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ABSTRACT

Background: Phosphorylated-tau181 (p-tau181), a specific marker of Alzheimer's disease (AD) pathology, was found elevated in plasma but not in cerebrospinal fluid (CSF) of patients with amyotrophic lateral sclerosis (ALS). We expanded these findings in a larger patient cohort, exploring clinical/electrophysiological associations, prognostic value, and longitudinal trajectories of the biomarker.

Methods: We obtained baseline plasma samples from 148 ALS, 12 spinal muscular atrophy (SMA), and 88 AD patients, and 60 healthy controls. Baseline CSF and longitudinal plasma samples were from 130 and 39 ALS patients. CSF AD markers were measured with the Lumipulse platform, and plasma p-tau181 with SiMoA.

Results: ALS patients showed higher plasma p-tau181 levels than controls ($p<0.001$) and lower than AD participants ($p=0.02$). SMA patients had higher levels than controls ($p=0.03$). In ALS patients, CSF p-tau and plasma p-tau181 did not correlate ($p=0.37$). Plasma p-tau181 significantly increased with the number of regions showing clinical/neurophysiological LMN signs ($p=0.007$) and correlated with the degree of denervation in the lumbosacral area ($Rho=0.51$, $p<0.0001$). Plasma p-tau181 levels were higher in classic and LMN-predominant than in bulbar phenotype ($p=0.004$ and $p=0.006$). Multivariate Cox regression confirmed plasma p-tau181 as an independent prognostic factor in ALS (HR 1.90, CI 1.25-2.90, $p=0.003$). Longitudinal analysis showed a significant rise in plasma p-tau181 values over time, especially in fast progressors.

Conclusions: Plasma p-tau181 is elevated in ALS patients, independently from CSF levels, and is firmly associated with LMN dysfunction. The finding indicates that p-tau181 of putative peripheral origin might represent a confounding factor in using plasma p-tau181 for AD pathology screening, which deserves further investigation.

Key messages

What is already known on this topic: A recent study showed that p-tau181 levels in plasma, but not in CSF, are elevated in ALS patients and correlate with lower motor neuron (LMN) dysfunction, albeit only clinically assessed.

What this study adds: In the most significant cohort studied to date, we confirmed that plasma p-tau181 is higher in ALS patients than in controls and show that it has a solid prognostic value and increases along the disease course, especially in patients with a faster disease progression. The biomarker is related to the spatial extent of clinical/neurophysiological LMN dysfunction and the denervation degree in the lumbosacral region assessed through neurophysiological studies.

How this study might affect research, practice, or policy: Plasma p-tau181 is a biomarker of LMN dysfunction in ALS. The finding also implies that the marker is not fully specific to the Alzheimer's Disease (AD) pathologic process. Future use of plasma p-tau181 as a screening tool for AD in the general population should consider the peripheral source of p-tau as a possible confounding factor.

1. INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the progressive degeneration of motor neurons in the brain and spinal cord, leading to widespread muscle wasting and weakness.[1] Clinically, ALS is a highly heterogeneous disease, ranging from classical to rarer phenotypes with the predominant involvement of upper motor neurons (UMN) or lower motor neurons (LMN), potentially challenging the diagnostic process. The possible association of cognitive impairment, sometimes leading to full-blown frontotemporal dementia [2], further broadens the clinical spectrum of ALS. Neuropathologically, ALS belongs to the TDP-43 proteinopathies characterized by TDP-43 enriched inclusions in affected neurons.[3,4]

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, namely amyloid-beta, phospho-tau (p-tau), and total tau (t-tau), and those detecting neuroaxonal degeneration, as neurofilament light chain (NfL) have provided the most substantial impact.[5,6] 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.[7,8] In particular, the measurement of blood p-tau is increasingly considered a realistic, cost-effective, and noninvasive assay that will help the diagnostic process for patients with cognitive decline.[7,9,10] Nevertheless, whether plasma measures of these biomarkers exclusively reflect their CSF concentration or are also influenced by peripheral sources remains to be fully explored.

