Significance of CSF NfL and tau in ALS

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Abstract

Cerebrospinal fluid (CSF) neurofilament light chain (NfL) has emerged as putative diagnostic biomarker in amyotrophic lateral sclerosis (ALS), but it remains a matter of debate, whether CSF total tau (ttau), tau phosphorylated at threonine 181 (ptau) and the ptau/ttau ratio could serve as diagnostic biomarker in ALS as well. Moreover, the relationship between CSF NfL and tau measures to further axonal and (neuro)degeneration markers still needs to be elucidated.

Our analysis included 89 ALS patients (median (range) age 63 (33-83) years, 61% male, disease duration 10 (0.2–190) months) and 33 age- and sex-matched disease controls (60 (32-76), 49%). NfL was higher and the ptau/ttau ratio was lower in ALS compared to controls (8,343 (1,795–35,945) pg/ml vs. 1,193 (612–2,616), $H(1)=70.8$, $p<0.001$; mean [SD] 0.17 [0.04] vs. 0.2 [0.03], $F(1)=14.3$, $p<0.001$), as well as in upper motor neuron dominant (UMND, n=10) compared to classic (n=46) or lower motor neuron dominant ALS (n=31; for NfL: 16,076 (7,447–35,945) vs. 8,205 (2,651–35,138) vs. 8,057 (1,795–34,951), $Z\geq2.5$, $p\leq0.01$; for the ptau/ttau ratio: (0.13 [0.04] vs. 0.17 [0.04] vs. 0.18 [0.03], $p\leq0.02$). In ALS, NfL and the ptau/ttau ratio were related to corticospinal tract (CST) fractional anisotropy (FA) and radial diffusivity (ROI-based approach and whole-brain voxelwise analysis). Factor analysis of mixed data (FAMD) revealed a co-variance pattern between NfL (factor load -0.6), the ptau/ttau ratio (0.7), CST FA (0.8) and UMND ALS phenotype (-2.8). NfL did not relate to any further neuroaxonal injury marker (brain volumes, precentral gyrus thickness, peripheral motor amplitudes, sonographic cross-sectional nerve area), but a lower ptau/ttau ratio was associated with whole-brain gray matter atrophy and widespread white matter integrity loss. Higher NfL baseline levels were associated with greater UMN disease burden, more rapid disease progression, a 2fold to 3fold greater hazard of death and shorter survival times.

The findings that higher CSF NfL levels and a reduced ptau/ttau ratio are more associated with clinical UMN involvement and with reduced CST FA offer strong converging evidence that both are markers of central motor degeneration. Furthermore, NfL is a marker of poor prognosis, while a low ptau/ttau ratio indicates extramotor pathology in ALS.

Introduction

Cerebrospinal fluid (CSF) neurofilament light chain (NfL) has emerged as putative diagnostic biomarker in several neurodegenerative conditions [1, 2], such as amyotrophic lateral sclerosis (ALS), and ALS patients reveal significantly higher levels compared to controls or disease mimics [3–9]. CSF neurofilaments seem also to aid as a prognostic biomarker [4, 5, 8–11] and have been found to be higher in ALS patients with dominant upper motor neuron (UMN) involvement [4, 5, 12]. In the meanwhile, several studies have also taken account of serum NfL which has additionally proven great potential to discriminate between ALS and controls or disease mimics [7, 13, 14]. Compared to CSF, serum neurofilament might, however, be less sensitive towards clinical and electrophysiological measures of motor neuron degeneration [9, 15], emphasizing the pivotal role of CSF neurofilaments when taking account of the extent of neuroaxonal damage despite less convenient sampling. With the exception of few studies focusing on the corticospinal tract's (CST) integrity applying diffusion tensor imaging (DTI) there is, however, nearly no data available how CSF neurofilaments relate to other biomarkers of peripheral (PNS) and central nervous system (CNS) neuroaxonal injury in ALS [6, 16]. Understanding their certain biomarker associations would indeed even improve the leading candidate role of CSF neurofilaments to aid as a promising outcome measure in future ALS therapeutic trials [17].

In contrast to CSF NfL, it is a matter of debate whether CSF total tau (ttau) and tau phosphorylated at threonine 181 (ptau) could serve as diagnostic biomarker in ALS as well, and there are just as many studies reporting elevated (abnormal) or normal levels in ALS when compared to controls [12, 18–25]. Two studies further proposed a reduction of the ptau/ttau ratio in ALS [23, 24]. While CSF tau seems not to universally correlate with disease progression in ALS [12, 19, 21, 26], few studies indeed found a relationship between higher CSF ttau or ptau at baseline and worse motor function [6, 23]. And, as for CSF NfL, with the exception of two DTI studies reporting an association between CST integrity and the

ptau/ttau ratio, but not with ttau or ptau, there are no data available how tau measures relate to further neuroaxonal injury markers in ALS [6, 23]. Continuing studies are thus indeed needed to determine the biomarker role of ttau and ptau in ALS.

We thus here conducted several analyses taking especially into account how CSF NfL, ttau, ptau and the ptau/ttau ratio distribute within certain ALS subgroups, relate to further axonal and (neuro)degeneration markers such as motor amplitudes or precentral gyrus thickness and the ALS patients' long-term outcome using a retrospective approach.

