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(Article begins on next page)

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Diagnostic-prognostic values and electrophysiological correlates of CSF biomarkers of neurodegeneration and neuroinflammation in amyotrophic lateral sclerosis.

Samir Abu-Rumeileh[1,*], Veria Vacchiano[1,*], Corrado Zenesini[2], Barbara Polischi[2], Silvia de Pasqua[1], Enrico Fileccia[1], Angela Mammana[2], Vitantonio Di Stasi[2], Sabina Capellari[1,2], Fabrizio Salvi[2], Rocco Liguori[1,2], Piero Parchi[2,3], BoReALS.

BoReALS group: I. Bartolomei, R. Plasmati, F. Pastorelli, C. C. Quarta, V. Reale, V. Mariano, D. Milletti, R. Nasca, F. Rizzi, F. Baracchini, A. Cherici, D. Rusolo, L. Valeriani, F. Anzolin, L. Zoni, E. Fantoni, A. Fiorito, L. Andrini, P. Avoni, V. A. Donadio, S. Capellari, S. De Pasqua, G. Rizzo, R. Infante, V. Vacchiano, E. Fileccia, L. Morandi, F. Marliani, L. Albini Riccioli, P. Parchi, M. P. Foschini, A. Pession, S. Battaglia, R. Poda, D. Valenti, S. Asioli, L. Vignatelli, F. Oppi, M. Stanzani Maserati, A. Bartoletti-Stella, C. Colombo, S. Maselli, M.P. Giannoccaro, M. Morescom, F. Salvi, R. Liguori

[1] Department of Biomedical and NeuroMotor Sciences (DIBINEM), University of Bologna, 40139 Bologna, Italy

[2] IRCCS Istituto delle Scienze Neurologiche di Bologna, 40139 Bologna, Italy

[3] Department of Experimental Diagnostic and Specialty Medicine (DIMES), University of Bologna, 40138 Bologna, Italy

[*] These authors contributed equally to this work

Corresponding Author address: Piero Parchi, IRCCS Istituto delle Scienze Neurologiche di Bologna, Ospedale Bellaria, 40139 Bologna, Italy. Tel.: +390514966740, Fax.: +390514966208. E-mail: piero.parchi@unibo.it.

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AUTHORS CONTRIBUTIONS

Conceptualization: Samir Abu-Rumeileh, Veria Vacchiano, Rocco Liguori and Piero Parchi; Methodology – acquisition and analysis of data: all authors; Writing - original draft preparation: Samir Abu-Rumeileh, Veria Vacchiano, Rocco Liguori and Piero Parchi; Writing - review and editing: Piero Parchi based on the critical revision of all authors - Supervision: Piero Parchi.

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ABSTRACT

Neurofilament light chain protein (NfL) is currently the most accurate cerebrospinal fluid (CSF) biomarker in amyotrophic lateral sclerosis (ALS) in terms of both diagnostic and prognostic values, but the mechanism underlying its increase is still a matter of debate. Similarly, emerging CSF biomarkers of neurodegeneration and neuroinflammation showed promising results, although further studies are needed to clarify their clinical and pathophysiological roles. In the present study we compared the diagnostic accuracy of CSF NfL, phosphorylated (p)-tau/total (t)-tau ratio, chitinase-3-like protein 1 (YKL-40) and chitotriosidase 1 (CHIT1), in healthy controls (n = 43) and subjects with ALS (n = 80) or ALS mimics (n=46). In ALS cases, we also investigated the association between biomarker levels and clinical variables, the extent of upper motor neuron (UMN) and lower motor neuron (LMN) degeneration, and denervation activity through electromyography (EMG). ALS patients showed higher levels of CSF NfL, YKL-40, CHIT1, and lower values of p-tau/t-tau ratio compared to both controls and ALS mimics. Among all biomarkers, NfL yielded the highest diagnostic performance (> 90% sensitivity and specificity) and was the best predictor of disease progression rate and survival in ALS. NfL levels showed a higher correlation with the extent of LMN involvement, whereas YKL-40 levels increased together with the number of areas showing both UMN and LMN damage. EMG denervation activity did not correlate with any CSF biomarker change. These findings confirm the highest value of NfL among currently available CSF biomarkers for the diagnostic and prognostic assessment of ALS and contribute to the understanding of the pathophysiological and electrophysiological correlates of biomarker changes.

INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is a heterogeneous neurodegenerative disease characterized by the progressive degeneration of both upper and lower motor neurons. According to current criteria, the diagnosis of ALS relies on the detection of clinical upper motor neuron (UMN) signs, clinical and electrophysiological lower motor neuron (LMN) signs, and the exclusion of ALS mimicking diseases [1,2].

In the past decade, there have been several attempts to exploit cerebrospinal fluid (CSF) candidate biomarkers to improve the diagnostic and prognostic assessments of ALS and to better stratify and monitor the patients in clinical trials [3]. To date, neurofilament light chain protein (NfL) represents the most promising molecular marker in terms of both diagnostic accuracy and prediction of clinical progression, also having the advantage of being easily measured in both CSF and blood [4-9]. Although recent studies suggested that NfL levels may reflect the extent of the UMN degeneration more than that of the LMN [8-10], the pathogenetic mechanism underlying the raise of NfL concentration in biological fluids is still a matter of debate. The fact that only one study to date [8] correlated NfL values with electromyographic findings, the most reliable source of information regarding the extent of LMN involvement, may be relevant in this context. Moreover, although the phosphorylated (p)-tau/total (t)-tau ratio constitutes an established marker within the frontotemporal dementia (FTD) spectrum [11-13], only a few studies assessed its clinical value in ALS [8,14,15].

