

Title: Diagnostic and prognostic value of serum NfL and p-Tau 181 in frontotemporal lobar degeneration

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Abstract

Objective To assess the diagnostic and prognostic value of serum neurofilament light (NfL) and serum phospho-Tau181 (p-Tau181) in a large cohort of patients with frontotemporal lobar degeneration (FTLD).

Methods In this retrospective study, performed on 417 participants, we analysed serum NfL and p-Tau181 concentrations with an ultrasensitive single molecule array (Simoa) approach. We assessed the diagnostic values of serum biomarkers in the differential diagnosis between FTLD, Alzheimer's disease (AD) and healthy ageing; their role as markers of disease severity assessing the correlation with clinical variables, cross-sectional brain imaging and neurophysiological data; their role as prognostic markers, considering their ability to predict survival probability in FTLD.

Results We observed significantly higher levels of serum NfL in patients with FTLD syndromes, compared with healthy controls, and lower levels of p-Tau181 compared with patients with AD. Serum NfL concentrations showed a high accuracy in discriminating between FTLD and healthy controls (area under the curve (AUC): 0.86, $p < 0.001$), while serum p-Tau181 showed high accuracy in differentiating FTLD from patients with AD (AUC: 0.93, $p < 0.001$). In FTLD, serum NfL levels

correlated with measures of cognitive function, disease severity and behavioural disturbances and were associated with frontotemporal atrophy and indirect measures of GABAergic deficit. Moreover, serum NfL concentrations were identified as the best predictors of survival probability.

Conclusions The assessment of serum NfL and p-Tau181 may provide a comprehensive view of FTLN, aiding in the differential diagnosis, in staging disease severity and in defining survival probability.

Introduction

Frontotemporal lobar degeneration (FTLD) encompasses a series of early onset progressive neurodegenerative conditions for which, in the last decade, the diagnostic workup has substantially changed with the publication of revised clinical criteria (Gorno-Tempini *et al.*, 2011; Rascovsky *et al.*, 2011). The careful characterization of clinical features of the behavioural variant frontotemporal dementia (bvFTD), the agrammatic or the semantic variant of primary progressive aphasia (avPPA and svPPA), and the spectrum of FTLD with extrapyramidal symptoms, such as corticobasal syndrome (CBS) and progressive supranuclear palsy (PSP), has enabled a better understanding of the heterogeneity of FTLD phenotypes (Bang *et al.*, 2015; Van Mossevelde *et al.*, 2018).

The pattern of brain atrophy and hypometabolism (Rosen *et al.*, 2002; Le Ber *et al.*, 2006), and the results of new positron emission tomography tracers (Makaretz *et al.*, 2017; Passamonti *et al.*, 2017; Tsai *et al.*, 2019), have assisted in increasing the diagnostic accuracy of FTD, while A β ₁₋₄₂ or tau measurements in cerebrospinal fluid (CSF) have been proven to be key in ruling out Alzheimer's disease (AD) (Olsson *et al.*, 2016). Furthermore, the identification of monogenic FTLD, due to pathogenetic mutations within the *granulin (GRN)*, *chromosome 9 open reading frame 72 (C9orf72)* or *microtubule-associated protein tau (MAPT)*, has undoubtedly contributed to the diagnostic work-up (Borroni and Padovani, 2013).

Considering the possible drawbacks of these supportive biomarkers due to invasiveness, availability or expensiveness, there is an urgent need to identify robust and accessible screening tests to be used even in the earliest disease stages (Borroni *et al.*, 2015), in a disorder that is much more frequent than previously thought (Logroscino *et al.*, 2019).

Along with recently proposed neurophysiological markers, measuring FTLD-related neurotransmitter deficits by Transcranial Magnetic Stimulation (TMS) (Benussi *et al.*, 2017, 2020c), a giant step forward towards potentially useful biomarkers for AD-related pathologies has been made with the new ultrasensitive Single molecule array (Simoa) approach (Rissin *et al.*, 2010). It has been reported that concentrations of neurofilament light chain (NfL), a marker of axonal