Methods

ALS sample

Our study comprised 89 ALS patients recruited from the Departments of Neurology, Otto-von-Guericke University, Magdeburg and Hannover Medical School, Hannover, Germany. Patients were diagnosed by one of two experienced neurologists (S.V., S.P.) according to the revised El Escorial criteria comprising the assessment of the number of regions (bulbar, thoracic, upper limb, lower limb) with UMN (clinically) or lower motor neuron (LMN) involvement (clinically or via electromyography) [27]. Similar to previous studies, we also included patients presenting with LMN signs only ("suspected ALS") [14]. The Penn UMN score was recorded to assess the UMN disease burden in the bulbar segment as well as in each of the four limbs [28] **(**see **Supplemental)**. ALS clinical phenotypes were classified in line with operational definitions as specified previously [29, 30] **(**see **Supplemental)**. At baseline patients underwent a clinical and diagnostic work-up (ALS functional rating scale (ALSFRS-R) total score, genetic testing, CSF measures of NfL, ttau, ptau, total protein and the CSF albumin/serum albumin ratio ($Q_{alb} \times 10^{-3}$); for methodological details regarding CSF measurements and the performance of the NfL assay see **Supplemental** and **Supplemental Table 1**). Measures of neuroaxonal injury comprised those PNS and CNS markers commonly found to be altered in ALS: median and ulnar nerve compound motor action potential (CMAP) amplitudes [31, 32] and sonographic cross-sectional nerve area (CSA) [29, 33] (for methodological details of PNS measures see [29]), precentral gyrus thickness, cortical and subcortical cerebral gray matter (GM) volumes and CST DTI metrics (e.g. fractional anisotropy (FA)) [34–37]. Disease duration was the time in months between symptom onset and a patient's baseline visit. Disease progression rate (DPR) was determined as (48−ALSFRS-R)/disease duration (points per month). Patients underwent follow-up ALSFRS-R measurements within a mean [SD] time interval of 6 [8] months. Please see the **Supplemental** and **Supplemental Figure 1** for the detailed demonstration of the availability of all measures.

Controls

Cross-sectional CSF NfL, ttau and ptau measures were additionally conducted in a hospital-based cohort of 33 neurologic patients (non-motor neuron disease controls), comprising cases with non-specific complaints who underwent lumbar puncture in terms of a diagnostic work up to rule out any neurologic condition. None of those disease controls suffered from any neuromuscular disorders (i.e. peripheral polyneuropathies, muscle or motor neuron disease) nor did they display any specific abnormalities on the neurological exam. CSF NfL data were available in all subjects, while tau measures have been conducted in 16 out of the 33 control cases only (please see **Supplemental Table 2** for further details).

Standard protocol approvals, registrations, and patient consents

The study was approved by the local ethics committee (No. 150 / 09, No. 07 / 17, No. 16 / 17), and all subjects gave written informed consent.

3T MRI measures of the brain

All MRI sessions were performed on the same Siemens Verio 3 T system (Siemens Medical Systems, Erlangen, Germany) at the same site (Magdeburg), and all patients underwent exactly the same MRI protocol. 3D MPRAGE images were acquired (for bilateral precentral gyrus thickness, GM (GMV) and white matter (WMV) and total brain (TBV) volumes). Diffusion MRI data were used to compute the maps of DTI scalars (FA, mean diffusivity (MD), radial diffusivity (RD), axial diffusivity (AD)). Applying tract-based spatial statistics [38] whole-brain regression analysis with white matter hyperintensities (WMH) as covariate of no interest (assessed in a T2-weighted FLASH sequence according to the Fazekas scale [39]) was conducted (with the Randomise tool version 2.9 available in FSL, 5000 permutations, threshold-free cluster enhancement (TFCE), 2D optimization for tract-based DTI analysis). Individual median values of bilateral CST DTI scalars were additionally extracted (region of interest (ROI) analysis). For the in-depth demonstration of all imaging analyses see the **Supplemental**.

Statistics

Gaussian distribution of data was assessed using the Shapiro-Wilk test. For group comparisons, for non-normally distributed data Kruskal-Wallis one-way analysis of variance (ANOVA) with *post hoc* pairwise Mann-Whitney U testing, and for normally distributed data ANOVA with Bonferroni *post hoc* testing was conducted. Relationship between distinct variables was calculated using bivariate correlations. Left- and right-sided CMAP amplitude and CSA measures were averaged, as there were no side differences. Compared to the left side, the right motor cortex was significantly thinner ($Z=-5.0$, $p<0.001$, Wilcoxon signed-rank test), which is a common finding in ALS [40]; left- and right-sided measures were thus considered separately.

We then applied a factor analysis for mixed (quantitative and qualitative) data (FAMD) using FactoMineR version 1.27 [41] to capture co-variance patterns between distinct measures related to CSF NfL. We included CSF NfL, the ptau/ttau ratio, CST FA (which is the most sensitive DTI metrics in ALS [42]) and ALS phenotype into that model and extracted 1 component with an eigenvalue > 1, which explained 40% of the variance in the data.

Random intercept mixed effects linear models with CSF NfL (ttau, ptau, ptau/ttau ratio) median-split (main effect) and time (disease duration) in months (main effect) were calculated to assess CSF NfL (ttau, ptau, ptau/ttau ratio) × time interaction effects on longitudinal ALSFRS-R total score, and estimates (e) are given. In addition, Kruskal-Wallis one-way ANOVA or ANOVA was calculated to assess group effects of slow (averaged ALSFRS-R points lost per month <0.4 from disease onset to last available ALSFRS-R), intermediate ($\geq$0.4, $\leq$1.4) and fast (>1.4) disease progressors [43] on baseline CSF NfL (ttau, ptau, ptau/ttau ratio).

Cox proportional hazard models giving the hazard ratio (Exp(B)) and Kaplan-Meier analysis using a pairwise log rank test were conducted to compare survival rates and times between

ALS patients revealing low, medium or high CSF NfL (ttau, ptau, ptau/ttau ratio) levels (terciles) at baseline, and censoring was done at the date of the last follow-up.

P-values <0.05 were deemed to be statistically significant. Analyses were performed using the IBM SPSS Statistics 23.0 software.