Beside NfL and p-tau/t-tau ratio, markers of glial activation, such as chitinase-3-like protein 1 (YKL-40) and chitotriosidase-1 (CHIT1), have been explored as possible diagnostic and prognostic biomarkers for ALS, but with conflicting results regarding their diagnostic performance in comparison to classical neurodegenerative markers [3,7, 16-19].

Taking into account all these unresolved issues, we aimed to comprehensively investigate the diagnostic value of well-established CSF biomarkers of neurodegeneration (NfL and p-tau/t-tau) and neuroinflammation (YKL-40 and CHIT1) in the discrimination between ALS, ALS mimics and controls. Furthermore, we evaluated whether CSF biomarker levels are associated with the clinical hallmark features of ALS, namely disease progression rate (DPR), survival, and the extent of UMN and LMN degeneration, with the latter evaluated through electromyography (EMG).

METHODS

Case classification

We analyzed 169 CSF samples from patients admitted at the Clinica Neurologica, Bellaria Hospital (Bologna, Italy) between 2009 and 2019. The cohort comprised 80 cases with ALS, 46 ALS mimics, and 43 healthy controls.

Patients with suspected ALS were prospectively enrolled and underwent a clinical examination, a neurophysiological study including EMG, lumbar puncture (LP), and ancillary tests to exclude ALS mimicking diseases. The maximum delay

between clinical examination, EMG, and LP was three months. Patients were divided into the following clinical phenotypes: classic, bulbar, prevalent UMN, and prevalent LMN (including flail-arm and flail-leg variants) as described [20]. Patients were also classified as definite ALS (N= 19), probable ALS (N=27), probable laboratory-supported ALS (N=16), and possible ALS (N=18) according to the revised El Escorial criteria [1].

DPR was defined as [48 - ALS Functional Rating Scale Revised (ALSFRS-R) score at LP]/disease duration at LP in points per month. Patients were divided into slow, intermediate and fast progressors according to DPR [slow if below the 25th percentile (< 0.24), fast if above the 75th percentile (> 1.15), intermediate if between or equal to 0.24 and 1.15] as previously described [3, 9].

In ALS patients, the extent of UMN degeneration was defined as the number of regions (bulbar, cervical and lumbosacral regions) displaying UMN degeneration as clinically assessed according to the revised El Escorial criteria [1]. The extent of LMN degeneration was defined as the number of regions (bulbar, cervical and lumbosacral regions) displaying LMN degeneration as assessed through EMG based on the Awaji criteria [2]. In detail, LMN involvement was defined by the presence of neurogenic changes (i.e. increased duration of motor unit potential with or without increased amplitude and/or polyphasia assessed quantitatively) plus denervation potentials (DP) and/or fasciculations. Thoracic UMN and LMN involvement were not assessed routinely. Subjects were then divided into categories, according to the presence of 1) none or one region 2) two regions, 3) three regions showing signs of UMN and/or LMN degeneration [3, 9].

Given that denervation activity has been previously described as an EMG marker associated with poor prognosis in ALS [21, 22], we chose to investigate the correlations, if any, between CSF biomarker changes and a denervation score (DS). The latter was defined after the revision of all EMG examinations of ALS patients included in the study and it was assigned based on the muscle with the highest denervation activity in a specific region (i.e. bulbar, cervical or lumbosacral). The score was derived from the number of sites per muscle showing DP. Each muscle was explored in 10 different sites; if DP (sharp waves or fibrillations) were identified in 6 or more sites, the DS was classified as high (DS=2 points). For muscles showing DP in a number of sites between 3 and 5 the DS was classified as low (DS=1 point). In cases where signs of denervation were limited to 2 sites or less out of 10, the muscle was considered not denervated (DS=0 points). DP were accepted for calculation of DS only if the examined muscle disclosed neurogenic changes.

The ALS mimics group comprised patients suspected of having ALS at the time of LP, who received an alternative clinical diagnosis at last follow-up by an expert neurologist (RL, FS or VV) (Supplementary table 1). The control group included age- and sex-matched subjects lacking any clinical or neuroradiologic evidence of central nervous system disease, such as tension-type headache, subjective cognitive or sleep complaints, and having normal values of p-tau/Amyloid-β (Aβ)42 and t-tau/Aβ42 ratios according to our in-house cut-offs [12, 13].

The study was conducted according to the revised Declaration of Helsinki and Good Clinical Practice guidelines. Informed consent was given by study participants or the next of kin. The present study was approved by the ethics committees of "Area Vasta Emilia Centro".