damage which is measurable in CSF, plasma or serum, are increased in FTLD and may be related to parameters of disease severity and prognosis (Pijnenburg *et al.*, 2015; Meeter *et al.*, 2016; Rohrer *et al.*, 2016; Wilke *et al.*, 2016; Foiani *et al.*, 2018; Steinacker *et al.*, 2018; Heller *et al.*, 2020; Katisko *et al.*, 2020). Furthermore, a Meso-Scale Discovery (MSD) assay for plasma phospho-Tau₁₈₁ developed by Lilly Research Laboratories was found to differentiate AD from healthy controls, suggesting its ability to identify mixed 3R/4R tau pathology (Mielke *et al.*, 2018). Two recent studies have further highlighted the usefulness of this biomarker assay in the differential diagnosis between FTLD and AD, and in monitoring disease progression (Janelidze *et al.*, 2020; Thijssen *et al.*, 2020). A paper employing a Simoa assay developed at University of Gothenburg, also found a marked increase in plasma p-Tau₁₈₁ in AD, correlating with tau PET ligand retention, while levels were normal in other tauopathies including FTLD and progressive supranuclear palsy (Karikari). This retrospective study aimed at confirming and extending previous literature data, comprehensively assessing the clinical value of serum NfL and serum phospho-Tau₁₈₁ in a large cohort of FTLD patients. We discuss when either serum NfL or serum phospho-Tau₁₈₁ should be considered on clinical grounds on the basis of specific clinical questions and defined outcomes. We analysed three main aims: *a*) the role of serum NfL and serum phospho-Tau₁₈₁ as diagnostic markers, evaluating the accuracy in the differential diagnosis between FTLD and both AD and healthy ageing, and, most importantly, their usefulness in the earliest disease stages; *b*) their role as markers of disease severity, assessing the correlation with clinical variables, cross-sectional brain imaging and neurophysiological data; *c*) their role as prognostic markers, considering their ability to predict survival probability in FTLD.

Materials and Methods

Subjects

This retrospective study included 417 participants from two independent cohorts, 307 from the Centre for Neurodegenerative Disorders, University of Brescia, Italy and 110 from the IRCCS Istituto San Giovanni di Dio Fatebenefratelli, Brescia, Italy.

The cohort consisted of 291 patients meeting probable clinical criteria for a syndrome in the FTLD spectrum, namely 134 bvFTD, 48 avPPA, 27 svPPA, 51 CBS and 31 PSP (Gorno-Tempini *et al.*, 2011; Rascovsky *et al.*, 2011; Armstrong *et al.*, 2013; Höglinger *et al.*, 2017). Moreover, 63 AD patients fulfilling current clinical criteria (Jack *et al.*, 2018) and 63 healthy controls (HC), recruited among spouses or caregivers, were included as well.

Each FTLD patient underwent a neurological evaluation, routine laboratory examination and a neuropsychological and behavioural assessment (Borroni *et al.*, 2015). In all cases, the diagnosis was supported by brain structural imaging, while cerebrospinal fluid (CSF) dosage of tau, phospho-tau₁₈₁ and A β ₁₋₄₂ was performed in a subset of cases (45.7%), to rule out AD, as previously reported (Borroni *et al.*, 2014). Furthermore, in familial cases (based on the presence of at least one dementia case among the first-degree relatives) and early onset sporadic cases, genetic screening for *GRN*, *C9orf72* and *MAPT* P301L mutations was performed (given the low frequency of *MAPT* mutations in Italy (Binetti *et al.*, 2003), we considered only the P301L mutation; we sequenced the entire *MAPT* gene only in selected cases).

Each participant underwent blood collection for measurements of serum NfL and phospho-Tau₁₈₁ biomarkers, and a subset of FTLD patients underwent standardized brain Magnetic Resonance Imaging (MRI) at baseline (n=132) to evaluate the correlation between serum biomarkers and imaging data. Moreover, a subgroup of patients underwent TMS protocols (n=113) to assess the correlation between serum biomarkers and neurophysiological data. For the purpose of the present study, we considered TMS measures that partially and indirectly reflect the activity of several neurotransmitters, including GABA_A by short interval intracortical inhibition (SICI), glutamate by

intracortical facilitation (ICF), GABA_B by long interval intracortical inhibition (LICI), and acetylcholine by short latency afferent inhibition (SAI) (Rossini *et al.*, 2015; Ziemann *et al.*, 2015).

Full written informed consent was obtained from all subjects according to the Declaration of Helsinki. The Brescia Ethics Committee approved the study protocol.

Clinical evaluation

At baseline patients underwent a standardized neuropsychological battery which included the Mini-Mental State Examination (MMSE) (Magni *et al.*, 1996), the Short Story Recall test (Novelli *et al.*, 1970), the Rey Complex Figure (copy and recall) (Caffarra *et al.*, 2002), phonemic and semantic fluencies (Novelli *et al.*, 1986), the Token test (De Renzi and Vignolo, 1962), the Clock-Drawing Test (Sunderland *et al.*, 1989), and Trail Making Test (part A and part B) (Giovagnoli *et al.*, 1996). Disease severity was assessed with the FTLD modified Clinical Dementia Rating (FTLD-modified CDR) sum of boxes scale (Knopman *et al.*, 2008), while the level of functional independence was assessed with the Basic Activities of Daily Living (BADL) (Katz *et al.*, 1963) and the Instrumental Activities of Daily Living (IADL) (Lawton and Brody, 1969) questionnaires. Furthermore, neuropsychiatric and behavioural disturbances were evaluated with the Frontal Behaviour Inventory (FBI) (Alberici *et al.*, 2007; Cosseddu *et al.*, 2020).