Results

Sample

Table 1 demonstrates the demographics and the clinical data of the whole sample. **Supplemental Table 3** depicts the demographics and clinical data separately for the ALS phenotypes.

CSF NfL, ttau, ptau and ptau/ttau ratio in controls and ALS

CSF NfL lacked symmetry (controls: D(33)=0.9, p=0.02; ALS: D(89)=0.9, p<0.001) but revealing a significant positive skew distribution instead with most measures clustering at the lower end of the scale (controls: $z_{skewness}$=2.6, p<0.01; ALS: $z_{skewness}$=5.6, p<0.001; **Figure 1A**). In ALS, distribution was the same for CSF ttau and ptau (ttau: D(88)=0.9, p<0.001, $z_{skewness}$=4.4, p<0.001; ptau: D(88)=0.9, p<0.001, $z_{skewness}$=4.1, p<0.001), while the ptau/ttau ratio was normally distributed (D(88)=1.0, p=0.4, $z_{skewness}$=1.5, p>0.05). In controls all CSF tau measures were normally distributed (ttau: D(14)=0.9, p=0.5, $z_{skewness}$=-0.7, p>0.05; ptau: D(14)=1.0, p=1.0, $z_{skewness}$=-0.04, p>0.05; ptau/ttau ratio: D(14)=0.9, p=0.3, $z_{skewness}$=1.3, p>0.05) (**Figure 1B**).

In ALS compared to controls, NfL was higher (median (range) 8,343 (1,795–35,945) pg/ml vs. 1,193 (612–2,616), H(1)=70.8, p<0.001) and the ptau/ttau ratio was lower (mean [SD] 0.17 [0.04] vs. 0.2 [0.03], F(1)=14.3, p<0.001; **Figure 1A&B**). There were no group differences for ttau and ptau (ALS vs. controls, ttau: 236 (96-666) pg/ml vs. 260 (122-373), ptau: 40 (17-99) pg/ml vs. 53 (20-80)).

In ALS, NfL was related to Q_{alb} (rho=0.2, p=0.04), and ttau and ptau were related to age (rho=0.4, p<0.001, respectively). There was no association with sex, disease duration, or onset site; NfL did relate to the ptau/ttau ratio (rho=-0.4, p<0.001; **Figure 1C**), but not to ttau and ptau.

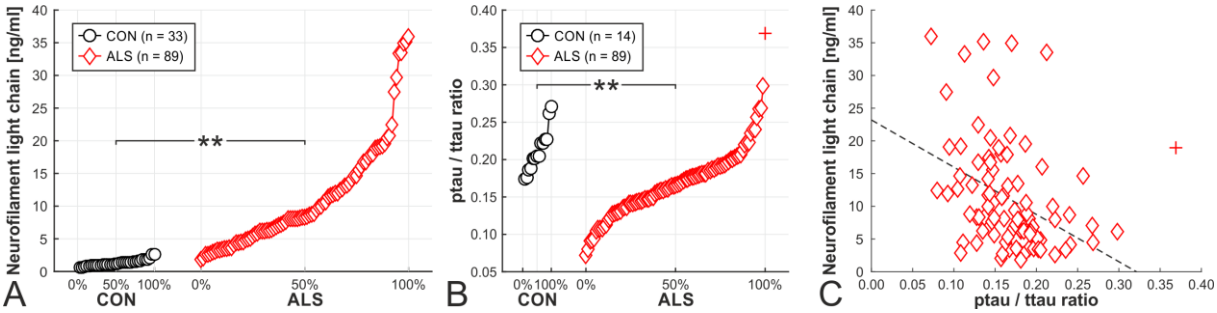

Figure 1. Quantile function of CSF NfL values and the ptau/ttau ratio within the samples under investigation

Graph demonstrates CSF NfL concentrations (**A**) and the ptau/ttau ratio (**B**) in controls (CON) and ALS. ALS patients compared to controls revealed significantly higher CSF NfL concentrations and a significantly lower ptau/ttau ratio. **C** demonstrates the significant relationship between CSF NfL and the ptau/ttau ratio in ALS. **p≤0.001.

There was a significant effect of clinical phenotype on CSF NfL ($H(2)=7.9$, $p=0.02$) and on the CSF ptau/ttau ratio ($F(2)=6.6$, $p=0.002$). Pairwise comparisons revealed group differences between classic and upper motor neuron dominant (UMND) (NfL: $Z=2.5$, $p=0.01$; ptau/ttau ratio: $p=0.02$) or lower motor neuron dominant (LMND) and UMND ALS (NfL: $Z=2.6$, $p=0.008$; ptau/ttau ratio: $p=0.001$), with UMND compared to classic or LMND patients displaying higher NfL (16,076 (7,447–35,945) vs. 8,205 (2,651–35,138) vs. 8,057 (1,795–34,951); **Figure 2A**) and a lower ptau/ttau ratio (0.13 [0.04] vs. 0.17 [0.04] vs. 0.18 [0.03]; **Figure 2C**). In PLS, NfL was lower than in ALS (7,043 (6,454–7,632)), and the ptau/ttau ratio was rather similar to the ratio in classic/LMND, but higher than in UMND ALS (0.16 [0.02]); as there were only 2 PLS cases (**Table 1**), they were, however, not considered for group and pairwise subgroup comparisons.