CSF and genetic analyses

CSF samples were obtained by LP at the L3/L4 or L4/L5 level following a standard procedure, centrifuged in case of blood contamination, divided into aliquots, and stored in polypropylene tubes at −80 °C until analysis. CSF NfL, t-tau, p-tau, Aβ42, YKL-40 and CHIT1 and levels were analyzed using commercially available enzyme-linked immunosorbent assay (ELISA) kits (IBL, Hamburg, Germany; INNOTEST htau-Ag, INNOTEST phosphorylated-Tau181, INNOTEST , Innogenetics/Fujirebio Europe, Ghent, Belgium; R&D ELISA, R&D Systems, Minneapolis, MN, USA; MBL, Belgium) as previously described [12,13]. The mean intra- and inter-assays coefficients of variation (CVs) were ≤ 5% and < 20% for all biomarkers as previously reported [13]. We previously demonstrated that storage time did not affect biomarker results [13,23].

In the large majority of our ALS cases (75 out of 80) we screened for pathogenetic mutations in ALS/FTD spectrum-associated genes as previously described [12]. Major screened genes included superoxide dismutase 1 (*SOD1*), TAR DNA-binding protein (*TARDBP*) and fused in sarcoma (*FUS)*. In the same patient group, we also screened for the presence of the hexanucleotide repeat expansion on chromosome 9 open reading frame 72 gene (*C9orf72 RE)* using the 2-step strategy with southern blotting confirmation, as previously described [12].

The expression and activity of CHIT1 is significantly reduced in subjects homozygous for a polymorphic 24 bp duplication in exon 10 of the *CHIT1* gene (rs3831317 polymorphism) which has a high prevalence in European populations (35%–50%) [13,17]. To exclude any effect of *CHIT* genotype on our results, we genotyped all cases with CHIT1 levels around the detection limit of the assay (280 pg/ml) (10 ALS patients, 2 controls and 4 mimics) as described [13]. Ten cases were indeed homozygotes (7 ALS patients, 2 controls and 1 mimics), four cases were heterozygotes and, unexpectedly, two were wild type for the duplication. Consequently, we performed all the analyses regarding CHIT1 twice, namely in all cases (n=169), and after the exclusion of the homozygotes for the 24 bp duplication (n=159).

Statistical analyses

Statistical analysis was performed using IBM SPSS Statistics version 21 (IBM, Armonk, NY, USA), Stata Stata SE version 14.2 (StataCorp LLC, Texas, USA) and GraphPad Prism 7 (GraphPad Software, La Jolla, CA) softwares. For continuous variables, depending on the data distribution, the Mann-Whitney *U* test or the *t*-test were used to test differences between two 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 applied for multiple group comparisons. Chi-Square test was adopted for categorical variables. Receiver operating characteristic (ROC) analyses were performed to establish the diagnostic accuracy, sensitivity, and specificity of each biomarker. The optimal cut-off value for biomarkers was chosen using the maximized Youden index. For the analysis of covariance (ANCOVA) biomarker values were transformed into a square root scale or into a logarithmic scale to obtain a normal data distribution. We used the square root- or log-transformed biomarker value as dependent variable and the extent of (1) UMN involvement, (2) LMN involvement, (3) UMN and LMN involvement as independent variables. We tested by univariate models the contribution of each possible covariate and then added to the multivariate model only those with significant associations. Final included covariates were age at LP, sex, DPR, disease duration at LP and the site of onset. Spearman's correlations were used to test the possible associations between CSF biomarkers levels and clinical variables, and between biomarkers values and DS. For the latter analysis, we calculated a partial score for each region (bulbar, cervical and lumbosacral) and a total score (i.e. sum of partial scores). 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 lumbar puncture, and the time of the endpoint was the date of death or the date of the last follow-up information (truncated at 6 years of follow-up), whichever came first. We performed univariate and multivariate Cox regression analysis to study the association between time to death and prognostic factors in ALS [24], namely tertiles of each CSF biomarker, DPR, age at LP, sex, the number of regions with UMN and LMN degeneration, the forced vital capacity, the disease duration at LP, the presence of C9orf72 mutation, the presence of FTD and the site of onset. The results are presented as Hazard Ratios (HRs) and 95% confidence intervals (95% CIs). The assumption of proportional hazard was assessed by Schoenfeld residuals. Differences were considered statistically significant at $p<0.05$.

RESULTS

Diagnostic value of CSF biomarkers of neurodegeneration and neuroinflammation in ALS

Demographic, clinical, and genetic data of ALS patients are reported in Table 1. There were no significant differences regarding age and sex distribution between groups (Table 2), and no effect of sex and age on biomarker values was detected except for age on YKL-40 in controls (Spearman's rho=0.549, $p<0.001$) [13].

ALS patients showed higher levels of CSF NfL, YKL-40 and CHIT1 compared to both controls ($p<0.001$ for each comparison) and ALS mimics ($p<0.001$ for each comparison); at variance t-tau differed only between ALS and controls ($p<0.001$), whereas p-tau levels were similar in all groups. Moreover, p-tau/t-tau ratio values were lower in ALS compared to both mimics and controls ($p<0.001$ for both comparisons) (Table 2 and Fig.1). The inter-correlations between CSF biomarkers in ALS patients are shown in supplementary results. NfL yielded the highest diagnostic accuracy in the

discrimination between ALS and both controls [area under the curve (AUC) 0.981±0.011] or ALS mimics (AUC 0.922±0.031) (Table 3).

