proportional hazard models. The last two rows are referred to the subgroup with both muscles assessment.

	Time to death/tracheostomy		**Time to NIV**		**Time to PEG/parenteral nutrition**		**Time to moderate dysphagia**		**Time to loss of verbal communication**	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
Bulbar LMN signs (28/103 pts)	1.864 (0.938 – 3.70)	0.075	0.946 (0.452 – 1.983)	0.884	1.33 (0.59 – 2.96)	0.49	3.516 (1.776 – 6.962)	**0.0001**	1.856 (0.880 – 3.915)	0.104
EMG Genioglossus involvement (45/103 pts)	2.654 (1.410 – 4.996)	**0.002**	1.627 (0.838 – 3.161)	0.151	1.453 (0.742 – 2.847)	0.276	4.727 (2.513 – 8.892)	**0.0001**	2.455 1.218 – 4.951	**0.012**
EMG Genioglossus involvement (27/55 pts)	3.013 (1.070 – 8.490)	**0.037**	2.260 (0.618 – 8.262)	0.218	12.974 (1.312 – 128.332)	**0.028**	4.521 (1.561 – 13.098)	**0.005**	7.555 (1.288 – 44.300)	**0.025**
EMG Masseter involvement (19/55 pts)	2.273 (0.792 – 6.520)	0.127	3.115 (0.983 – 9.870)	0.053	0.31 (0.06 – 1.46)	0.14	1.968 (0.753 – 5.139)	0.167	2.489 (0.694 – 8.928)	0.162

Key: CI, confidence interval; EMG, electromyography; HR, Hazard ratio; LMN, lower motor neuron signs; NIV, non-invasive ventilation; PEG, percutaneous endoscopic gastrostomy; pts, patients.

Patients with EMG genioglossus involvement showed a shorter survival (p = 0.0001), a shorter time to NIV and PEG/parenteral nutrition indication (p = 0.017 and p = 0.033 respectively), an earlier onset of moderate dysphagia (p = 0.0001) and severe dysarthria (p = 0.002) as compared to patients without (Table 2). Accordingly, Cox proportional hazard models (Table 3, Fig. 1ABC) showed a strong association of the EMG genioglossus involvement with a shorter survival (p = 0.002), an earlier onset of moderate dysphagia (p = 0.0001), and a shorter time to loss of intelligible verbal communication (p = 0.012). Considering only ALS patients without clinical LMN signs in the bulbar region (N = 75), we confirmed the prognostic value of the EMG genioglossus involvement as regard to a shorter time to survival (HR 4.137, CI 1.436 – 11.920, p = 0.09), a shorter time to NIV indication (HR 3.461, CI 1.330 – 9.006, p = 0.011), a shorter time to dysphagia (HR = 5.796, CI 2.165 – 15.516, p = 0.0001) and a shorter time to loss of verbal communication (HR 4.891, CI 1.299 – 18.413, p = 0.019), without any association with time to PEG (p = 0.311).

EMG masseter involvement was found in 19 out 55 patients (34.55%). Patients with EMG masseter involvement had significantly shorter mean disease duration (p = 0.032), median time to NIV indication (p = 0.047), and median time to onset of moderate dysphagia (p = 0.037) compared to patients without. Conversely, mean time to indication for PEG/parental nutrition and median time to loss of intelligible verbal communication was not significantly different between the two groups (p = 0.527 and p = 0.238 respectively), Table 2. In order to compare the prognostic value of the genioglossus and the masseter muscles, we used the same survival models exploring the prognostic value of the EMG genioglossus and masseter involvement only in the subgroup of patients with both muscles explored at diagnosis (N = 55). We did not find any association between the EMG masseter involvement and the survival (p = 0.127), time to NIV indication (p = 0.053), time to onset of moderate dysphagia (p = 0.167), PEG/- parenteral nutrition indication (p = 0.139) or loss of communication (p = 0.162), Table 3. Otherwise, we confirmed also in this subgroup a significant association between EMG genioglossus involvement and survival (p = 0.037), time to dysphagia (p = 0.005), time to PEG/parenteral nutrition (p = 0.028), time to loss of communication (p = 0.025), while there were no significant association with time to NIV indication (p = 0.218), Table 3.