There was, moreover, a significant relationship between higher NfL or a lower ptau/ttau ratio and greater UMN disease burden according to the Penn UMN score ($rho=0.4$, $p<0.001$, $rho=-0.2$, $p=0.03$; **Figure 2B&D).**

265 Ttau and ptau did not differ across ALS phenotypes, and did not relate to the Penn UMN
266 score.

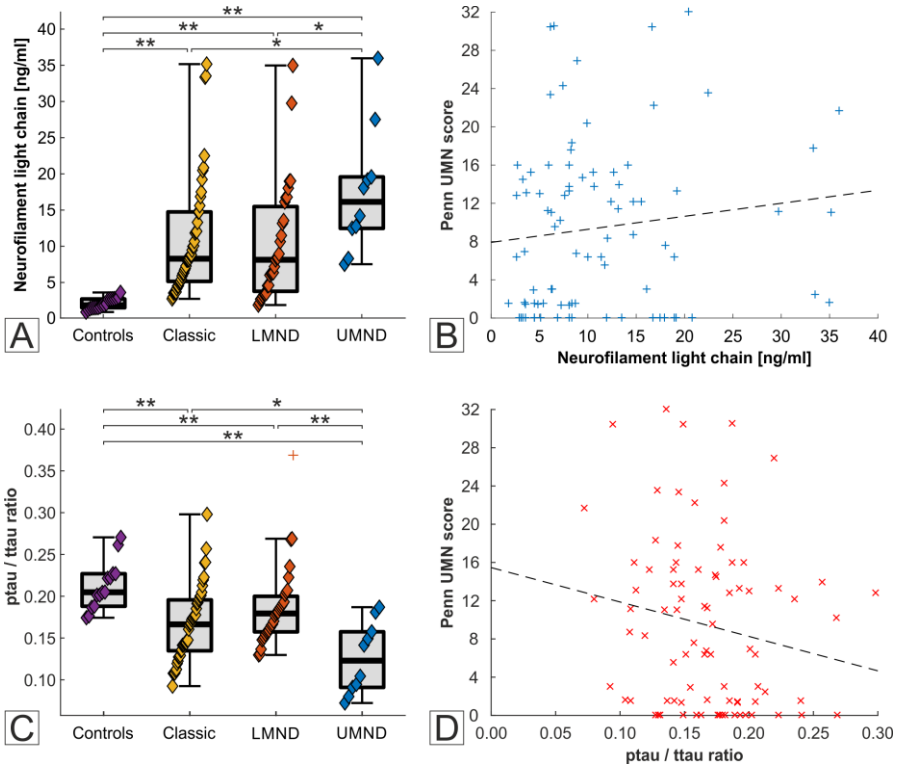

Figure 2. CSF NfL concentrations and the ptau/ttau ratio across the ALS disease spectrum

When compared to classic and lower motor neuron dominant (LMND) ALS, upper motor neuron dominant (UMND) ALS phenotype was related to significantly higher CSF NfL concentrations (**A**) and a significantly lower ptau/ttau ratio (**C**). Higher CSF NfL levels and a smaller ptau/ttau ratio were moreover associated with a greater UMN disease burden as assessed by the Penn UMN score (**B&D**). *p≤0.05, ** p≤0.001.

267 **CSF NfL, ttau, ptau, the ptau/ttau ratio and biomarkers of neuroaxonal injury in ALS**

268 There was a medium-effect size relationship between NfL and median CST FA, MD and RD
269 (ROI-based approach, rho=-0.5, p=0.001, rho=0.3, p=0.02, rho=0.5, p<0.001; **Figure 3A&C**).
270 Correlations between NfL and median CST FA and RD remained significant when solely

considering the classic ALS patients (rho=-0.4, p=0.03, rho=0.4, p=0.05; please see the **Supplemental** and **Supplemental Figure 2**). For the whole ALS cohort, strong correlation between NfL and FA along the cortical spinal pathway additionally emerged from an unbiased whole-brain analysis and it was statistically significant at the stringent threshold of p<0.05 FWE corrected **(Figure 3B)**. At the same statistical threshold, the regression analysis revealed also a positive correlation between NfL and RD which spatially overlapped with the distribution of the significant results in the FA analysis **(Figure 3D)**.

