Title

Cerebrospinal fluid neurofilament light levels in neurodegenerative dementia: diagnostic accuracy in the differential diagnosis of prion diseases.

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Research in context

SYSTEMATIC REVIEW: The authors reviewed the literature using traditional (PubMed) sources and meeting abstracts and presentations. Alterations in the levels of neurofilament light (NFL) protein in the cerebrospinal fluid (CSF) of several neurological conditions have been reported.

INTERPRETATION: The present work assesses the diagnostic accuracy of NFL quantification in the differential diagnosis of neurodegenerative dementias, with special focus on sporadic and genetic forms of prion diseases, the diagnostic group showing the highest NFL levels.

FUTURE DIRECTIONS: Further studies using independent large study populations and addressing the analytical reliability of the findings (ring trial) will help to define the accuracy of NFL quantification in the differential diagnosis of neurodegenerative dementias. Since NFL is considered a surrogate marker of neurodegeneration, a comprehensive comparative analysis of NFL levels in brain and CSF will shed light into the pathological features associated to the neurodegenerative process.

Abstract

INTRODUCTION: Neurofilament light (NFL) levels in the cerebrospinal fluid (CSF) are increased in several neurodegenerative dementias. However, the diagnostic accuracy of NFL in the differential diagnostic context is unknown.

METHODS: CSF NFL levels were quantified in neurological controls (NC, n=123), cognitive impairment/dementia (CI/DEM, n=63), mild cognitive impairment (MCI, n=48), Alzheimer's disease (AD, n=108), dementia with Lewy bodies/Parkinson's disease dementia (DLB/PDD, n=53), vascular dementia (VaD, n=46), frontotemporal dementia (FTD, n=41), Creutzfeldt-Jakob disease (sCJD, n=196) and genetic prion diseases (n=182).

RESULTS: The highest NFL levels were detected in sCJD, followed by VaD, FTD, DLB/PDD and AD. In sCJD, NFL levels correlated with CSF tau and disease duration and were able to differentiate sCJD from NC (AUC=0.99, 95%CI: 0.99-1) and CI/DEM cases (AUC=0.90, 95%CI: 0.87-0.92). NFL was also elevated in genetic prion diseases associated to prion protein gene E200K, V210I, P102L and D178N mutations.

DISCUSSION: Increased NFL levels are a common feature in neurodegenerative dementias and their quantification may support the diagnosis of prion diseases.

Keywords

Neurofilament light, cerebrospinal fluid, neurodegenerative dementias, prion diseases, Alzheimer's disease, dementia with Lewy bodies, Parkinson's disease dementia, vascular dementia, frontotemporal dementia.

BACKGROUND

Neurofilament light (NFL) protein is currently under examination as a candidate biological fluid biomarker for the diagnosis and prognosis of several neurological conditions. NFL levels have been reported to be increased in the CSF of several neurodegenerative diseases, such as mild cognitive impairment (MCI), Alzheimer's disease (AD), vascular dementia (VaD), sporadic Creutzfeldt-Jakob disease (sCJD) and in the spectrum of frontotemporal lobar degeneration-related syndromes, as well as in motor neuron diseases [1–5]. Beyond its potential role as diagnostic biomarker, NFL levels may be a useful marker predicting progression of disease [6], disease severity and survival [2,7–9], and differentiating between disease subtypes [10,11]. In the context of neuroinflammation-mediated axonal injury, CSF NFL is a proven marker of treatment response to effective disease-modifying drugs (PMIDs: 21280078 and 25809304).

The presence of NFL is generally associated to white mater involvement [43]. Additionally, the recent characterization of NF proteins in the postsynaptic terminal associated to terminal dendritic branches, where they play a role in neurotransmission [44], would suggest a role for NFL as a surrogate marker of synaptic degradation. Therefore, the differential analysis of its levels in the spectrum of dementias might shed light into the specific pathological singularities among these conditions.

Alterations of CSF NFL are disease-type dependent [2,12] with higher levels in VaD and frontotemporal dementia (FTD). Recently, highly increased NFL levels have been reported in the serum and CSF of sCJD patients [3], which is in line with a previous report showing elevated CSF NFL in sCJD compared to controls and AD cases [1]. This finding suggests a prominent role of NFL in the pathology of prion diseases. However, lack of systematic studies analyzing different diagnostic groups including prion diseases impedes the understanding of the precise accuracy of CSF NFL quantification in the differential diagnostic context of dementia, hampering its potential introduction in clinical routine.

The aim of this study was to thoroughly investigate the ability of CSF NFL to discriminate neurodegenerative dementias from different etiologies, to report the precise accuracy of NFL quantification in the differential diagnosis of prion diseases considering demographic and genetic factors, and to study the potential role of NFL as a prognostic marker of prion diseases.