HC underwent a brief standardized neuropsychological assessment (Mini-Mental State Examination $\geq 27/30$); psychiatric or other neurological illnesses were considered exclusion criteria.

Serum biomarkers

Serum was collected by venipuncture, processed and stored in aliquots at -80°C according to standardised procedures. Serum NfL and serum phospho-Tau₁₈₁ were measured using the multiplex Neurology 4-Plex A kit (Quanterix Corporation, Lexington, USA) and the Human Total Tau kit (Quanterix, Boston Massachusetts, USA), respectively, on the Simoa HD-1 Analyzer (Quanterix, Boston Massachusetts, USA) following manufacturer's instructions as previously described

(Gisslén *et al.*, 2016; Foiani *et al.*, 2018). The lower limits of detection of the assay for serum NfL and phospho-Tau₁₈₁ were 0.104 pg/mL and 0.019 pg/mL, respectively. Measurements were carried out at the same study site on consecutive days, using the same batch of reagents, and the operator was blinded to all clinical information. Quality control samples had a mean intra-assay and inter-assay coefficient of variation of less than 10%

MRI acquisition, processing and analysis

Brain images were collected using 1.5 Tesla (Siemens Symphony and Avanto, Erlangen, Germany) or 3 Tesla scanner (Siemens Skyra, Erlangen, Germany) equipped with a circularly polarized transmit-receive coil to obtain 3D magnetization-prepared rapid gradient echo (MPRAGE) T1-weighted scans. At 1.5T, sequences were acquired with the following parameters: repetition time 2100-2050 ms, echo time 2.95-2.56 ms, inversion time 1100 ms, slice thickness 1 mm, voxel size 1×1×1 mm, in-plane field of view 256 mm, flip angle = 15°. At 3T, sequences were acquired with the following parameters: repetition time 2000 ms, echo time 2.92 ms, inversion time 850 ms, slice thickness 1.1 mm, voxel size 1.1 × 1.1 × 1.1, field of view 282 mm, flip angle 8°.

T1 scans were visually inspected and excluded from subsequent analyses if excessive motion blurring or artifacts were present. Then, images were processed and analyzed with the fully automated surface-based morphometry pipeline in the Computational Anatomy Toolbox (CAT12.6) (<http://www.neuro.uni-jena.de/cat/>) for Statistical Parametric Mapping (SPM12 v. 7771) (<http://www.fil.ion.ucl.ac.uk/spm/software/spm12/>), running on MATLAB 9.2 (The MathWorks, Inc, Natick, MA USA). Cortical meshes were resampled to the Human Connectome Project mesh and smoothed with a 15 mm filter.

Smoothed cortical thickness meshes were included in a multiple regression model, in which serum NfL and serum phospho-Tau₁₈₁ values represented the independent variables. Age, gender, clinical phenotype and MRI scanner type were considered as confounding factors. The statistical threshold

was set at 0.05 and corrected for multiple comparisons using false discovery rate (FDR) at whole-brain level.

Transcranial Magnetic Stimulation

A TMS figure-of-eight coil (each loop diameter 70 mm – D70² coil) connected to a monophasic Magstim Bistim² system (Magstim Company, Oxford, UK) was employed for all TMS paradigms, as previously reported (Benussi *et al.*, 2019b). Electromyographic (EMG) recordings were performed from the first dorsal interosseous (FDI) muscles using 9 mm diameter, Ag-AgCl surface-cup electrodes. The active electrode was placed over the muscle belly and the reference electrode over the metacarpophalangeal joint of the index finger. Responses were amplified and filtered at 20 Hz and 2 kHz with a sampling rate of 5 kHz.

Resting motor threshold (RMT) was determined on the left motor cortex as the minimum intensity of the stimulator required to elicit motor evoked potentials (MEPs) with a 50 μ V amplitude in 50% of 10 consecutive trials, recorded from the right first dorsal interosseous muscle during full muscle relaxation.

SICI-ICF, LICI and SAI were studied using a paired-pulse technique, employing a conditioning-test design. For all paradigms, the test stimulus (TS) was adjusted to evoke a MEP of approximately 1 mv amplitude in the right first dorsal interosseous muscle.