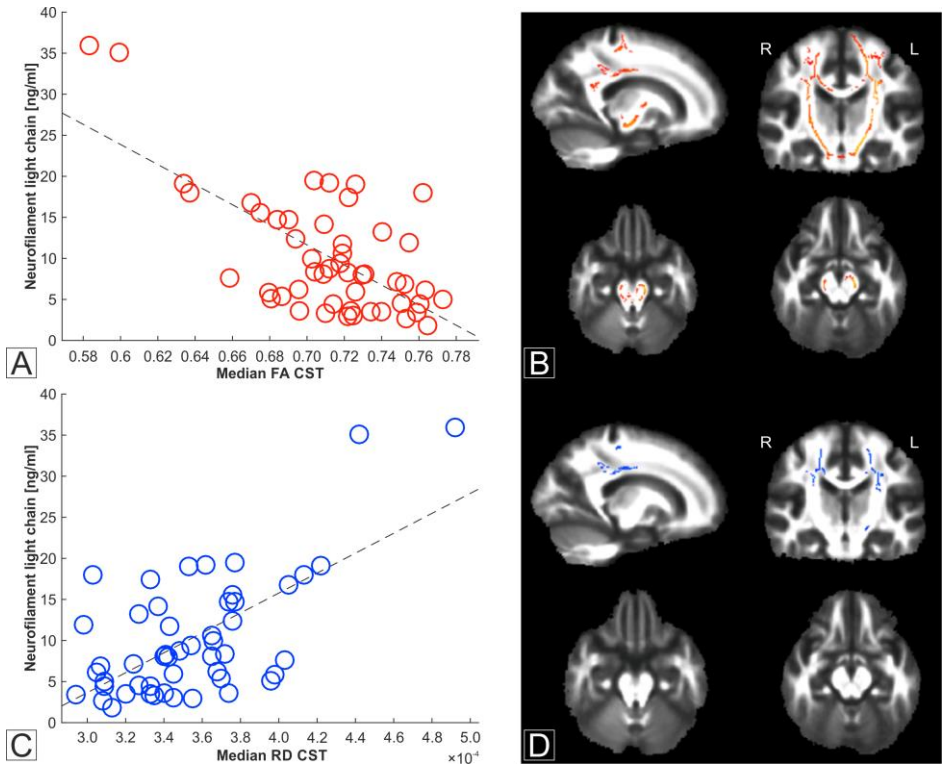

Figure 3. Relationship between CSF NfL and DTI metrics in ALS

The relationship depicted between CSF NfL and median fractional anisotropy (FA) or median radial diffusivity (RD) of the corticospinal tract (CST) using a ROI-based approach is demonstrated in **A&C**. The results of the skeletonized whole-brain regression analysis for FA (panel **B** in red) and RD (panel **D** in blue) are overlapped to the mean FA map. The statistical threshold is set at p<0.05 FWE corrected. The images are displayed following

the radiological convention.

Likewise, there was a medium-effect size relationship between the ptau/ttau ratio and median CST FA and RD (ROI-based approach, rho=0.4, p=0.01, rho=-0.3, p=0.03; **Figure 4A&C**). Considering the whole-brain analysis, the correlation between the ptau/ttau ratio and DTI metrics survived the stringent FWE correction for multiple comparison ($p<0.05$) and partially overlapped with the results of the correlation analysis between NfL level and DTI metrics **(Figure 3B&D and Figure 4B&D)**. In both cases the CST was involved (please see also the results of the ROI analysis, **Figure 3A&C**, **Figure 4A&C**), but the whole-brain analysis revealed that the ptau/ttau ratio was also related to a FA decrease in the genu of the corpus callosum, in the anterior portion of the corona radiata (bilateral), in the anterior portion of the cingulum WM (right), in the external capsule (left) and in anterior limb of the internal capsule (left) **(Figure 4B)**. The ptau/ttau ratio was also correlated with increased RD values in all sections of the corpus callosum **(Figure 4D)**.

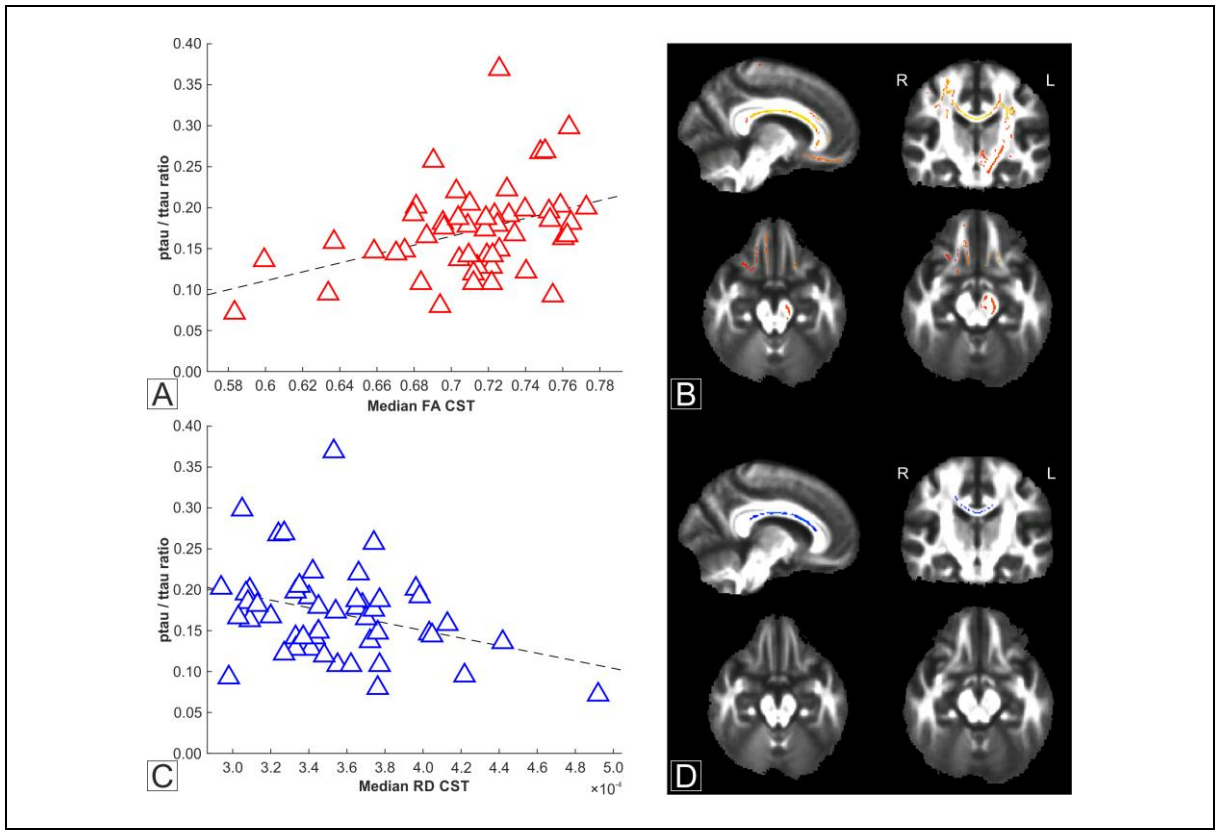

Figure 4. Relationship between the CSF ptau/ttau ratio and DTI metrics in ALS

The relationship depicted between the CSF ptau/ttau ratio and median FA or RD of the CST using a ROI-based approach is demonstrated in **A&C**. The results of the skeletonized whole-brain regression analysis for FA (panel **B** in red) and RD (panel **D** in blue) are overlapped to the mean FA map. The statistical threshold is set at p<0.05 FWE corrected. The images are displayed following the radiological convention.