5.1.4 Discussion

In this study we investigated the prognostic value of bulbar neurophysiological involvement at diagnosis in a cohort of ALS patients. We first explored the prognostic value of clinical LMN signs in the bulbar region, showing a significant association only with an early onset of moderate dysphagia. Therefore, we evaluated the prognostic burden of EMG genioglossus involvement according to the Awaji criteria (de Carvalho et al., 2008) and then the prognostic value of the EMG masseter and genioglossus abnormalities separately in a subset of patients in whom both muscles were analysed. In the diagnosis of ALS, the detection of both denervation and re-innervation in muscles of the craniobulbar region is important to assure that there is widespread lower motor neuron involvement, and to reach an early diagnosis (Tankisi et al., 2013). In the present study we found that the neurophysiological involvement of the genioglossus muscle at the diagnosis was a prognostic factor for a shorter survival, an earlier onset of dysphagia and severe dysarthria, at variance with the sole clinical bulbar LMN signs which was only significantly associated with the time to onset of dysphagia. These findings were strongly confirmed also in the subgroup of patients without clinical LMN signs in the bulbar region, where even the time to NIV indication was significantly associated with the presence of genioglossus abnormalities at diagnosis. Our results supported the strong prognostic value of the subclinical involvement of the bulbar region in ALS patients, in line with the evidence that the denervation activity is recognized as a neurodegeneration sign before the appearance of clinical signs such as muscle weakness and muscle atrophy in ALS (Mills 2005). Regarding the

time to PEG/parenteral nutrition, we did not find any significant association with clinical or neurophysiological bulbar involvement at diagnosis, with the only exception of the subgroup of 55 ALS patients undergoing both masseter and genioglossus study. These results could be explained by the fact that the indication for PEG is influenced by the presence of respiratory contraindications during the course of the disease. Therefore, the time for proposing PEG in the clinical setting results strictly limited and sometimes the presence of respiratory failure in a very early phase of the disease prevents the PEG indication in several ALS patients. On the other hand, parenteral nutrition is often proposed in a later phase of the disease in patients not eligible for PEG position. However, we have overcome this limitation by using the time to onset of moderate dysphagia needing dietary consistency changes as a clinical milestone, resulting in a significant association with both clinical and neurophysiological bulbar involvement at diagnosis. In this study we showed that masseter muscle had a lower prognostic role in predicting clinical milestones as compared to genioglossus in a subgroup of the population study. Although our results could be influenced by the relatively small sample of patients undergoing the masseter study, we can reasonably conclude that this muscle, probably less involved in this disease at least in the early phase (Lawyer et al., 1953; Preston et al., 1997), also has a minor prognostic role in ALS. Our results are in line with our previous study (Fileccia et al., 2020), showing a significant association between the denervation findings in the bulbar region (calculated only when also reinnervation was present in the region) and the time to NIV/tracheostomy and survival. However, a drawback of these results was that not all patients underwent the same muscle examination in the bulbar region. In fact, although the EMG genioglossus study can cause discomfort to the patients and can be marred by the technical difficulties mainly in relaxing the muscle, we are aware that the genioglossus muscle has high diagnostic accuracy in detecting LMN involvement of the bulbar region (Vacchiano et al., 2021; Cappellari et al., 1999) as compared to the masseter muscle, also in patients without bulbar clinical signs (Vacchiano et al., 2021; Finsterer et al., 1998). Moreover, in the current study the sample has been significantly expanded due to the longer time of observation: 55 patients had both muscles explored (vs 22 in the previous study) and 48 patients had only EMG genioglossus assessment (vs 30 in the previous one). In this way, we selectively focused on the more frequently and severely affected bulbar muscle in ALS, exploring its prognostic burden. Other studies (Preston et al., 1997; Sato et al, 2015; Zhang et al., 2016) had explored the possible prognostic role of EMG findings at diagnosis, particularly the association between the degree of denervation findings and the prognosis in ALS. In particular, one study (Sato et al, 2015) evaluated the association between the denervation findings in cranial, cervical and lumbosacral regions and the progression from mild to severe ALS forms and the deterioration of daily life activities based on loss of speech function, loss of upper limb function, and loss of walking

ability as sub outcomes. The authors demonstrated a significant association between the denervation findings in the cranial region and the time to loss of speech, accordingly with our results. However, they did not specify the muscles explored in the craniobulbar region in order to define the specific prognostic value of each muscle. Other studies focused on the correlation between the presence of denervation potentials in paraspinal (de Carvalho et al., 2010) or abdominal muscles (Zhang et al., 2016) and the ventilation dysfunction or on the general presence of denervation activity (in many or all muscles explored for patient), and survival (Krarup et al., 2011). However, these studies did not focus on the role of the bulbar region and did not explore the impact of EMG abnormalities on clinical milestones such as needing ventilation or PEG implantation/parenteral nutrition. To the best of our knowledge, this was the first study exploring and comparing the prognostic value of the neurophysiological involvement of genioglossus and masseter muscles at diagnosis in ALS patients. Interestingly, our results have been recently confirmed in a larger cohort of 689 ALS patients (Colombo et al., 2023), where the amount of spinal denervation and reinnervation resulted associated with the functional disability (lower ALSFRS-R scores), while the amount of denervation in both bulbar and spinal regions turned out as a negative prognostic factor for survival in ALS.