For SICI and ICF, the conditioning stimulus (CS) was adjusted at 70% of the RMT, employing multiple interstimulus intervals (ISIs), including 1, 2, 3 ms for SICI and 7, 10, 15 ms for ICF (Kujirai *et al.*, 1993; Ziemann *et al.*, 1996). LICI was investigated by implementing two supra-threshold stimuli, with the CS adjusted at 130% of the RMT, employing ISIs of 50, 100 and 150 ms (Valls-Solé *et al.*, 1992). SAI was evaluated employing a CS of single pulses (200 μ s) of electrical stimulation delivered to right median nerve at the wrist, using a bipolar electrode with the cathode positioned proximally, at an intensity sufficient to evoke a visible twitch of the thenar muscles

(Tokimura *et al.*, 2000). Different ISIs were implemented (0, +4), which were fixed relative to the N20 component latency of the somatosensory evoked potential of the median nerve.

For each ISI and for each protocol, ten different paired CS-TS stimuli and fourteen control TS stimuli were delivered in all participants in a pseudo-randomized sequence, with an inter trial interval of 5 secs ($\pm 10\%$).

The conditioned MEP amplitude, evoked after delivering a paired CS-TS stimulus, was expressed as percentage of the average control MEP amplitude. Average values for SICI (1, 2, 3 ms ISI), ICF (7, 10, 15 ms ISI), LICI (50, 100, 150 ms ISI) and SAI (0, +4 ms ISI) were used for analysis.

Stimulation protocols were conducted in a randomized order. Audio-visual feedback was provided to ensure muscle relaxation during the entire experiment and trials were discarded if EMG activity exceeded 100 μV in the 250 ms prior to TMS stimulus delivery. Less than 5% of trials were discarded for each protocol. All of the participants were capable of following instructions and reaching complete muscle relaxation; if, however the data was corrupted by patient movement, the protocol was restarted and the initial recording was rejected.

Statistical analysis

Linear regression and stepwise multiple regression analysis (including all variables with a $p < 0.001$ at univariate analysis) were used to characterize the relationship between serum biomarkers and demographic characteristics (age, age at onset, sex and mutational status).

Differences in clinical variables and biomarker concentrations were assessed with one-way analysis of covariance (ANCOVA), corrected for age, sex and mutational status, with Bonferroni multiple comparisons correction. Pearson's correlations were used to assess associations between serum biomarkers, age and education corrected clinical variables and TMS measures.

Receiver operating characteristics (ROC) curve analyses were used to determine the ability of serum NfL and phospho-Tau₁₈₁ to differentiate between diagnostic groups. The area under the curve

(AUC) including 95% confidence interval (CI) values are reported, with cut-off points set to achieve highest levels of sensitivity and specificity (Youden's index).

Survival was calculated as time from symptom onset to time of death from any cause (outcome=0) or censoring date (outcome=1). Survival analysis was carried out by the Kaplan-Meier method with log rank *post hoc* testing and by means of univariate and multivariate stepwise Cox proportional-hazard regression analysis; hazard ratios (HR) are provided with their respective 95% confidence intervals (CIs).

A two-sided p -value <0.05 was considered significant and corrected for multiple comparisons using false discovery rate (FDR) when appropriate (Pike, 2011). Statistical analyses were performed using SPSS (v.24; SPSS, IBM).

Data availability

All study data, including raw and analysed data, and materials will be available from the corresponding author, B.B., upon reasonable request.

Results

Participant characteristics

Baseline demographics, clinical variables and fluid biomarker levels are reported in **Table 1**.

In the FTLD group, serum NfL concentrations did not correlate with age ($\beta=-0.07$, $p=0.272$), age at onset ($\beta=-0.03$, $p=0.614$), or gender ($\beta=-0.08$, $p=0.193$), but correlated with the presence of a pathogenic mutation at both the linear regression ($\beta=0.48$, $p<0.001$) and at the stepwise multiple regression model ($\beta=0.48$, $p<0.001$). Serum NfL concentrations were higher in patients with a pathogenic mutation (mean \pm SE, *GRN* mutations $n=30$, 86.2 ± 5.0 ; *MAPT* mutations $n=3$, 43.0 ± 15.9) compared with patients without a pathogenic mutation (no mutation/unknown $n=258$, 36.0 ± 1.7 pg/L). Serum phospho-Tau₁₈₁ concentrations also did not correlate with age ($\beta=0.07$, $p=0.209$), age at onset ($\beta=0.08$, $p=0.159$), or gender ($\beta=-0.08$, $p=0.200$), but correlated inversely with the presence of a pathogenic mutation in both the linear regression ($\beta=-0.16$, $p=0.006$) and in the stepwise multiple regression model ($\beta=-0.13$, $p<0.021$). Serum phospho-Tau₁₈₁ concentrations were lower in patients without a pathogenic mutation (no mutation/unknown, 3.9 ± 0.4) compared with patients with a pathogenic mutation (*GRN* mutations, 0.6 ± 1.1 ; *MAPT* mutations, 2.3 ± 3.6).