There was no relationship between NfL, ttau, ptau and the ptau/ttau ratio and WMH.

FAMD revealed a co-variance pattern between CSF NfL (factor load -0.6), the ptau/ttau ratio (0.7), CST FA (0.8) and UMND ALS phenotype (-2.7), which has to be interpreted this way, that high NfL together with a lower ptau/ttau ratio and CST FA decrease is found in patients with dominant UMN involvement.

A lower ptau/ttau ratio was, moreover, related to smaller GMV (r=0.3, p=0.02). There was no association between CSF NfL, ptau, ttau and the ptau/ttau ratio and any further PNS and CNS axonal or (neuro)degeneration ALS marker (e.g. nerve CSA, CMAP amplitudes, cortical thickness of the precentral gyrus).

CSF NfL, ttau, ptau and the ptau/ttau ratio and long-term prognosis in ALS

There was a small-effect size inverse relationship between NfL and baseline ALSFRS-R total score (rho=-0.2, p=0.03): ALS patients with higher compared to lower NfL (median-split) revealed lower ALSFRS-R total scores (H(1)=4.6, p=0.03).

Mixed effects linear models displayed a significant NfL main effect on longitudinal ALSFRS-R total score (e=-4.9, p=0.01), while there was no significant NfL × time interaction effect. This means that when averaging the ALSFRS-R total score across all available time points, ALS patients with higher compared to lower baseline NfL (median-split) show a -4.9 points lower mean value.

There was a trend-level group effect of slow, intermediate and fast progressors on baseline CSF NfL (H(2)=5.0, p=0.08). Posthoc analysis revealed that fast compared to intermediate progressors displayed significantly higher NfL (Z=2.3, p=0.02) **(Figure 5A)**. In line with this, there was a small-effect size correlation between NfL and DPR (rho=0.2, p=0.07, trend-level).

Cox proportional hazard modelling depicted a 2fold to 3fold greater hazard of death for patients with high CSF NfL compared to patients having medium or low NfL (Exp(B) [95%CI]=0.5 [0.3, 0.9], p=0.01, Exp(B) [95%CI]=0.3 [0.1, 0.9], p=0.02). Hazard remained after model adjustment for age, sex, onset site, sporadic vs. familial ALS and baseline ALSFRS-R total score (Exp(B) [95 %CI]=0.4 [0.2, 0.8], p=0.007, Exp(B) [95 %CI]=0.3 [0.1, 0.9], p=0.03; **Figure 5B**).

Kaplan-Meier analysis was in line with those results, displaying significantly shorter median [SE] survival times of patients showing high (upper tercile, 34 [3] months) compared to medium (medium tercile, 48 [3], χ^2=6.1, p=0.01) or low baseline NfL (lower tercile, 45 [22], χ^2=5.1, p=0.02; **Figure 5C**).

There was no relationship between ttau, ptau or the ptau/ttau ratio and the patients' functional scoring (ALSFRS-R), disease progression and survival.

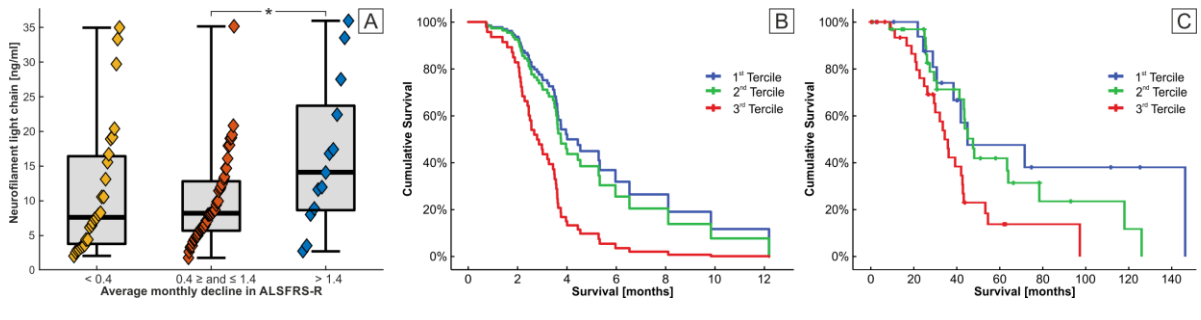

Figure 5. Disease progression and survival as a function of baseline CSF NfL values in ALS

Fast disease progression (averaged ALSFRS-R points lost per month >1.4) was related to higher baseline CSF NfL levels (**A**). **B** depicts predicted survival curves after covariate adjustment (age, sex, onset site, sporadic vs. familial ALS, baseline ALSFRS-R total score) for CSF NfL terciles (Cox proportional hazard modelling). ALS patients with high (3rd tercile) compared to medium (2nd tercile) and low (1st tercile) baseline NfL levels display a 2fold to 3fold greater hazard of death. **C** demonstrates Kaplan-Meier analysis; median survival time was significantly shorter in ALS patients exhibiting baseline CSF NfL levels within the upper (3rd) tercile compared to patients revealing baseline CSF NfL concentrations within the medium (2nd) or lower tercile (1st). *p<0.05.