In motor neuron diseases, NfL levels in cerebrospinal fluid (CSF) and plasma have been shown to accurately distinguish ALS patients from their mimics[11-15], correlate with disease severity, and predict survival.[8,11,13-16]

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 ALS patients with cognitive dysfunction[17-20] and, to a lesser extent,

in those with pure motor ALS.[21] 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.[14,22-24] Unexpectedly, a recent study by Cousins et al.[25] showed that ALS patients exhibit significantly increased levels of plasma p-tau phosphorylated at residue 181 (p-tau181) compared to controls. Intriguingly, the authors found that plasma p-tau181 levels do not correlate with CSF p-tau181 levels and AD post-mortem 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 [26,27] we aimed to expand the current data on the plasma p-tau181 levels in ALS patients. Furthermore, we explored for the first time the associations 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).

2. METHODS

2.2 Inclusion criteria and clinical assessment

Our cohort comprised 148 patients with a clinical diagnosis of ALS according to the Revised El Escorial criteria,[28] 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.[29] We also included 18 ALS patients with only plasma samples available because their age at sampling (less than 60, median 54.5, interquartile range 47.25-57) made a concomitant AD pathology unlikely.[30] Finally, we included

20 ALS patients with CSF evidence of underlying amyloid co-pathology (A+), 12 SMA patients, 88 AD patients, 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. AD patients fulfilled the criteria for "probable AD dementia with evidence of the AD pathophysiological process" according to the 2011 NIAAA criteria.[31]

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[32], clinical phenotype[33], ALS Functional Rating Scale-revised (ALSFRS-R) score, Medical Research Council (MRC) scale of 0 to 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[34], Milan-Torino (MiToS)[35], and Fine'til 9 (FT9) clinical stages.[36] Patients were stratified according to the validated clinical classification [33] 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 (i.e., PUMN and PLS), and LMN-predominant (PLMN, including flail arm/leg and PMA).

Details on cognitive function assessment in ALS patients are provided in Supplementary Materials.

One hundred forty-two (96%) patients underwent genetic screening for the most frequent ALS-associated genes (i.e., *SOD1, FUS*, *TARDBP,* and the repeats-expansion of *C9Orf72*)[35], Supplementary Materials]. UMN involvement was evaluated semiquantitatively by the number of regions (bulbar, cervical, and lumbosacral region) showing UMN signs at clinical examination. In contrast, we used clinical and EMG assessments according to the Awaji criteria to define the extent of the LMN involvement[36].

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.[14] 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 ten 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).[11] Accordingly, patients were divided into slow (DPR < 0.5), intermediate (DPR 0-5 – 1), and fast progressors (DPR > 1).[11]

Thirty-nine of the 148 ALS patients had plasma samples from two or more follow-up visits. Repeated sampling was performed at non-standardized 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, 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).[15]

2.3 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 ALS patients, were determined with the Single molecule array (Simoa) technology on a SR-X instrument using commercially available kits (Quanterix, Billerica, MA, USA). The mean intra- and inter-assay coefficients of variation (CVs) were below 15% for both biomarkers. CSF NfL in AD patients was quantified by a validated commercial enzyme-linked immunosorbent 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 intra- and inter-assay CVs for these markers were <8%. The Aβ42/Aβ40 was calculated as described.[35] Pathological values for the AD core markers were determined according to in-house validated cutoffs. Specifically, a CSF Aβ42/Aβ40x10 ratio <0.68 was considered supportive of amyloid deposition (i.e., A+ according to the ATN classification).

2.4 Statistical analyses

Statistical analysis was performed using Stata SE version 14.2 (StataCorp LLC, Texas, USA) and GraphPad Prism 7 (GraphPad Software, La Jolla, CA) 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 (ANOVA) (followed by Tukey's post hoc test) were used for multiple group comparisons. The Chi-Square test was applied for categorical variables.

For the cross-sectional analysis, Spearman's rho 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% 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, 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, presence of FTD, b-DPR, and King's score. 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 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 ALS patients stratified into fast, intermediate, and slow progressors, according to both basal and longitudinal DPR.[15] The results are presented as ß coefficients and 95% CI.