Serum NfL and serum phospho-Tau₁₈₁ concentrations in FTLD subgroups

Serum NfL concentrations were significantly increased in most FTLD subgroups (age- and sex-corrected ANCOVA, $F(6,408)=11.97$, $p<0.001$, $\eta^2=0.15$). In Bonferroni-corrected *post hoc* tests, we observed a significant increase in serum NfL levels in bvFTD, avPPA and CBS, and in AD patients compared with HC. Patients with avPPA had significantly higher levels of serum NfL compared with svPPA, CBS, PSP and AD (see **Table 1**).

After correcting also for mutation status, considering the unbalanced distribution of pathogenic mutations across FTLD subgroups (see **Table 1**) and the increased NfL concentrations in mutation carriers, we observed a significant increase in NfL levels in all the FTD variants (bvFTD, avPPA

and svPPA) compared with HC (age, sex and mutation corrected ANCOVA, $F(6,408)=7.00$, $p<0.001$, $\eta^2=0.09$), without significant differences between avPPA and the other subgroups (see **Figure 1, panel A**).

Serum phospho-Tau₁₈₁ concentrations were significantly reduced in all FTLD subgroups compared with AD (age and gender corrected ANCOVA, $F(6,408)=21.35$, $p<0.001$, $\eta^2=0.24$) (see **Table 1** and **Figure 1, panel B**). No significant differences between FTLD subgroups were found except for higher values in CBS compared with bvFTD (see **Figure 1, panel B**). Serum phospho-Tau₁₈₁ was also significantly increased in AD patients compared with HC (see **Figure 1, panel B**). Comparable results were observed also after adjusting for age, gender and mutation status (ANCOVA, $F(6,408)=20.21$, $p<0.001$, $\eta^2=0.23$).

Diagnostic accuracy of serum NfL and serum phospho-Tau₁₈₁

To differentiate FTLD patients from HC, we applied a ROC curve analysis on serum NfL concentrations, observing an AUC of 0.862 ($p<0.001$, 95% CI 0.818-0.906); the serum NfL cut-off of 22.5 pg/mL differentiated FTLD from HC with a sensitivity of 71.5% and a specificity of 92.1% (see **Figure 2, panel A**). In patients with a mild disease stage (FTLD-modified CDR ≤ 5), a serum NfL cut-off of 19.1 pg/mL differentiated mild FTLD from HC with a sensitivity of 74.8% and specificity of 74.6%, with an AUC of 0.813 ($p<0.001$, 95% CI 0.753-0.874) (see **Figure 2, panel B**).

To differentiate FTLD from AD patients, we applied a ROC curve analysis on serum phospho-Tau₁₈₁ concentrations, observing an AUC of 0.930 ($p<0.001$, 95% CI 0.903-0.956); a serum phospho-Tau₁₈₁ cut-off of 5.88 pg/mL differentiated FTLD from AD with a sensitivity of 81.4% and a specificity of 93.5% (see **Figure 2, panel C**).

In patients with a mild disease stage (FTLD with an FTLD-modified CDR ≤ 5 and AD with a MMSE ≥ 19), the serum phospho-Tau₁₈₁ cut-off of 6.11 pg/mL differentiated mild FTLD from mild

AD with a sensitivity of 80.0% and specificity of 91.7%, with an AUC of 0.907 ($p < 0.001$, 95% CI 0.862-0.951) (see **Figure 2, panel D**).

Serum NfL and serum phospho-Tau₁₈₁ associations with cognitive function and disease severity in FTLD