Limitations of our study are related to its retrospective nature and the relatively small number of patients undergoing both cranial muscles EMG investigations.

In conclusion, EMG assessment in the bulbar region, especially of the genioglossus muscle, may be important for an early diagnosis but also for its intrinsic prognostic value, providing additional information about the rate of progression of the disease.

5.2 MScanFit MUNE as a biomarker of motor unit loss in ALS

5.2.1 State of art and objectives of the study

The implementation of motor unit number estimation (MUNE) methods has long been of interest to estimate the lower motor neurons loss in motor neuron diseases. Indeed, in muscles with denervation, measurement of muscle strength does not directly reflect the number of surviving motor units because of collateral sprouting, namely the phenomenon whereby healthy axons take over the muscle territory of axons that have been lost. Likewise, the amplitude of the compound muscle action potential (CMAP) does not decrease until at least 50% of motor units due to the same phenomenon, therefore conventional nerve conduction studies cannot provide accurate information about the amount of motor units lost. Accordingly, the degree of denervation in EMG does not correlate with the number of motor unit loss. Besides, we are measuring the reinnervation rather than directly loss of motor unit with motor unit potential (MUP) analysis and in patients with fast disease progress such as amyotrophic lateral sclerosis, MUPs may look normal despite the severe loss of motor units. Thus, MUNE might be better suited than any other electrophysiological test to study the degree and time course of lower motor unit loss (Gooch et al., 2014).

The first MUNE method based on incremental stimulation was implemented in 1971 by McComas et al. (McComas et al., 1971). Since then, a number of different MUNE methods have been developed, with their strengths and limitations. Most of these, such as multiple point stimulation (Doherty et al., 1993) and spike-triggered averaging (Bromberg et al., 1993) are based on determining the size of an average surfaced-recorded motor unit potential (MUP) and dividing that value in to maximal CMAP. More recent methods have used statistical techniques based on the probabilistic nature of the firing of a motor unit in response to a stimulus. Another recent method, the motor unit number index (MUNIX), uses the surface interference patterns recorded during voluntary contraction to quantify the average size of surface-recorded MUPs, being therefore considered a fast and non-invasive method (Neuwirth et al., 2011). However, MUNIX values extracted from surface recorded MUPs were found to be highly correlated with CMAP amplitude, suggesting that MUNIX technique may not be much more useful than the simple CMAP amplitude measurements (Bostock et al., 2019).

Overall, common criticisms of these MUNE methods are the presence of subjectivity in the estimation process, the defeat to obtain a representative sample of units, and the failure to incorporate all potential causes of uncertainty.

In 2016 professor Hugh Bostock (Bostock, 2016) introduced the most recent MUNE method, the MScanFit MUNE (MScan), which is based on the estimation of MUNE values from CMAP Scans by taking into account the probabilistic nature of motor unit firing. Thus, it has overcome the above-mentioned criticisms, taking into account all the motor units contributing to the maximal CMAP and

their variability, and avoiding subjectivity. First, a preliminary model is generated based on the change in the mean and standard deviation of response as a function of stimulus in the main scan and then the model is progressively improved by changing individual motor unit parameters (Bostock, 2016). MUNE analyses require a specific QTRACW© (Institute of Neurology, University College London, UK, distributed by Digitimer Ltd.).

MScan has shown a higher reproducibility and sensitivity than two more traditional methods, MUNIX and multipoint stimulation, and a better determination of disease progression in ALS (Jacobsen et al., 2019). MScan has also the advantages of being semi-automated and fast to perform, not requiring skilled operating staff.

Interestingly, a recent study (Jacobsen et al., 2018) investigated MScan on the abductor pollicis brevis muscle (APB) muscle and compared its diagnostic utility with MUP parameters derived from quantitative EMG analyses (qEMG), showing a higher sensitivity of MScan in detecting abnormalities compared to MUP duration and amplitude.

The objective of the present study was to examine the diagnostic utility of MScan performed on APB, abductor digiti minimi (ADM) and tibialis anterior (TA) muscles of ALS patients compared to the quantitative MUP analyses performed in the same muscles. We also aimed to investigate if MScan parameters correlated with qEMG variables and clinical measures.

5.2.2 Methods

5.2.2.1 Subjects and clinical variables

Patients diagnosed with ALS (Brooks et al., 2000) at the IRCCS Institute of the Neurological Sciences of Bologna from January 2021 to October 2023 were prospectively enrolled in the study.