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Discussion

Our analysis argues that CSF NfL and the ptau/ttau ratio act as diagnostic biomarkers which at once relate to one another, to UMN involvement and DTI white matter signature of cerebral CST degeneration. A smaller ptau/ttau ratio was further indicative of whole-brain gray matter atrophy and widespread microstructural white matter pathology. Neither NfL nor CSF tau measures were related to peripheral motor axon involvement. Our results, moreover, demonstrate a particular relationship between higher baseline CSF NfL and greater disease severity, more rapid disease progression, greater hazard of death and shorter survival in ALS. These findings suggest that elevated CSF NfL and a lower ptau/ttau ratio are particularly biomarkers of central motor degeneration that together with measures emerging from microstructural white matter neuroimaging could be used to stratify ALS patients and to monitor their disease progression presumably allowing to assess the efficacy of future neuroprotective therapies.

Irrespective of ALS pathophysiology, neurofilaments are structural constituents of the neuroaxonal cytoskeleton and integral components of synapses; they are essential for axonal growth, transport and signaling pathways. Neurofilaments are highly abundant in the large Betz cells of the motor cortex and in large-caliber myelinated axons, e.g. of the CST [44–47]. White matter and cortical injury is related to elevated CSF NfL that represents a downstream effect of neuroaxonal loss [48–51]. Tau is a microtubule-associated protein that is highly expressed in neuronal axons, e.g. in thin unmyelinated axons of the neocortical gray matter, providing axonal transport and maintenance of the neurons' structure/morphology [52, 53]. Neuroaxonal degeneration results in increased release of tau from the brain into the interstitial fluid/CSF; and – like CSF NfL – elevation of CSF ttau likely reflects unspecific neuronal and axonal damage, as observed in many chronic neurodegenerative diseases [54]. High CSF ptau, however, specifically relates to the occurrence of neurofibrillary tangle formations and is one hallmark diagnostic biomarker of Alzheimer's disease (AD) [55].

This work replicates the results of several studies showing significantly higher CSF NfL levels in ALS compared to controls (e.g. [4, 6, 14]). Our findings, moreover, support recent data that ALS relates to a lower ptau/ttau ratio, indicating a shifted proportion of ttau and ptau which might be based on three constellations: ALS patients compared to controls reveal (i) higher CSF ttau, or (ii) lower CSF ptau, or both – (iii) higher CSF ttau together with lower CSF ptau. Recent studies reporting a reduced ptau/ttau ratio in ALS or ALS with frontotemporal dementia (ALS-FTD), either disclosed higher CSF ttau [24, 56, 57] or lower ptau [23]. Because phosphorylation of tau occurs mainly in AD and not so much in other neurodegenerative disorders, one may suspect that a lower ptau/ttau ratio probably reflects severe neuroaxonal damage in rapidly progressive diseases favoring the hypothesis of increased CSF ttau rather than a ptau reduction [57]. In our ALS sample, however, both ttau and ptau were unaltered, indicating that the ptau/ttau ratio seems to be a more sensitive candidate biomarker in ALS than CSF ttau or ptau alone.

As shown before, in ALS CSF NfL is skewed towards lower levels, leaving the pivotal question what drives the substantial CSF NfL increase found in a certain ALS subgroup. Our data reveal that in ALS the variability of NfL depends on cerebral CST degeneration and UMN involvement, extending the findings of previous studies [4, 5, 16] by showing for the first time that those variables co-vary, implying that they are altered together in the same patient. This is supported by the fact that in ALS the strong DTI white matter signature in terms of CST FA decrease also relates to UMN pathology [28, 34, 58–60]. Constellation of high CSF NfL, CST degeneration and dominant UMN involvement additionally goes along with a lower ptau/ttau ratio. Supposing that a smaller ptau/ttau ratio indicates neuroaxonal injury, our findings emphasize that CSF NfL and the ptau/ttau ratio together stand for the extent and the severity of upper motor neuron degeneration in ALS. However, in contrast to NfL, a reduced ptau/ttau ratio further indicates gray matter atrophy and white matter integrity loss beyond upper motor neuron pathology. This suggests that the ratio could also serve as

a marker for extramotor involvement in ALS, which needs to be elucidated within future studies.

In our sample CSF NfL and the ptau/ttau ratio were not only related to FA, but also to various DTI metrics (MD, RD). FA and RD alterations seem to be sensitive against demyelination, Wallerian-type myelin degeneration and axonal integrity loss, especially in chronic diseases with extended axonal damage; MD changes have, moreover, been speculated to relate to augmented cellularity (e.g. due to the loss of large myelinated axons) [61, 62]. As the biological underpinnings of differences in DTI variables are still unclear, especially in terms of co-existing underlying pathologies (i.e. axonal injury, demyelination, inflammation) [62, 63], we prefer to refrain from further interpretations of those particular findings.

Our results are in line with two recent studies relating higher CSF NfL and a lower ptau/ttau ratio to altered DTI metrics in ALS [16, 23]. They, however, contradict another ALS study that did not find any relationship between CSF NfL and DTI CST integrity [6]. Steinacker et al. therein combine participants scanned on two different MRI systems and with two different field strengths (2/3 underwent a 1.5T MRI, 1/3 underwent a 3T MRI). The authors have shown that the data obtained from the two systems were comparable and they thus combined all the DTI values in a single analysis. A lower field strength (1.5T compared to 3T) inherently entails a lower signal-to-noise ratio that could, potentially, mask the presence of an effect like the relationship between FA values and NfL levels. Our study, as well as the aforementioned study of Menke and colleagues [16], which also reported a correlation between both FA and RD values and levels of NfL in ALS patients, is based on data acquired on a single 3T scanner employing only one protocol. This kind of design is likely to enhance the sensitivity of the study to detect effects that could, otherwise, be masked by noise.

One might have expected to find an association between motor cortex thickness denoting UMN pathology and CSF NfL and the ptau/ttau ratio. As Betz cells and their gray matter

axons just represent a small fraction of the motor cortex their degeneration seems to be better mirrored by NfL levels or the ptau/ttau ratio instead of affecting the overall number/density of motor neurons/gray matter neuropil or precentral gyrus thickness, accounting for the absent relationship [42, 64].