Cognitive and behavioural assessment. Serum NfL concentrations showed significant associations with baseline BADL ($r=0.23$, $p < 0.001$), IADL ($r=0.23$, $p < 0.001$) and FTLD-modified CDR sum of boxes ($r=0.28$, $p < 0.001$), the higher the serum NfL levels, the greater impairment in functional activities and disease severity. Significant correlations were observed between serum NfL concentrations and MMSE scores ($r=-0.30$, $p < 0.001$), phonemic ($r=-0.24$, $p=0.001$) and semantic fluencies ($r=-0.24$, $p=0.001$), clock-drawing ($r=-0.24$, $p=0.001$), short story ($r=-0.25$, $p=0.002$), trail-making part B ($r=-0.22$, $p=0.011$), digit symbol ($r=-0.16$, $p=0.027$), and token test ($r=-0.17$, $p=0.035$), with higher levels of serum NfL correlating with poorer scores. No significant correlations were observed for the Rey figure copy ($r=-0.10$, $p=0.155$) and recall ($r=-0.09$, $p=0.222$), and Trail-making test part A ($r=0.11$, $p=0.117$). Neuropsychiatric and behavioural disturbances, evaluated with the FBI, significantly correlated with serum NfL levels ($r=0.18$, $p=0.007$). All tests were age- and education-corrected; FDR-adjusted p -values for multiple comparisons are reported for each test.

No significant correlations were observed between serum phospho-Tau₁₈₁ concentration and FTLD-CDR sum of boxes score or other neuropsychological, behavioural or functional measures.

Brain imaging. As reported in **Figure 3**, serum NfL concentration correlated with cortical thinning of the frontotemporal and parietal regions, mainly on the left side ($p < 0.05$ whole-brain FDR-corrected, cluster threshold = 200). There was no statistically significant association between serum phospho-Tau₁₈₁ and cortical thickness in patients with FTLD .

TMS measures. TMS measures were performed to evaluate average SICI, ICF, LICI and SAI. In the FTLD group (n=89), serum NfL levels were significantly associated with SICI ($r=0.464$, $p<0.001$) and LICI ($r=0.545$, $p<0.001$), but not with ICF or SAI (see **Figure 4, panel A and B**). No associations were observed between serum phospho-Tau₁₈₁ and TMS measures.

Interestingly, in the AD group (n=12), we observed a significant association between serum phospho-Tau₁₈₁ and average SAI ($r=0.720$, $p=0.048$) (see **Figure 4, panel C**). We did not observe any significant associations between serum NfL and TMS measures.

Reported p -values are FDR-adjusted for multiple comparisons.

Serum NfL and serum phospho-Tau₁₈₁ associations with prognosis in FTLD

Serum NfL concentration significantly predicted the survival rate in FTLD patients.

The univariate and stepwise multivariate Cox regression analysis showed a significant association between survival and serum NfL levels (HR 1.01 95%CI 1.00-1.02, $p=0.005$), but not with phospho-Tau₁₈₁, age, age at onset or mutation status (see **Figure 5, panel A**). Patients with high serum NfL levels (upper half of median values) had significantly shorter survival than those with low serum NfL levels (lower than median value) at the Kaplan-Meier survival curves ($p=0.034$) (see **Figure 5, panel B**).

Discussion

In this work, we confirmed and extended previous literature claiming a different usefulness of serum NfL and serum phospho-Tau₁₈₁ measurements in clinical practice, depending on specific clinical questions. Serum NfL concentrations showed high accuracy in identifying FTLD from cognitively unimpaired elderly, as well as in assessing FTLD severity and prognosis, while serum phospho-Tau₁₈₁ concentrations showed high accuracy in discriminating FTLD from AD.

Importantly, in this study we also further demonstrate high accuracy of these biomarkers even in the earliest disease stages.

The non-invasiveness and reliability of serum NfL and phospho-Tau₁₈₁ measurements make these markers extremely useful in clinical practice for the diagnosis of FTLD, even in the early disease stages, compared to CSF biomarkers or more expensive brain imaging modalities.

Serum NfL concentrations, as already demonstrated in other neurodegenerative disorders (Bridel *et al.*, 2019; Forgrave *et al.*, 2019; Zhao *et al.*, 2019), were associated with measures of disease severity, and are helpful in assessing disease stage. In fact, higher serum NfL levels were significantly associated with more pronounced cognitive impairment and behavioural disturbances.

We also observed an association with cortical thickness at brain imaging analysis. In particular, NfL concentrations were inversely correlated with cortical thickness values mainly in frontal, temporal and parietal regions, supporting the view that NfL is a neurodegeneration marker strongly related to FTLD (Ljubenkov *et al.*, 2018). These findings were also consistent with previous studies in FTLD that reported a correlation between brain structure and NfL concentrations, with a predominant involvement of the left frontotemporal area (Scherling *et al.*, 2014; Rohrer *et al.*, 2016; Falgàs *et al.*, 2020). To further corroborate the role of serum NfL as a marker of disease severity, we evaluated the association between serum NfL concentrations and indirect measures of GABAergic neurotransmission, which have been demonstrated to be impaired in FTLD (Burrell *et al.*, 2011; Benussi *et al.*, 2016, 2018, 2019a, 2020a, b; Murley and Rowe, 2018). We observed that the higher the serum NfL levels, the greater was the impairment in SICI and LICI, which are considered to

reflect short-lasting postsynaptic inhibition mediated through the GABA_A and GABA_B receptors at the level of local interneurons, respectively (Rossini *et al.*, 2015; Ziemann *et al.*, 2015).