Clinical variables of enrolled patients were collected at the time of diagnosis, which basically coincided with the timing of qEMG and MScan examinations. Clinical variables included: age, sex, time and type of onset (bulbar vs spinal), clinical phenotype (classical, bulbar, predominantly lower and upper motor neuron disease [Al-Chalabi et al., 2016; Chiò et al., 2011]), Revised Amyotrophic Lateral Sclerosis Functional Rating (ALSFRS-R) scale, the degree of the force assessed by the Muscle Research Council (MRC) scale, and the forced vital capacity (FVC) expressed as the percentage of the predicted normal for a person of the same sex, age and height. We also calculated the disease progression rate (DPR), defined as 48—ALSFRS-R score at MScan/disease duration at MScan in points per month.

Furthermore, 14 age-matched healthy volunteers were enrolled in the study.

Exclusion criteria for patients and healthy controls were: a history of diabetes, dementia, nerve entrapment syndromes, polyneuropathy or diseases that could induce polyneuropathy.

5.2.2.2 Neurophysiological examinations

Motor and sensory nerve conduction studies (NCS) using conventional surface electrode techniques were performed in median, ulnar and peroneal nerves, according to standard procedures (Stålberg et., 2019). The results were compared to our laboratory normal material.

qEMG was done using a concentric needle electrode and the EMG-equipment Keypoint version 5.11. Standard filter settings at the department (20 Hz–10 kHz), gain (100 mV/division) and sweep speed (10 ms/ division) were used. At least two muscles innervated by different nerves and roots in the cervical and lumbosacral regions, genioglossus and/or masseter and paraspinal myotome at T5 were examined with qEMG as a part of the diagnostic electrophysiological evaluation of patients referred for suspected ALS (data not presented). Furthermore, APB, ADM and TA muscles on the less affected side were systematically examined through qEMG in a subgroup of patients in order to perform comparisons between qEMG and MScan parameters.

The presence of fibrillation potentials (fibs), positive sharp waves (PSWs) and fasciculations was assessed in 10 different sites for 90 seconds at each site. We classified the EMG recordings based on a denervation score (Abu Rumeileh et al., 2020). Shortly, the denervation was considered high if PSWs or fibs were identified in more than five sites, and as low when they were observed between three and five sites. In cases where signs of denervation were limited to two sites or less out of ten, the muscle was considered not denervated (Tankisi et al., 2007).

Quantitative MUP analysis was done by sampling of 20 different MUPs during weak effort, corresponding to about 4% of maximal voluntary contraction (Fuglsang-Frederiksen, 1989). Mean duration, mean amplitude and percentage of polyphasic potentials were evaluated and compared to our laboratory controls (data not showed). Being the strongest parameter of reinnervation, MUP duration was graded as normal (0) if the value was within the 20% of variation from the mean normal value, mildly affected (1) if value was > 20 and $\leq 30\%$, moderate (2) if > 30 and $\leq 65\%$ and severely (3) if $> 65\%$ compared to the mean normal value.

Muscles were considered affected only if both signs of denervation (and/or fasciculations) and neurogenic changes were observed (de Carvalho et al., 2008).

MScan examinations were performed on APB, ADM and TA muscles. The dominant side was examined in healthy subjects and the less affected side in patients.

A DS5 bipolar stimulator, a HumBug 50 Hz noise eliminator, a D440 amplifier (Digitimer Ltd) and an analogue-to-digital (A/D) board NI6221 (National Instruments) were used with the set up. Recordings followed the MScan-R2 protocol (Sørensen et al., 2023).

The subject was instructed to relax and not to move the arms and the legs during the recordings.

The median and the ulnar nerves were stimulated at the wrist, while the peroneal nerve just below the fibular head. The stimulus duration was 0.2 ms for the median and ulnar nerves, and 0.5 ms for the peroneal nerve. The stimulus intensity was increased until supramaximal stimulation, from which point the CMAP scan was initiated. This sequence runs automatically with 20 pre-scan stimuli of supramaximal intensity. Then, the stimulus intensity was automatically reduced in 0.2% steps every 0.6 seconds until the motor response reached zero, and 20 post-scan stimuli with very low stimulus intensity were applied before the recording was terminated. Thus, we obtained a detailed stimulus response curve that describes the amplitude of the motor response as a function of the stimulus intensity due to recruitment of more motor units with increasing stimulus intensity.
By using the offline MScanFit component of the QtracP analysis program, a model was fitted to the recorded stimulus response curve (CMAP scan) to obtain an estimate of motor unit number and distribution of motor unit sizes and thresholds (Bostock, 2016).
From the automatic analysis, we derived the CMAP amplitude (peak-to-peak) in mV, and the followings variables:
Parameters reflecting the extent of the degeneration:
- MUNE: the estimated number of functional motor units;
- N50: the estimated number of larger units making up 50-100% of the amplitude of the CMAP.
Parameters reflecting the phenomenon of collateral reinnervation:
- HalfAmpAmp (A50): the size of the motor units at the 50% mark of the cumulative amplitude expressed in % (it is an amplitude measure designed to be less sensitive to the size limit than mean or median amplitude).
- Largest SMUP (LSMUP): the amplitude of the largest unit, expressed as a percentage of CMAP amplitude.
EMG and MScan examinations were performed by two different examiners who were blind to each other.
The protocol was approved by the local Medical Ethical Committee. Informed consent was obtained from all participants.