Associations between CSF biomarkers and the extent and severity of UMN and/or LMN degeneration

There was a tendency for both NfL (Kruskal Wallis p=0.083) and YKL-40 levels (Kruskal Wallis p=0.057) to correlate with the number of regions with both UMN and LMN involvement (Table 4). After adjustment for covariates, both UMN and LMN involvement had an effect on YKL-40 [three regions vs. zero or one region: ß= 1.852 (95% CI 1.418-3.562), p=0.034] and a borderline association with NfL [three regions vs. zero or one region: ß= 13.262 (95%CI 0.295-26.494), p=0.050] (Table 4). Moreover, after the exclusion of the homozygotes for the 24 bp duplication, the analysis showed a tendency for CHIT1 (Kruskal Wallis, p=0.053), to significantly increase together with the number of regions showing UMN involvement, which was not confirmed after the adjustment for covariates [three regions vs. zero or one region: ß= 0.610 95% CI (-0.167-1.387), p=0.122] (Table 4). NfL, p-tau/t-tau, and YKL-40 levels did not correlate with the extent of UMN involvement. In addition, CSF NfL, but not p-tau/t-tau, YKL-40 and CHIT1 levels, significantly increased together with the number of regions showing LMN degeneration as assessed through EMG (Kruskal Wallis p=0.008) and also this finding was confirmed after adjustment for covariates (three regions vs. zero or one region: ß= 16.561 (95% CI 3.343-29.779), p=0.015) (Table 4).

The analysis of the effect of DS on CSF biomarkers did not reveal significant correlations in any examined region (bulbar, cervical, and lumbosacral). Moreover, there was no significant association between total DS and CSF values for each biomarker.

Prognostic value of CSF biomarkers in ALS

Median CSF biomarker values did not significantly differ among ALS clinical phenotypes. In ALS patients, the disease duration at LP (mean±SD= 20.32±18.14 months) slightly inversely correlated with NfL (Spearman's rho=-0.362, p=0.001) but not with other biomarkers. The results could be explained by the statistically significant difference in disease duration at LP among fast (n=18), intermediate (n=42), and slow (n=20) progressors, with the former showing a shorter disease duration compared to the other groups ($p<0.001$ for each comparison). Accordingly, DPR correlated with both NfL (Spearman's rho =0.391, $p<0.001$), p-tau/t-tau (Spearman's rho =-0.238, p=0.034), but not with other biomarkers. Moreover, fast progressors, according to DPR1, showed higher NfL levels compared to slow (p=0.004) progressors and lower values of p-tau/t-tau compared to intermediate (p=0.006) and slow progressors (p=0.041). Otherwise, no differences in other biomarker levels were detected between groups with different DPR.

Based on univariate Cox regression analysis (73 ALS patients; 22 dead, 1368 person-month) age, sex, DPR, presence of FTD, CSF NfL and CSF p-tau/t-tau ratio were identified as predictors of the mortality hazard ratios in ALS patients (Table 5, Fig.2 and supplementary results). In the multivariate Cox regression, only NfL but not p-tau/t-ratio remained a significant predictor of ALS survival (p=0.036) together with age, sex, and FTD status (Table 5 and supplementary results). Accordingly, ALS patients with higher baseline CSF NfL were associated with shorter survival (highest tertile of NfL vs. lowest tertile of NfL, HR (CI 95%): 3.943 (1.097-14.167), p=0.036). Other CSF biomarkers were not associated with survival in ALS patients.

DISCUSSION

In the present study, we have compared the diagnostic and prognostic performances of a large panel of CSF biomarkers of neurodegeneration and neuroinflammation in ALS. Our results confirm NfL as the best available CSF marker in terms of diagnostic value, and prediction of survival and disease progression. However, the data also provide some new insights into the clinical correlates and pathophysiological mechanisms underlying the increase of CSF NfL in ALS.

In our cohort, ALS patients showed higher levels of CSF NfL and lower p-tau/t-tau ratio values compared to both controls, and ALS mimics, reflecting the ongoing massive neurodegeneration occurring in the symptomatic phase of ALS [4-8,14]. Moreover, in full agreement with the notion that neuroinflammation has an important role in ALS pathogenesis [25], we confirmed the significant increase of CSF astroglial and microglial proteins, such as YKL-40 [3,7,17-19,26] and CHIT1 [3, 16-19, 26]. Thus, CSF YKL-40 and CHIT1 measurement could contribute knowledge regarding the timing, type, and extent of immune response that occur in ALS.