Altogether, these findings strongly support the notion that serum NfL concentrations may be useful to stage disease severity, in a disorder where there is urgent need to find not only diagnostic but also prognostic markers, in light of the near onset of new pharmacological clinical trials. Compared with AD, FTLD is clinically heterogeneous, with patients presenting a combination of behavioural disturbances, impairment of executive functions or language deficits. Available standardised neuropsychological and clinical assessments may not be ideal in detecting the effects of future treatments, particularly in the early disease stages and across different FTLD subtypes. A non-invasive and easy to perform peripheral biomarker may represent a practical and valuable choice to assess disease severity, to monitor outcomes and to categorize patients into disease subgroups.

Most importantly, this study has demonstrated that serum NfL concentrations are able to predict survival rates. Indeed, several studies have now shown the prognostic value of NfL in patients with FTLD; however, concentrations were evaluated in CSF, or in small group of patients or in patients with monogenic disease (Skillbäck *et al.*, 2014; Donker Kaat *et al.*, 2018; Meeter *et al.*, 2019; van der Ende *et al.*, 2019). These confirmatory results observed using serum NfL concentrations in a large cohort of FTLD subjects are key to clearly prove that patients with higher NfL levels show decreased survival. These findings further prove that NfL, a major component of neuronal cytoskeleton involved in axonal and dendritic growth, signaling and transport (Yuan *et al.*, 2015), reflect the ongoing neuronal loss also in FTLD (Meeter *et al.*, 2019).

Conversely, serum phospho-Tau₁₈₁ levels, besides being very accurate in discriminating AD from FTLD, were not helpful in monitoring disease severity or predicting prognosis in FTLD. Indeed, according to previous data, serum phospho-Tau₁₈₁ may detect mixed 3R/4R neuropathology, *i.e.*, AD, but not other tauopathies, such as 4R tauopathy (*i.e.*, Pick's disease) or 3R tauopathy (*i.e.*, PSP or CBS) (Mielke *et al.*, 2018) + Karikari. For these reasons, serum phospho-Tau₁₈₁ was not able to identify FTLD subtypes. The modest increase in phospho-Tau₁₈₁ concentrations observed in CBS

patients could be secondary to a concomitant AD neuropathology, which has been frequently observed in these patients (Schneider *et al.*, 1997; Boeve *et al.*, 1999). Accordingly, in patients carrying a *MAPT P301L* mutation, phospho-Tau₁₈₁ concentrations were not significantly higher than in other FTLN subtypes (data not shown), as they have a pure 4R tau pathology. It is however noteworthy that patients carrying *GRN* mutations, and consequently with FTLN-TDP43 pathology, showed decreased serum phospho-Tau₁₈₁ compared with patients without a pathogenetic mutation. The related pathological mechanism needs to be further explored. Finally, SAI, a TMS measure of cholinergic dysfunction widely associated with AD (Di Lazzaro *et al.*, 2002, 2006), correlated harmoniously with serum phospho-Tau₁₈₁ levels, further confirming the reliability of peripheral phospho-Tau₁₈₁ in detecting AD, as previously reported (Mattsson *et al.*, 2016).

Major strengths of our study are the large series of FTLN patients and the comprehensive approach in correlating clinical, imaging and neurophysiological data with fluid biomarkers, carried out at the same study site to minimize variability. A weakness of the study is the lack of autopsy confirmation, which prevented correlations between biomarkers and FTLN-related proteinopathies. Secondly, longitudinal serum NfL measurements were not available, and we were not able to draw conclusions on possible changes throughout disease progression.

In conclusion, our results show the usefulness of both peripheral NfL and phospho-Tau₁₈₁ assessment, with different and specific purposes in clinical practice. Assessing both blood-based biomarkers may provide a comprehensive view of FTLN, aiding in the differential diagnosis, in staging disease severity and in defining survival probability.

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Disclosures

HZ has served at scientific advisory boards for Denali, Roche Diagnostics, Wave, Samumed and CogRx, has given lectures in symposia sponsored by Fujirebio, Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg, all unrelated to the work presented in this paper. KB has served as a consultant or at advisory boards for Abcam, Axon, Biogen, Lilly, MagQu, Novartis and Roche Diagnostics, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Venture-based platform company at the University of Gothenburg, all unrelated to the work presented in this paper.