5.2.2.3 Statistical Analysis

Statistical analysis was performed using the software QtracP (©Institute of Neurology, University College London) and IBM SPSS Statistics version 27 (IBM, Armonk, NY, USA). For continuous variables the Mann-Whitney U test was used to investigate differences between groups. Chi-Square test was adopted for categorical variables. Receiver operating characteristic (ROC) analysis was performed to evaluate the ability of MScan to discriminate between ALS patients and healthy controls by means of MUNE and LSMUP (%) values. Sensitivity, specificity and the area under curve (AUC)

were calculated. Spearman's correlations were used to test the possible associations between neurophysiological and clinical variables. P values < 0.05 were considered as statistically significant.

5.2.3 Results

5.2.3.1 Clinical features of the study population

We enrolled 37 ALS patients and 14 healthy controls.

There were no statistical differences in age (p=0.066) and sex (p=0.35) between ALS and healthy controls. ALS patients were diagnosed according to the Revised El Escorial criteria (Brooks et al., 2000) in clinically possible (N=6), clinically probable laboratory-supported (N=10), clinically probable (N=15) and clinically definite (N=5) ALS, while one patient did not show any sign of upper motor neuron involvement and was therefore categorized as progressive muscular atrophy (PMA).

Demographic and clinical features of the study population are detailed in Table 1.

Table 1. Demographic and clinical features of the study population.

ALS patients - clinical characteristics	**N (tot. 37)**	**%**
Sex	22 (M)	59.5
Type of onset		
Bulbar	10	27
Spinal	27	73
ALS phenotype		Median (IQR)
Classic	26	70.3
Bulbar	5	13.5
PLMN	4	10.8
PUMN	2	5.4
Age at enrolment (y)		63 (55-72)
Age at onset (y)		63 (54.5-71)
DD from first symptom to MScan (m)		10 (6-20)
ALSFRS-R score		42.5 (40-45)
ALSFRS-R motor subscore		20 (17-22)
MRC score APB muscle		4 (4-5)
MRC score ADM muscle		4 (4-5)
MRC score TA muscle		5 (4-5)
FVC		90 (74.5-110)
Healthy controls	**N (tot. 14)**	**%**
Sex	6 (M)	42.9
Age at enrolment (y)		
Median (IQR)		59 (54.5-61.25)

ALS, amyotrophic lateral sclerosis; APB, abductor pollicis brevis; ADM, abductor digiti minimi; ALSFRS-R, Revised Amyotrophic Lateral Sclerosis Functional Rating; DD, disease duration; FVC, forced vital capacity; IQR, interquartile range; M, males; m, months; MRC, Medical Research Council; PLMN, predominant lower motor neuron; PUMN, predominant upper motor neuron; TA, tibialis anterior; y, years.

5.2.3.1 MScan parameters in ALS patients and healthy controls

APB, ADM and TA muscles were examined through MScan in all enrolled subjects.
CMAP and MUNE values were significantly lower and motor unit sizes were higher in ALS patients compared to healthy controls in all three muscles ($p < 0.05$), Table 2.

Table 2: Neurophysiological parameters derived from MScan of ABP, ADM and TA muscles in ALS patients compared to healthy controls

	APB		**p-value**	**ADM**		**p-value**	**TA**		**p-value**
	ALS	**HC**		**ALS**	**HC**		**ALS**	**HC**	
CMAP peak (mV)	4.2 (0.7-9.9)	8.7 (6.2-14.1)	**<0.001**	6.3 (2.5-12.9)	9.9 (8.1-12.1)	**0.001**	4.6 (0.7-8.5)	6.9 (4.8-10.7)	**<0.001**
MUNE	32 (10-125)	86 (64-154)	**<0.001**	77 (11-168)	132 (71-170)	**<0.001**	94 (12-200)	172 (151-197)	**<0.001**
N50	9.2 (1.2-36.9)	23.2 (3.9-48.4)	**<0.001**	19.5 (1.9-53.3)	41.4 (5.4-58.5)	**0.001**	23.3 (3.8-74.2)	53.1 (3.2-67.8)	**0.001**
A50 (%)	3.6 (0.9-19.6)	1.3 (0.7-2.1)	**<0.001**	1.6 (0.7-18.3)	0.8 (0.6-2)	**<0.001**	1.3 (0.5-11.1)	0.6 (0.5-0.8)	**<0.001**
LSMUP (%)	8.3 (3.9-46.5)	4.2 (1.9-7.2)	**<0.001**	5.6 (1.6-33.9)	2.5 (1.7-6.1)	**0.001**	4.8 (1.2-16.7)	2.1 (1.6-7.9)	**0.003**