The neuroanatomical correlates of CSF NfL changes in ALS remain unclear. In this regard, our data showed a tendency towards a positive correlation between CSF NfL values and the number of regions affected by both UMN and LMN damage. However, the correlation was significant only in the sub-analysis with the extent of LMN degeneration and not in that with the UMN involvement, suggesting that the damaged motor neurons in the anterior horn of the spinal cord, which is rich in large axons, significantly contribute to the release of NfL in CSF. The result is at variance with other studies [8-10], in which the finding of a positive correlation between CSF or blood NfL levels and the corticospinal tract degeneration assessed either clinically or by 3T-Magnetic Resonance Imaging (DTI measures) led to the claim that CSF NfL should be considered a marker of UMN damage. However, the fact that CSF NfL levels are also significantly increased in spinal muscular atrophy (SMA) type 1, which is characterized by a selective, rapidly progressive, LMN degeneration [27], and the positive correlation we found between NfL levels and DPR at LP, which is confirmatory of previous data [5,7,9] are also supportive of a role of LMN damage. In contrast, slowly progressive motor syndromes with either selective UMN degeneration, such as Hereditary Spastic Paraplegias [28] and primary lateral sclerosis [4, 9] or a

selective involvement of LMN such as SMA type 3 [29] typically show lower CSF or blood NfL levels than ALS. Considering these findings altogether, we speculate that both the rate/aggressiveness of motor neuron degeneration and the extent of motor neuron degeneration in the anterior horn of the spinal cord significantly contribute to the raise of NfL levels in ALS. A possible explanation for the above-mentioned conflicting results in the current literature may be related to the inter-rater variability in the clinical evaluation of UMN or LMN signs. Thus, our decision to use only EMG as a stringent criterion to define LMN involvement by reducing the inter-rater clinical variability and allowing the detection of subclinical LMN pathophysiological changes likely added strength to our results [21]. On the contrary, the clinical-based assessment of UMN signs represents a limit our study shares with previous investigations [3, 5, 8, 9, 19].

Regarding glial markers, our findings of an association between the extent of both UMN and LMN involvement and levels of YKL-40, in partial agreement with previous literature [3, 19], and of a tendency toward a correlation between the number of areas with UMN damage and CHIT1 levels as described [3] are supported by the findings of a significant upregulation of both YKL-40 and CHIT1 in ALS spinal corticospinal tract [16-18].

While we showed a positive correlation between CSF NfL level and the extent of LMN involvement as assessed using EMG, we failed to find any correlation between CSF biomarker values, and, in particular CSF NfL, and the quantitative score of denervation activity (DS), a known adverse prognostic factor in ALS [21,22]**.** It is, therefore, plausible that other neurophysiological parameters, such as the motor unit reinnervation rate and the loss of motor units, may show a positive correlation with CSF NfL. Future prospective studies are needed to clarify these issues, especially the application of new advanced methodologies, such as motor unit estimation (MUNE) and motor unit number index (MUNIX), which better characterize the loss of motor units and the degree of LMN involvement [30]**.**

In this study, we systematically compared, for the first time, the accuracy of YKL-40 and CHIT1 one hand, and NfL and p-tau/t-tau on the other in the discrimination between ALS and controls or mimics. The collected data demonstrated a relatively lower or similar performance of glial markers compared to NfL or p-tau/t-tau, respectively. Similarly, Thompson et al. showed that both glial markers do not contribute significantly to the performance of phosphorylated neurofilament heavy chain protein (NfH) for ALS diagnosis [19]. Moreover, while in our cohort, YKL-40 and CHIT1 AUC values overlapped significantly with those reported by other studies [3,16,19,26], the p-tau/t-tau ratio showed lower diagnostic accuracy in comparison to a previous study [14].

Finally, our data suggested that NfL is a strong independent predictor of survival in ALS patients [5,8,9,31], even after taking into account other prognostic factors. Given that higher NfL levels are associated with a faster disease progression and shorter survival, this biomarker currently represents the primary candidate to stratify patients in clinical trials for future neuroprotective treatments. However, at variance with previous findings **[**3,7,19,26] we failed to reveal a similar prognostic value for YKL-40 and CHIT1.

The principal limitation of our study consists of its cross-sectional nature, which did not help in tracking biomarker longitudinal evolution according to the disease stage. Further studies are needed to evaluate the changes in CSF biomarkers according to disease progression. On the other side, the single-center prospective design, together with the inclusion of a large panel of CSF biomarkers, detailed clinical evaluations, follow-up information, and electromyographic data, allowed a more accurate patient characterization and added robustness to the findings. Finally, the fact that we did not analyze the data concerning cognitive status in ALS cases due to lack of a standardized cognitive assessment might be considered an additional limit.

In conclusion, we confirmed the best performance of NfL in terms of diagnostic value, prediction of survival, and disease progression rate compared to CSF markers of neuroinflammation and the p-tau/t-tau ratio. Moreover, we showed a positive correlation between CSF NfL, YKL-40 and CHIT1 levels and the extent of LMN or UMN motor neuron degeneration or both, depending on the specific marker. Finally, we failed to find a significant association between CSF biomarker levels and the denervation activity. This latter finding, also considering that our DS score still needs to be validated warrant confirmation by multicentric studies, which should evaluate other EMG markers to explore the neurophysiological correlates of CSF biomarkers better.