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Table 1. Demographic and clinical characteristics of FTLD patients and controls

Variable	FTLD					Controls	
	bvFTD	avPPA	svPPA	CBS	PSP	AD	HC
Number	134	48	27	51	31	63	63
Age, years	64.5± 8.0	67.7± 8.8	64.0± 8.2	65.8± 7.6	72.9± 7.4	75.5± 8.1	65.4± 12.1
Sex, female %	58.2	43.8	59.3	52.9	51.6	31.7	20.6
Age at onset, years	61.5±7.8	64.9±8.6	60.5±8.0	63.2±7.5	68.8±7.3	74.0 ±8.3	-
Monogenic disease, %	14.9	25.0	0.0	2.3	0.0	0.0	-
Serum NfL (pg/mL)							
mean±SE	43±2.4	54.6±3.9	33.3±5.2	36.5±3.8	30.4±4.9	32.7±3.6	14.2±3.5
lower-upper bound	38.3-47.8	46.9-62.3	23.0-43.6	29.1-44.0	20.7-40.1	25.6-39.9	7.4-21.1
Serum phospho-Tau₁₈₁ (pg/mL)							
mean±SE	2.5±0.7	3.3±1.1	3.8±1.5	7.1±1.1	3.9±1.4	16.4±1.1	5.4±1.0
lower-upper bound	1.1-3.8	1.1-5.5	0.8-6.8	4.9-9.3	1.0-6.7	14.3-18.5	3.5-7.5

FTLD = Frontotemporal Lobar degeneration; bvFTD = behavioural variant frontotemporal dementia; avPPA = agrammatic variant of primary progressive aphasia; svPPA = semantic variant of primary progressive aphasia; CBS = corticobasal syndrome; PSP = progressive supranuclear palsy; AD = Alzheimer's disease; HC = healthy controls; NfL = Neurofilament Light Chain; SE = standard error. Results are expressed as mean ± standard deviations, unless otherwise specified. Monogenic disease: all *GRN* mutations, but 3 *MAPT* mutations (2 bvFTD and 1 CBS).

Legend to Figures

Figure 1. Serum biomarkers concentrations in participants by clinical diagnosis.

(A) Serum NfL and (B) serum phospho-Tau₁₈₁ concentrations in participants by clinical diagnosis. bvFTD = behavioural variant frontotemporal dementia; avPPA = agrammatic variant of primary progressive aphasia; svPPA = semantic variant of primary progressive aphasia; CBS = corticobasal syndrome; PSP = progressive supranuclear palsy; AD = Alzheimer's disease; HC = healthy controls. Bar graphs represent mean values and error bars represent 95% confidence intervals. * $p < 0.050$; ** $p < 0.010$; *** $p < 0.001$ after Bonferroni corrected *post hoc* tests.

Figure 2. ROC curves for serum biomarkers in differentiating FTLD from HC and AD.

ROC curves for serum NfL in differentiating (A) FTLD and (B) mild FTLD patients from HC. Serum phospho-Tau₁₈₁ in differentiating (C) FTLD from AD and (D) mild FTLD from mild AD patients. ROC = receiver operating characteristic; AUC = area under the curve; FTLD = frontotemporal lobar degeneration; mild FTLD = FTLD patients with FTD-CDR ≤ 5 and AD; FTD-CDR = frontotemporal dementia clinical dementia rating scale; HC = healthy controls; AD = Alzheimer's disease; mild AD = AD patients with MMSE $\geq 19/30$; MMSE = Mini-Mental State Examination.

Figure 3. Significant association between serum NfL and whole-brain cortical thickness.

The significant clusters (inverse relationship) from the multiple regression model where serum NfL values were considered as independent variable (age, gender, clinical phenotype and MRI scanner type included as confounding factors). The statistical threshold was set at $p < 0.05$ and corrected for multiple comparisons using false discovery rate (FDR) at whole-brain level. The significant clusters were superimposed on a 3-dimensions T1 standardized template.

Figure 4. Significant associations between serum biomarkers and neurophysiological measures.

Association between serum NfL and (A) average SICI (ISI 1, 2, 3 ms ISI), (B) average LICI (ISI 50, 100, 150 ms ISI) and (C) average SAI (0, +4 ms ISI).

SICI = short-interval intracortical inhibition; LICI = long-interval intracortical inhibition; SAI = short latency afferent inhibition; ISI = interstimulus interval.

Figure 5. Survival curves

(A) Survival probability curves and (B) Kaplan-Meier survival curves in FTLD patients for serum NfL subgroups (upper half vs lower half of median values).