Values are expressed as median (range min-max). Key: APB, abductor pollicis brevis; ADM, abductor digiti minimi; ALS, amyotrophic lateral sclerosis; A50, the size of the motor units at the 50% mark of the cumulative amplitude expressed in %; CMAP, compound motor amplitude potential; HC, healthy controls; MUNE, motor unit number estimation; LSMUP, amplitude of the largest unit, expressed as a percentage of CMAP amplitude; mV, millivolt; N50, estimated number of larger units making up 50-100% of the amplitude of the CMAP; TA, tibial anterior.

The ability of MScan to discriminate between ALS patients and healthy controls by means of MUNE values and LSMUP (%) is reported in Table 3. From the ROC analyses we derived the area under curve (ROC AUC) as well as the best cut-off value to maximize the accuracy (best sensitivity and specificity) for discriminating patients from controls.

Table 3: ROC curve analyses showing the area under curve (ROC AUC) as well as the best cut-off value of MUNE values and largest unit (%) to maximize the accuracy (best sensitivity and specificity) for discriminating ALS patients from controls.

	ABP MUNE value	**ADM MUNE value**	**TA MUNE value**
ROC AUC (95% CI)	0.884 (0.795-0.973)	0.845 (0.73-0.959)	0.907 (0.827-0.988)
Cut-off value	61	112	149
Sensitivity	73%	78%	81%
Specificity	100%	79%	100%
	ABP Largest unit %	**ADM Largest unit %**	**TA Largest unit %**
ROC AUC (95% CI)	0.913 (0.83-0.996)	0.803 (0.683-0.923)	0.769 (0.636-0.902)
Cut-off value	5,27	3,58	3,19
Sensitivity	95%	70%	68%
Specificity	79%	86%	93%

Key: APB, abductor pollicis brevis; ADM, abductor digiti minimi; AUC, area under curve; CI, confidence interval; MUNE, motor unit number estimation; ROC, receiver operating characteristic analysis; TA, tibial anterior.

5.2.3.2 EMG parameters and correlations between neurophysiological measures.

APB muscle was studied through qEMG in 36 out 37 ALS patients. The qEMG analysis revealed abnormalities (i.e. the presence of both denervation and reinnervation signs) in 31 out 36 (86.1%) of cases. To define a muscle studied by MScan as affected, we used the above calculated cut-offs for MUNE values, that showed a general higher accuracy in the three muscles than LSMUP %.

MUNE values were less frequently abnormal (27/37) than qEMG (72.9% *vs* 86.1%).

28 out 37 ALS patients performed qEMG analysis on the ADM muscle, which showed abnormal values in 82.1% of cases, compared to 29 out 37 patients (78.4%) with pathological MUNE values.

Finally, among 36 TA muscles with available qEMG analyses, 32 (88.9%) resulted pathological compared to 30 out 37 (81%) with abnormal MUNE values.

For details about findings of muscles studied through both MScan and qEMG see Table 4.

Looking at the few muscles with MUNE values still in the normal range but with abnormalities in qEMG, we found that among seven APB, three fell in the category “not denervated” and four in the “mild denervated” one. Likewise, of two normal ADM, one belonged to the “not denervation” category and the other one to the “mild denervation”. This was also true for five TA muscles with normal MUNE values, with one falling in the category of “not denervation” and four in the one with “mild denervation”. In other words, no one of these muscles was highly denervated (Table 4).

Table 4. Distribution of muscles studied through both MScan and qEMG, according to MUNE values and EMG abnormalities.

	Normal qEMG findings	**Abnormal qEMG: Denervation degree**		
APB muscle		Not denervated (with fasciculations)	Mild denervation (2-5 sites)	High denervation (> 5 sites)
Normal MUNE	2	3	4	**0**
Abnormal MUNE	3	4	18	2
ADM muscle				
Normal MUNE	2	1	1	**0**
Abnormal MUNE	3	4	16	1
TA muscle				
Normal MUNE	2	1	4	**0**
Abnormal MUNE	2	2	14	11

qEMG abnormalities are graded based on the denervation degree. Key: APB, abductor pollicis brevis; ADM, abductor digiti minimi; MUNE, motor unit number estimation; TA, tibial anterior.