ETHICAL STANDARD

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

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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FIGURE CAPTIONS

Fig.1 CSF biomarkers of neurodegeneration and neuroinflammation in ALS, mimics and controls. NfL (**a**), p-tau/t-tau ratio (**b**), CHIT1 (**c**) and YKL-40 levels (**d**) in the disease groups. Horizontal lines represent medians. NfL and CHIT1 values are expressed in logarithmic scale. Only statistically significant differences are displayed (Kruskal-Wallis followed by Dunn-Bonferroni post hoc test). Abbreviations: ALS, amyotrophic lateral sclerosis; CHIT1, chitotriosidase 1; NfL, neurofilament light chain protein; p-tau, phosphorylated tau; t-tau, total tau protein; YKL-40, chitinase-3-like protein 1

Fig.2 Survival curves in ALS patients according to the values of NfL and p-tau/t-tau ratio. Biomarker levels were stratified in low, mid and high tertiles. Kaplan Meier analyses were created for NfL (**a)** and p-tau/t-tau ratio (**b**). Abbreviations : NfL, neurofilament light chain protein; p-tau, phosphorylated tau; t-tau, total tau protein

TABLES

Table 1. Demographic, clinical and genetic characteristics of ALS patients.

Demographic and clinical characteristics	Mean±SD
Age at onset (years); age at diagnosis (LP) (years)	60±12; 62±12
Disease duration from onset to LP (months)	20±18
ALSFRS-R at LP (points)	39±6
Disease duration from LP to death (months)	28±28
Site of onset	N (%)
Spinal	61 (76.25)
Bulbar	19 (23.75)
ALS clinical phenotypes	N (%)
Spinal	54 (67.50)
Bulbar	12 (15.0)
Prevalent upper motor neuron	7 (8.75)
Prevalent lower motor neuron + flail arm	7 (8.75)
Genetic data	N (%)
Family history for ALS/FTD	19
Genetic cases	14
C09orf72	11*
SOD1	2*
TARDP	2
FUS	-

*One patient with pathogenetic mutations in two genes

Abbreviations : ALS, amyotrophic lateral sclerosis; ALSFRS-R, ALS Functional Rating Scale Revised; *C9orf72 RE*, hexanucleotide repeat expansion on chromosome 9 open reading frame 72 gene; FTD, frontotemporal dementia; FUS, fused in sarcoma gene; LP, lumbar puncture ; SD, standard deviation; SOD1, superoxide dismutase 1 gene; TARDBP, TAR DNA-binding protein gene

Table 2. Demographics and CSF biomarker results in the diagnostic groups.

	ALS	ALS mimics	Controls	P
N	80	46	43	
Age at LP (years±SD)	62.21±12.41	62.37±11.93	63.93±10.60	0.807
Female (%)	42.5	32.6	48.8	0.289
NfL (pg/ml) Median (IQR)	5250 (3339-8750)	873 (450-1309)	574 (416-775)	<0.001
t-tau (pg/ml) Median (IQR)	254 (186-364)	206 (159-264)	173 (138-219)	<0.001
p-tau (pg/ml) Median (IQR)	39 (28-49)	39 (32-46)	39 (27-44)	0.675
p-tau/t-tau Median (IQR)	0.144 (0.117-0.180)	0.189 (0.160-0.217)	0.201 (0.166-0.231)	<0.001
YKL-40 (ng/ml) Median (IQR)	253 (193-327)	154 (119-228)	136 (104-162)	<0.001
CHIT1 (pg/ml) Median (IQR) All	6572 (2508-19050)	1387 (902-2959)	1374 (753-2440)	<0.001
CHIT1 (pg/ml) Median (IQR) WT + Het	8088 (3526-21200)	1504 (990-2998)	1382 (762-2472)	<0.001

Abbreviations : ALS, amyotrophic lateral sclerosis; CHIT1, chitotriosidase 1; Het, heterozygotes for CHIT1 24bp duplication; IQR, interquartile range; LP, lumbar puncture; NfL, neurofilament light chain protein; p-tau, phosphorylated tau: t-tau, total tau protein; YKL-40, chitinase-3-like protein 1; WT, wild type for CHIT1 24bp duplication

Table 3. Diagnostic accuracy of CSF biomarkers in the differential diagnosis of ALS

	ALS vs controls					ALS vs mimics				
	AUC		**cut-off**	**sens (%)**	**spec (%)**	**AUC**		**cut-off**	**sens (%)**	**spec (%)**
NfL	0.981±0.011	>	1207 pg/ml	96.3	97.7	0.922±0.031	>	1955 pg/ml	91.7	91.3
t-tau	0.736±0.046	>	191 pg/ml	75.0	62.8	0.630±0.051	>	228 pg/ml	57.5	56.5
p-tau/ t-tau	0.813±0.039	<	0.166	76.7	68.7	0.750±0.046	<	0.170	69.6	71.2
YKL-40	0.879±0.034	>	184 ng/ml	81.3	90.7	0.740±0.048	>	199 ng/ml	72.5	63.0
CHIT1 All	0.817±0.039	>	2411 pg/ml	80.0	76.2	0.796±0.040	>	1719 pg/ml	83.8	65.2
CHIT1 WT + Het	0.893±0.030	>	2411 pg/ml	87.7	75.6	0.862±0.034	>	2311 pg/ml	87.7	68.2

Abbreviations: ALS, amyotrophic lateral sclerosis; AUC, area under the curve; CHIT1, chitotriosidase 1; Het, heterozygotes for CHIT1 24bp duplication; Het, heterozygotes for CHIT1 24bp duplication; IQR, interquartile range; NfL, neurofilament light chain protein; p-tau, phosphorylated tau; t-tau, total tau protein; YKL-40, chitinase-3-like protein 1; WT, wild type for CHIT1 24bp duplication