3. RESULTS

3.1 Demographic values 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.

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 ALS patients

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	88 (59.4)		**N (%)**
Bulbar	18 (12.2)	**King's staging**	
Respiratory	1 (0.7)	1	7 (4.7)
PUMN	13 (8.8)	2	47 (31.7)
PLS	2 (1.3)	3	79 (53.4)
Flail arm syndrome	11 (7.4)	4	15 (10.1)
Flail leg syndrome	8 (5.4)	**MiToS staging *(n=140)***	
PMA	7 (4.7)		

Deceased/with tracheostomy	67 (45.3)	0	118 (84.3)
Genetic status *(n=142)*		1	18 (12.8)
C9Orf72 RE carriers	15 (10.6)	2	3 (2.1)
SOD1 mutation carriers	3 (2.1)	3	1 (0.7)
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.

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 ALS and AD patients had substantially higher p-tau181 levels than controls ($p<0.001$), with significantly lower levels in ALS compared to 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, ALS patients (main group) had significantly lower plasma p-tau181 values than A+ ALS patients ($p=0.005$). In contrast, ALS patients showed significantly lower CSF p-tau values than AD

participants (p<0.001). The distribution of CSF core AD biomarkers is shown in Supplementary Table 1.

3.2 Association between biomarkers and clinical variables in ALS patients

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

Plasma p-tau181 levels were weakly correlated with age at sample collection (Rho=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 (Rho=-0.21, p=0.01) and MRC (Rho=-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 (Rho=0.16, p=0.052), creatinine values (p=0.46), CVF (p=0.22) or DPR (p=0.78). The disease duration correlated weakly with only a trend of significance (Rho=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 to 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 to *C9ORF72* (p=0.04) and *TARDBP*-mutated patients (p=0.04), Table 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 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 the 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, Supplementary Table 2). Notably, the association was still significant after adding plasma NfL levels in the multivariate model (Supplementary Table 3).

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).

3.4 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 (Supplementary Table4). 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, Supplementary Table 5).

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

Plasma p-tau181 levels were also confirmed as an independent prognostic factor after including plasma NfL levels in the model (Supplementary Table 6). 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).

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).

4. DISCUSSION

In this study, we confirmed in a large cohort that ALS patients show significantly elevated plasma p-tau181 levels that in most cases is unrelated to AD pathology. The biomarker change is likely 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, [25] 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,[40] 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 fibers arising from the lumbosacral region, compared to 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 fibers. 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.,[25], we used a standardized phenotype classification based on the clinical longitudinal assessment of patients by expert neurologists besides the UMN- or LMN-onset anamnestic distinction.

We also confirmed that ALS patients with concomitant FTD display lower levels of plasma p-tau181 compared to those with pure motor ALS, making the sporadic reports on a limited tauopathy in ALS-FTD patients [17,41] unrelated 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 to *C9ORF72* and *TARDBP* patients. With the necessary caution related to the low sample size, these data, in line with those previously reported,[25] 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.[33]

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, must confirm these results. Further studies are 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 pre-clinical or prodromal stage.[26,27] 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 [25] 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 behavior of this biomarker in a subset of ALS patients, 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. [11,13,15] The longitudinal behaviour of p-tau181 in ALS patients could reflect the ongoing denervation until the final phase of the disease, with an addictive effect of damage of the peripheral fibers, initially insulted but still undergoing rearrangement of axonal fibers.[42]

CSF and blood NfL probably have a more robust predictive value on survival [15-20] than plasma p-tau181 in ALS patients. 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 pharmacodynamics targets implied in ALS research.[43]

Including a significant number of well-characterized ALS patients with available quantitative electromyographic data is the major strength of our study. The association with survival data and the availability of longitudinally repeatedly sampled ALS patients 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 standardized time points for the longitudinal sampling of ALS patients. Finally, the limited number of SMA patients included did not allow us to draw a definitive conclusion on the significance of the increased trend of plasma p-tau181 in these patients.