MScan parameters which reflect the loss of motor unit and collateral sprouting in all three muscles resulted well correlated with denervation degree and duration of motor unit potentials assessed by qEMG, as showed in Table 5.

Table 5. Spearman's correlation between Mscan and quantitative EMG parameters

	Spearman's Rho coefficient	**P-value**
APB muscle		
MUNE vs denervation	-0.56	**<0.001**
MUNE vs MUP duration	-0.45	**0.005**
LSMUP (%) vs denervation	0.47	**0.004**
LSMUP (%) vs MUP duration	0.46	**0.005**
A50 (%) vs denervation	0.48	**0.003**
A50 (%) vs PUM duration	0.41	**0.012**
ADM muscle		
MUNE vs denervation	-0.37	**0.052**
MUNE vs MUP duration	-0.52	**0.004**
A50 (%) vs denervation	0.32	0.1
A50 (%) vs PUM duration	0.51	**0.006**
LSMUP (%) vs denervation	0.38	**0.044**
LSMUP (%) vs MUP duration	0.41	**0.031**
TA muscle		
MUNE vs denervation	0.49	**0.002**
MUNE vs MUP duration	0.43	**0.009**
A50 (%) vs denervation	0.46	**0.004**
A50 (%) vs MUP duration	0.43	**0.008**
LSMUP (%) vs denervation	0.53	**0.001**
LSMUP (%) vs MUP duration	0.43	**0.009**

Denervation degree is expressed as an ordinal score from 0 to 2. Duration of PUM expressed as an ordinal score from 0 to 3.

Key: APB, abductor pollicis brevis; ADM, abductor digiti minimi; ALS, amyotrophic lateral sclerosis; A50, the size of the motor units at the 50% mark of the cumulative amplitude expressed in %; MUNE, motor unit number estimation; LSMUP, amplitude of the largest unit, expressed as a percentage of CMAP amplitude; MUP, motor unit potential; TA, tibial anterior.

5.2.3.3 Correlations between clinical variables and MScan measures

In ALS patients, MUNE values in all three muscles moderately correlated with the force measured by MRC scale (APB, Rho=0.42, p=0.010; ADM, Rho=0.57, p<0.001; TA, Rho=56, p<0.001).

MUNE in TA muscle was also correlated with the motor subscore of the ALSFRS-R scale (Rho=0.47, p=0.004).

No other associations were found between MUNE values in three muscles and clinical variables (age, disease duration, DPR, ALSFRS-R score, FVC), with the only exception of a mild correlation between MUNE values of ADM muscle and FVC (Rho=0.38, p=0.031).

5.2.4 Discussion

In this study we aimed to investigate the ability of MScan in discriminating ALS patients from healthy controls, and compared MScan values with parameters derived from qEMG analysis. We found that qEMG was more frequently abnormal in all three muscles than MScan, even though the concordance between the two techniques was quite high.

Our results are different from other studies (Jacobsen et al., 2018; Kristensen et al., 2019). In details, the study of Jacobsen et al. showed that MScan on APB muscle was more frequently abnormal than MUP duration and amplitude in 25 ALS patients. Similarly, Kristensen et al. evaluated the feasibility of MScan on the TA muscle in a cohort of 25 ALS patients, and confirmed that more patients presented pathological MScan MUNE values as compared to the chronic neurogenic changes assessed by qEMG. Possible explanations for this inconsistency might be the different populations, since our patients presented a shorter disease duration as compared to the others (17 months ranging from 3 to 60 months in the first study; 24 months with range 2-120 in the latter one). It is possible that patients with a lower disease duration presented a higher pool of surviving and still functioning motor units, with earlier signs of rearrangement. In line with this explanation, in our population muscles with MUNE values still in the normal range but with EMG abnormalities more frequently presented mild or even absent denervation with only fasciculations, together with the early presence of chronic neurogenic signs. Furthermore, we considered a qEMG exam as abnormal when subacute neurogenic changes (i.e. both denervation and reinnervation findings) were present and we did not consider the increase of duration by itself as a criterion to define a muscle as affected.

Besides, this was the first study which compared MScan values with qEMG parameters in the ADM muscle, confirming a higher sensitivity of the quantitative motor unit potentials analysis.