Table 4. CSF biomarker levels according to the extent of UMN and/or LMN degeneration

		N	NfL pg/ml Median (IQR)	p-tau/t-tau Median (IQR)	YKL-40 ng/ml Median (IQR)	CHIT1 pg/ml Median (IQR) All	N	CHIT1 pg/ml Median (IQR) WT+Het
UMN and LMN degeneration	**Zero or one region**	30	5015 (2698-8175)	0.146 (0.108-0.192)	212 (186-280)	5647 (2345-11300)	27	7069 (2503-13100)
	Two regions	28	5038 (3413-8325)	0.147 (0.129-0.173)	256 (184-310)	6427 (2966-20175)	26	7380 (4033-21000)
	Three regions	22	7388 (4297-11300)	0.140 (0.118-0.175)	287 (219-392)	8673 (3322-35625)	20	10092 (5017-38475)
UMN degeneration	**Zero or one region**	17	6200 (2847-8500)	0.142 (0.108-0.177)	214 (199-281)	5450 (2276-9962)	15	7069 (2499-10700)
	Two regions	26	4484 (2516-7662)	0.147 (0.134-0.181)	250 (184-314)	5825 (1380-11150)	24	6014 (2781-12450)
	Three regions	37	6800 (4009-9725)	0.142 (0.111-0.180)	273 (191-375)	9352 (3779-23750)	34	11350 (4673-26550)
LMN degeneration	**Zero or one region**	17	3412 (1960-7575)	0.167 (0.106-0.200)	221 (172-300)	5844 (1819-14100)	16	6822 (2739-14600)
	Two regions	29	4748 (2713-7425)	0.147 (0.129-0.184)	276 (199-325)	5785 (2016-15200)	24	6870 (3780-20175)
	Three regions	34	7388 (4611-11013)	0.140 (0.110-0.165)	251 (199-346)	8673 (2946-23550)	33	8984 (3930-23700)

Abbreviations: CHIT1, chitotriosidase 1; IQR, interquartile range; LMN, lower motor neuron, NfL, neurofilament light chain protein; p-tau, phosphorylated tau; t-tau, total tau protein; UMN, upper motor neuron, YKL-40, chitinase-3-like protein 1; WT, wild type for CHIT1 24bp duplication

Table 5. Univariate and multivariate Cox Regression analysis for NfL and possible clinical predictors of survival in patients with ALS.

Variable		Univariate COX regression		Multivariate COX regression	
		HR (95% CI)	p value	HR (95% CI)	p value
NfL	**Low tertile (<3900)**	Ref	Ref	Ref	Ref
	Mid tertile (3900 – 7350)	1.020 (0.319-3.259)	0.973	0.925 (0.1902-5.501)	0.923
	High tertile (>7350)	2.516 (0.931-6.800)	0.069	3.943 (1.097-14.167)	**0.036**
DPR	**Slow (<0.24)**	Ref	Ref	Ref	Ref
	Intermediate (0.24 – 1.15)	1.182 (0.416-3.342)	0.753	1.466 (0.397-5.418)	0.566
	Fast (>1.15)	3.806 (1.195-12.121)	**0.024**	3.416 (0.834-13.980)	0.088
Age	**1 quartile (<55 yrs)**	Ref	Ref	Ref	Ref
	2 quartile (56 – 65 yrs)	1.840 (0.426-7.958)	0.414	1.777 (0.310-10.196)	0.519
	3 quartile (66 – 71 yrs)	1.713 (0.407-7.211)	0.463	1.713 (0.301-9.760)	0.544
	4 quartile (> 71 yrs)	4.522 (1.187-17.232)	**0.027**	5.876 (1.303-26.496)	**0.021**
Sex	**Female**	Ref	Ref	Ref	Ref
	Male	2.526 (1.006-6.343)	**0.048**	3.737 (1.038-13.456)	**0.044**
FTD status	**Non-FTD**	Ref	Ref	Ref	Ref
	FTD	1.070 (0.390-2.936)	**0.895**	4.225 (1.521-11.738)	**0.006**

Abbrevations: CI, confidence interval; DPR, disease progression rate; FTD, frontotemporal dementia; HR, hazard ratio; NfL, neurofilament light chain protein; Ref, reference; yrs, years