In conclusion, our study provides evidence that plasma p-tau181 is elevated in ALS patients 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 ALS patients. 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.

CONTRIBUTORS

VV, AM, and PP: conceptualization and writing—original draft preparation. PP: writing—review, and editing based on the critical revision of all authors and supervision. All authors: methodology, formal analysis, and investigation.

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COMPETING INTERESTS

The authors declare no conflict of interest.

ETHICAL APPROVAL

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" (CE-AVEC -17151-17152).

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FIGURE LEGENDS

Figure 1 - Distribution of plasma p-tau181 values across the different diagnostic categories. Plasma p-tau181 values in the different diagnostic categories included. Thick lines represent medians and interquartile ranges. Key: p-tau, phosphorylated tau 181.

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.

Figure 3 – **Longitudinal trajectories of plasma p-tau181**: Overall and single-patient longitudinal plasma p-tau181 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.

Figure 1.

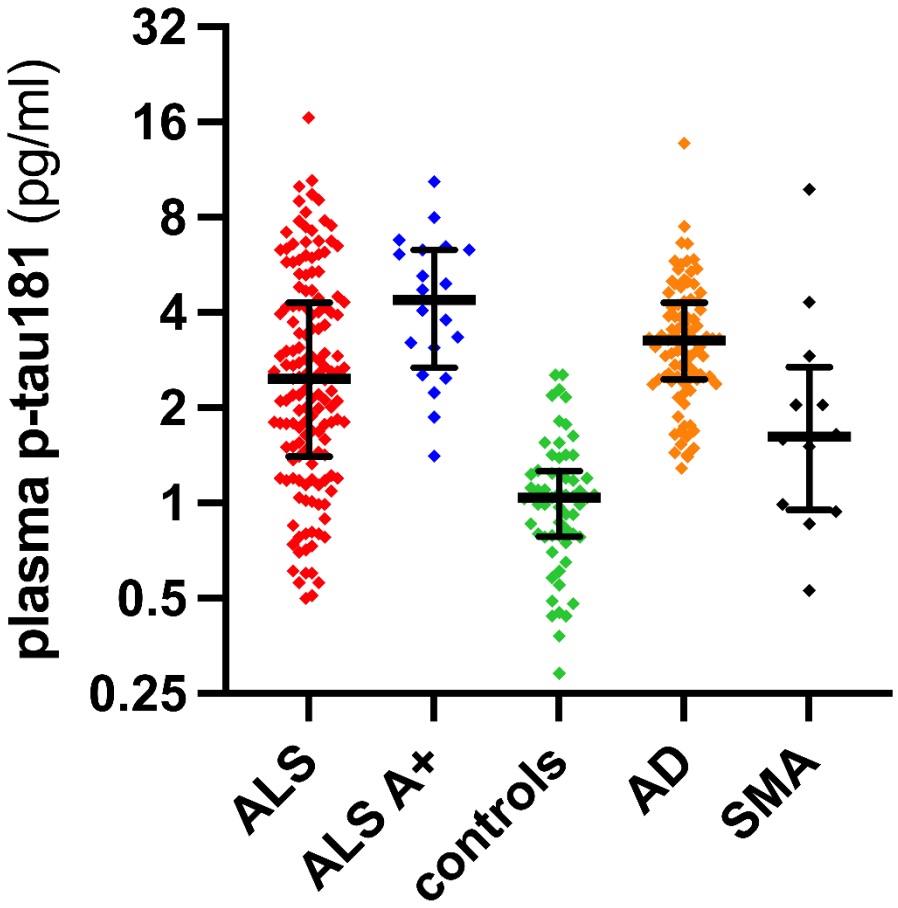


Figure 2.