Furthermore, we calculated the better cut-offs of MUNE to discriminate ALS patients from healthy controls and showed the diagnostic accuracy of this text on the three muscles. Our results were quite similar to those of other studies for APB (Jacobsen et al., 2017; Jacobsen et al., 2018; Sirin et al., 2019), while the calculated cut-off for ADM muscle was quite higher compared to other findings (Sirin et al., 2019), probably due to the different features of our population. However, similarly to previous results (Sirin et al., 2019), in our sample the diagnostic accuracy was better for APB than ADM muscle, probably reflecting the preferential involvement of the thenar group muscle with the relative preservation of the hypothenar region, as attested by the split-hand phenomenon (Corcia et al., 2021). Likewise, for TA muscle we found a much higher cut-off compared to the study of Kristensen et al., discrepancy which we could again explain with the different features of the population but also with the different Qtrac program, since we used the MScanFit-2 program, a more recently program developed to allow for the higher numbers of units in TA (Sørensen et al., 2023).

Interestingly, neurophysiological parameters reflecting neuronal loss in MScan showed a good correlation with both denervation degree and duration of motor unit potentials as assessed by qEMG. This actually means that the more a muscle is denervated and reinnervated, the more is the motor unit lost. Moreover, parameters mirroring reinnervation in MScan (A50 and LSMUP) and qEMG resulted well correlated, despite the two techniques sampled different motor units, giving strength to MScan measures.

We failed to find significant correlations between MUNE values and clinical features, except for the force degree which was related to MUNE values and TA muscle's MUNE which correlated with the motor subscore of the ALSFRS-R scale. Indeed, the correlation between MUNE values and the ALSFRS-R score has been reported in some studies (Jacobsen et al., 2018; Jacobsen et al., 2017) but not always confirmed (Sirin et al., 2019; Gunes et al., 2021), probably because the scale reflects a more general functional status coming from several body regions other than specific abilities related to the muscles neurophysiologically examined. Indeed, we tried to partially overcome this limitation by using the motor subscore, although for a more precise correlation we should have extrapolated the fine and gross motor domains.

Our study has some limitations. Firstly, our ALS cohort was relatively small and unbalanced for onset type and clinical phenotype. Also, ALS mimic diseases might have been enrolled to better test the sensibility of MScan. Furthermore, not all muscles were examined through both MScan and qEMG examinations, limiting the comparison between the two techniques. Finally, the cross-sectional design of the study prevented a longitudinal analysis, which would be helpful in clarify the role of MScan in defining the disease progression.

To conclude, we confirmed that MScan is a valid tool to discriminate ALS patients, but is not suggested to replace conventional diagnostic methods, but as a supplementary tool, which could aid in diagnosing and following pathophysiological disease progression in ALS patients.

6. Conclusions

ALS remains a challenge for patients and clinicians, due to the lack of therapeutic interventions which could arrest or at least significantly slow down the neurodegenerative process.

The still nebulous knowledge of the pathogenic mechanisms underlying the disease and its intrinsic high variability are the main causes of the unsuccess of clinical trials.

Biomarkers are essential to fill these gaps, and showed the potential to radically reduce the duration and cost of therapeutic trials, as well as to offer a first step towards the goal of more personalized disease monitoring for those living with amyotrophic lateral sclerosis.

In the first three studies we investigated biofluids biomarkers, and confirmed the diagnostic and prognostic values of neurofilament light chains, that have been recently implemented in clinical trials to measure the effect of experimental treatments (Miller et al., 2022). Then we explored the role of plasma p-tau181, a biomarker recently suggested as a promising diagnostic tool for the diagnosis of Alzheimer's disease (AD). We here demonstrated that plasma p-tau181 values are also related to the lower motor neuron dysfunction and are therefore increased in ALS, suggesting to be more cautious in proposing plasma p-tau181 as a screening tool for AD in the general population, being its peripheral source a possible confounding factor.

Subsequently, we focused on plasma GFAP, another biomarker underlying AD pathology, and showed that its increase in ALS mainly reflects the presence of AD co-pathology which can actually influence the cognitive phenotype of patients, covering by itself a practical and clinical role.

With the two last studies, we explored the role of neurophysiological biomarkers. In the first one, we confirmed that conventional EMG, which is the main tool for diagnosing ALS, also plays a prognostic role in discriminating patients with a faster disease progression and a shorter survival.

In the second one we perform an exploratory study on a novel neurophysiological tool, the MScan, comparing its diagnostic utility to more conventional neurophysiological parameters.

The strength of our studies is the deeply characterization of the population, since patients are systematically diagnosed and followed-up in our Neurological Clinic, allowing to collect homogeneous and systematic clinical and neurophysiological data. Our main limitation is the design of these studies, more frequently cross-sectional and with limited longitudinal observations. However, performing longitudinal observational studies in ALS patients could be challenging due to the different disease progression rates and the burden derived from the effort requested to patients in the absence of a therapeutic proposal.

To conclude, we confirmed the undiscussed utility of some biomarkers and explored the potential of others in revealing some other pathogenic aspects of the disease in the living human brain. Through

these studies we produced several pieces of evidence which significantly contribute to the field of biomarkers' research in ALS.

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