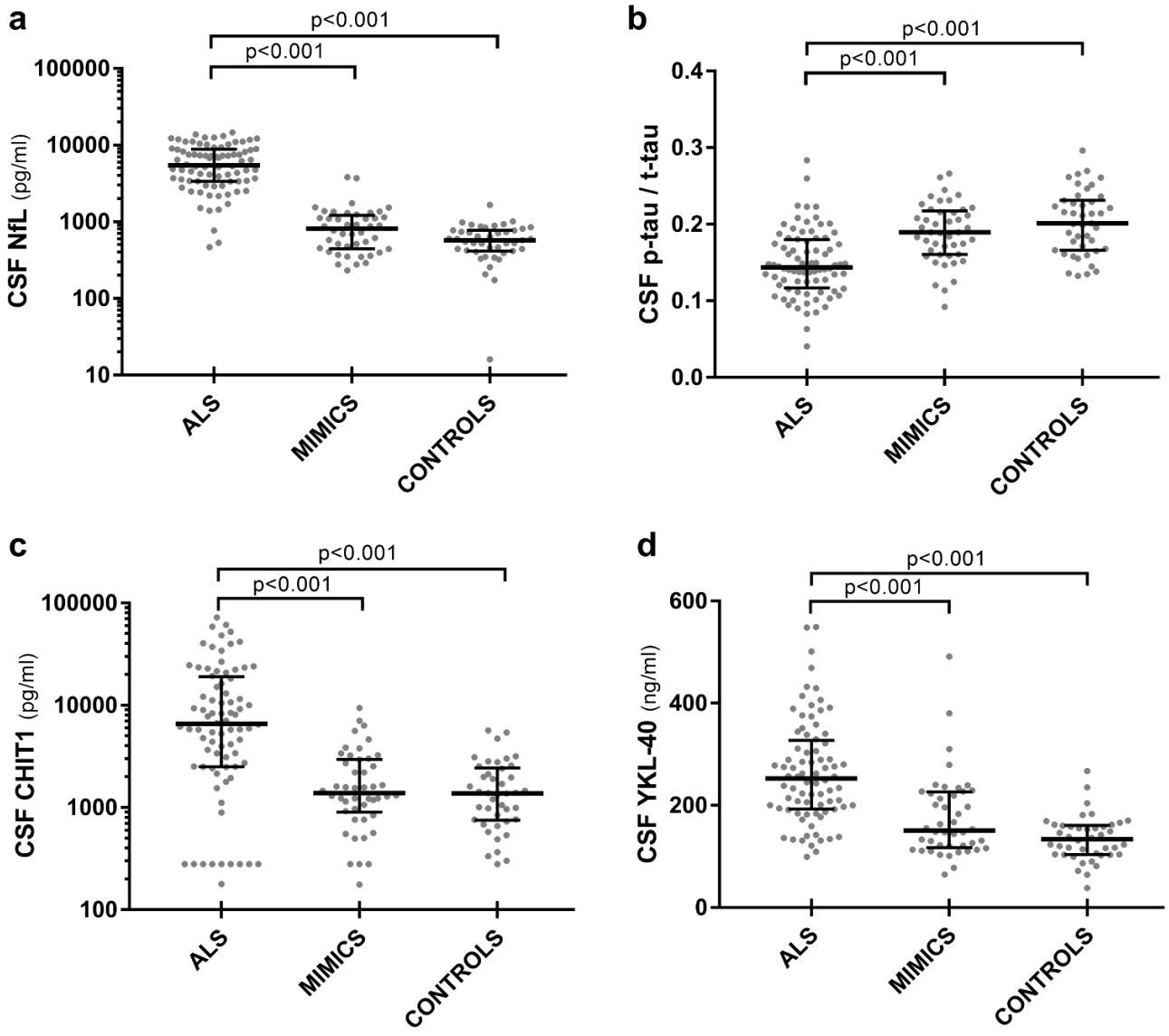
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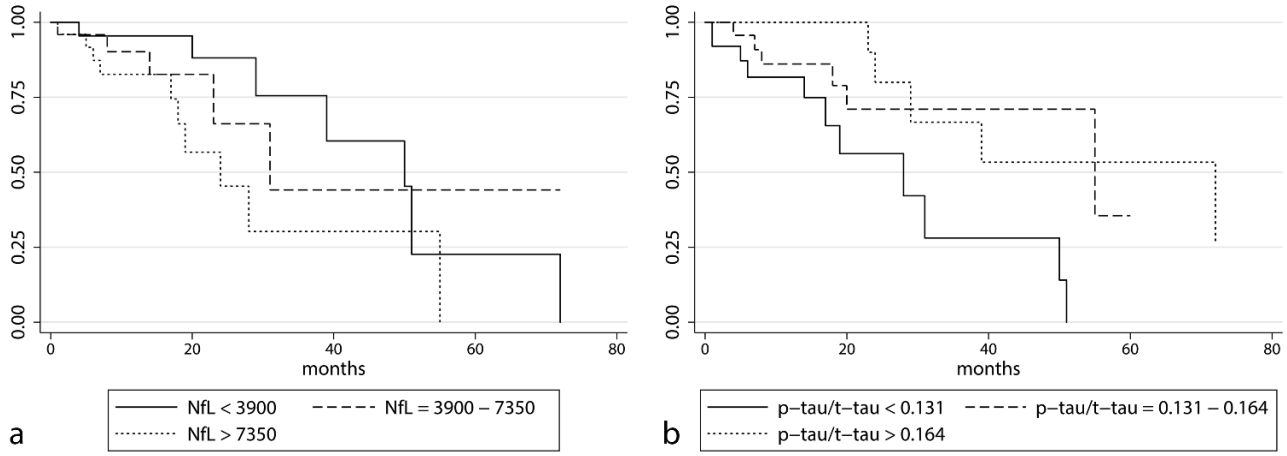
Figure 1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p–tau/t–tau < 0.131
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b

Figure 2