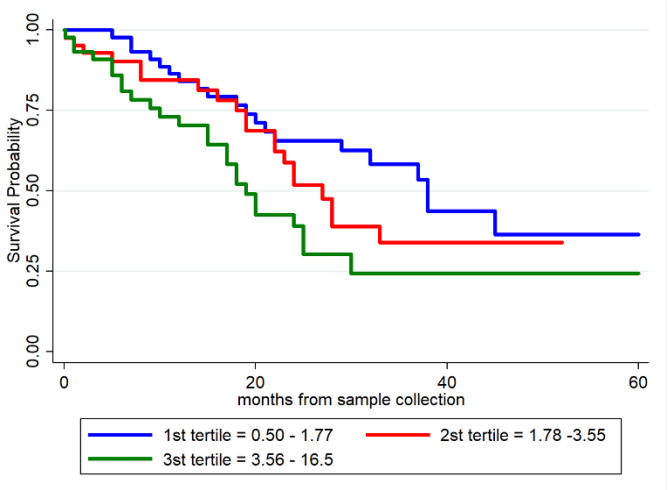
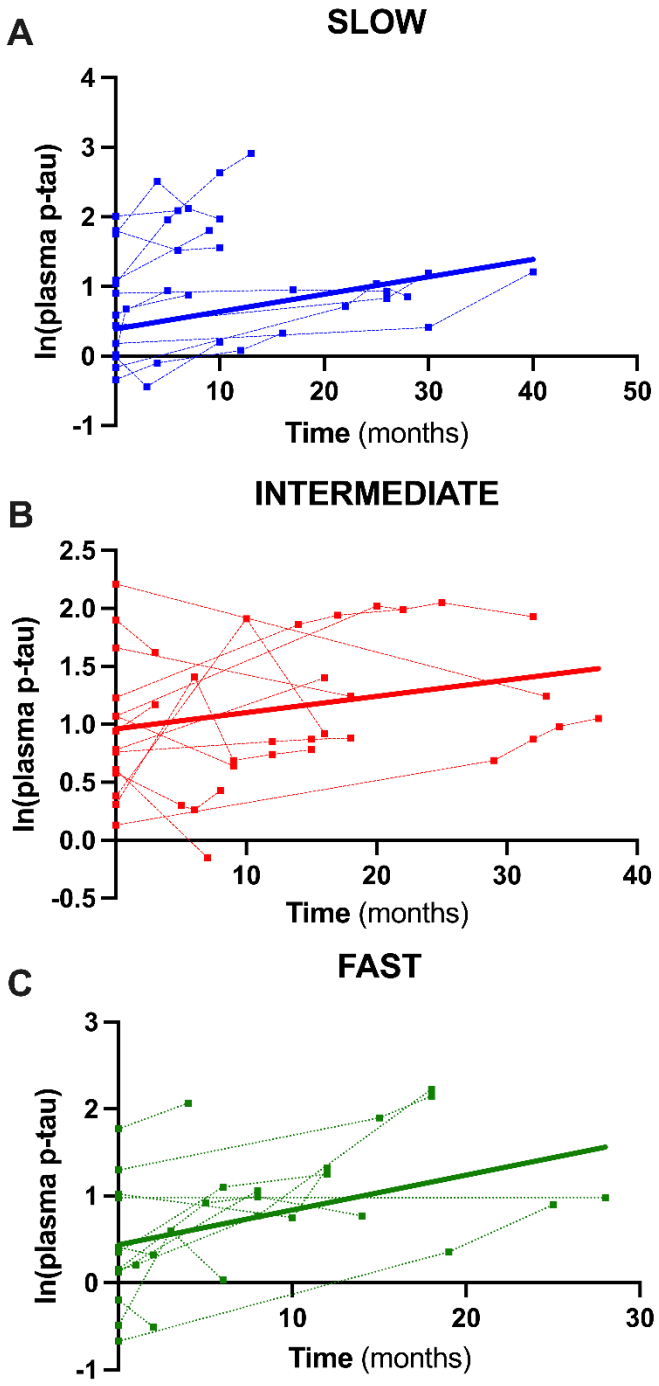


Figure 3.


A
SLOW
ln(plasma p-tau)
Time (months)
B
INTERMEDIATE
ln(plasma p-tau)
Time (months)
C
FAST
ln(plasma p-tau)
Time (months)