We failed to find a relationship between CSF NfL or the ptau/ttau ratio, clinical LMN involvement and LMN biomarkers of axonal injury (e.g. reduced CMAP amplitudes or nerve CSA indicating muscle and nerve atrophy, respectively). However, patients with LMND clinical phenotype presenting isolated LMN signs, likewisely displayed elevated NfL concentrations or a reduced ptau/ttau ratio within the range of classic ALS. ALS disease mimics with sole LMN involvement, i.e. e.g. Kennedy's disease or spinal muscular atrophy, do contrary not show abnormal neurofilament levels [5, 14]. Our findings of significant NfL increase across all clinical phenotypes are in line with previous observations in early symptom onset ALS [14], strengthening the role of NfL and ptau/ttau as biomarkers which enhance the diagnostic accuracy of ALS, especially in patients with predominant or isolated LMN signs.

Corroborating previous findings, these analyses also indicate that in ALS higher CSF NfL refers to greater disease severity at baseline and longitudinal follow-up. It conversely remains vague whether higher baseline NfL also relates to a steeper decline or a steady trajectory of overall motor function (group effect of slow, intermediate and fast progressors on NfL vs. non-significant time × median-split baseline NfL interaction effect on longitudinal ALSFRS-R total score). Considering the latter, it might be possible that a certain ALS subject just comes into the disease with an already determined signature of functional performance and related CSF NfL levels. This would be in line with recent analysis demonstrating no or just minimal change of CSF and highly related serum neurofilament concentrations over the course of disease [7, 11, 13]. Especially at the lower end of CSF NfL levels in ALS, there might be a continuous turnover of neurofilaments as a result of an equilibrium between

neurodegeneration and –regeneration, with the latter depending on individual variables (e.g. genetics, resilience, exposure to environmental (epigenetic) factors throughout life [43]). Due to such individual circumstances a certain ALS subgroup abandons that equilibrium (supposedly at an already preclinical state or rather suddenly indicating the onset of irreversible neuroaxonal damage [5, 65]), passing the threshold for disease development and CSF NfL increase which in turn relates to shorter survival. This model especially holds true for the ALS conversion of asymptomatic familial cases revealing normal CSF NfL at presymptomatic but highly elevated levels at symptomatic disease stages [4, 11, 65]. We, however, refrained from comparing NfL between sporadic and familial ALS, because of the small number of genetic variants. Impact of CSF NfL on long-term prognosis remains significant after the adjustment for several disease-modifying variables, replicating previous findings [4, 7] and suggesting NfL to aid as an independent prognostic biomarker.

The association of the same biomarker with both UMND ALS and worse prognosis is somewhat puzzling, as the UMND phenotype compared to classic ALS usually has a slower functional decline. Our results may thereby point to the existence of distinct groups displaying high CSF NfL: UMND ALS with longer survival despite high CSF NfL and ALS patients with combined UMN and LMN pathology (classic disease phenotype), high CSF NfL and worse prognosis [4] (see follow-up analysis in the **Supplemental** and **Supplemental Figure 3**). Further studies are indeed needed to disentangle the existence of such subgroups.

The strength of our study is the availability of a considerable set of locally well-established imaging biomarkers used to understand the co-variance patterns between CSF NfL, CSF tau measures and further measures of PNS and CNS neuroaxonal damage in ALS. Limitations comprise: (i) our cross-sectional and retrospective approach, (ii) the relatively small sample size of distinct clinical ALS subgroups (especially of UMND ALS, making up only 11% of our patient cohort), which kept us from performing extensive phenotype-wise analysis (of e.g. the

relationship between CSF measures and neuroaxonal injury markers), as well as (iii) the absence of measures of serum NfL and phosphorylated neurofilament heavy chain (pNFH) (e.g. to investigate the superiority of one marker to reflect disease severity and biomarker neuroaxonal injury). An additional limitation of the study is the heterogeneity among the number of patients who underwent each assessment (**Supplemental Figure 1**).

Nevertheless our findings overall strengthen the idea that combining CSF NfL, the ptau/ttau ratio, CST DTI metrics and clinical measures (of e.g. UMN pathology) improve the diagnostic accuracy and prognostic assessment in ALS.

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Tables

Table 1. Demographics and clinical data of the sample under investigation

	ALS (n=89)	**Controls (n=33)**	**P-value**
Age, in years	63 (33-83)	60 (32-76)*	0.06
Male sex, n (%)	54 (61)	16 (49)[#]	0.3
Sporadic ALS / Familial ALS, n (%)	63 (91) / 6 (9)[1]		
El Escorial na / suspected / possible / probable / definite, n (%)	1 (1) / 24 (27) / 34 (38) / 15 (17) / 15 (17)		
Clinical phenotypes classic / LMND / UMND / PLS, n (%)	46 (52) / 31 (35) / 10 (11) / 2 (2)		
Disease onset bulbar / limb, n (%)	29 (33) / 60 (67)		
Disease duration, in months	10 (0.2-190)		
Disease progression rate, in 1 / months	0.7 (0.04-3.3)		
ALSFRS-R total score / 48, baseline	41 (4-48)		

Unless otherwise reported, medians and (ranges) are given. ALS, amyotrophic lateral sclerosis; ALSFRS-R, revised ALS functional rating scale; LMND, lower motor neuron dominant; na, not applicable; PLS, primary lateral sclerosis; UMND, upper motor neuron dominant; [1]familial ALS comprised 2 cases with C9orf72 positivity and 4 patients with SOD1 positivity, *Mann-Whitney U test, [#]χ^2 test. P-values <0.05 were deemed to be statistically significant.