METHODS

Study population

The study included a total of 860 patients. For the analysis of the role of CSF NFL in the differential diagnosis of neurodegenerative dementias, two cohorts comprising neurological controls (NC), MCI, AD, dementia with Lewy bodies/Parkinson's disease dementia (DLB/PDD), VaD, FTD and sCJD cases were used. Cohort 1 (study cohort) was composed of samples collected at the Clinical Dementia Center in the University Medical Center of Göttingen (Germany). Cohort 2 (validation cohort) was composed of samples collected at the Dementia Clinic, Neurology Department of Coimbra University Hospital Portugal and at the National Referral Center for CJD in Portugal.

For the validation of the diagnostic accuracy of NFL quantification in the differentiation of sCJD from cognitive impairment/dementia (CI/DEM – no CJD) cases, two additional cohorts were used. Cohort 3 included samples collected at the Polish neurologic and psychiatric hospital departments further processed at the Department of Molecular Pathology and Neuropathology (Medical University of Lodz - Poland). Cohort 4 included samples collected at the Neurologic Clinics of Northern Greece hospitals further processed at the Laboratory of Pharmacology, School of Pharmacy (Aristotle University of Thessaloniki - Greece).

For the study of NFL levels in genetic prion disease cases, six cohorts were used: Cohort 1 (Clinical Dementia Center in the University Medical Center of Gottingen - Germany), Cohort 2 (National Referral Center for CJD, Coimbra - Portugal), Cohort 3 (Medical University of Lodz - Poland), Cohort 5 (Unit of Biodiagnostic of CJD and other prion diseases at the Hospital Clinical of Barcelona - Spain), Cohort 6 (National Centre of Microbiology at the Carlos III Institute of Health, Madrid - Spain) and Cohort 7 (Istituto Superiore di Sanità, Rome - Italy). Detailed information on the number of cases from each diagnostic group used in this study is supplied in Supplementary Table 1.

The NC group included psychiatric disorders, ischemic stroke, epilepsy, autoimmune diseases, meningitis, alcohol abuse, headache, vertigo, pain syndromes, acute hypoxia, encephalopathy, cerebral vasculitis, normal pressure hydrocephalus and alternative neurologic conditions. The CI/DEM – no CJD group was composed of subjects with cognitive impairment or dementia (of unknown etiology) suspected of CJD at a preliminary stage of diagnosis, where prion disease diagnosis was later excluded according to established clinical and/or neuropathological criteria [13,14] .

AD was diagnosed according to Dubois criteria [15] in Cohort 2 and the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders (NINCDS-ADRDA) [16,17] criteria was used in Cohort 2. FTLD was diagnosed according to the Lund and Manchester clinical criteria [18] revised by the International Behavioural Variant Frontotemporal Dementia Criteria Consortium for bvFTD [19]. MCI was diagnosed based on international criteria of the Petersen workgroup [20]. The diagnosis of DLB was based on the criteria of McKeith [21,22]. PDD was diagnosed as dementia in patients with PD. VaD diagnosis was based on clinical and radiological criteria as described by Roman et al. 1993 (NINDS-AIREN) [23]. All patients with sCJD were classified as probable or definite cases according to diagnostic consensus criteria [13] and neuropathological examination [14].

Lumbar punctures (LPs) were performed for diagnostic purposes at the time of diagnosis. CSF from serial lumbar punctures from 18 sCJD cases was used to study the influence of time of LP on NFL levels.

CSF tests

CSF NFL was centrally quantified (Clinical Dementia Center, Göttingen) using a commercially available enzyme-linked immunosorbent assay (NF-light; Uman Diagnostics) as described by the manufacturer. The kit was previously validated in a multicenter study showing good assay sensitivity and intra- and inter-assay precision [24]. Inter- and intra-assay coefficients of variation in our study

were below 15%. The analysts were masked to clinical data. CSF was locally analyzed for the presence of 14-3-3 protein by Western blot as described previously [25] in each of the participants labs. Total tau (tau) and phospho-tau (p-tau) were centrally quantified (Clinical Dementia Center, Göttingen) using the enzyme-linked immunosorbent assay kits INNOTEST® hTAU-Ag and INNOTEST® PHOSPHO-TAU(181P) from Fujirebio, according to the manufacturer's instructions, with the exception of tau levels in cohort 2, which were locally measured using the same commercial kit and analytical procedures.

Genetic tests

For detection of a prion disease–associated mutation, genetic testing was performed as described before [26].

Statistical tests

Mann-Whitney U tests were used to compare two groups of samples. For multiple comparisons Kruskal-Wallis test followed by Dunn's post-test was applied. In order to assess the diagnostic accuracy of NFL, receiver operating characteristic (ROC) curve analyses were carried out and areas under the curve (AUC) with 95% confidence intervals were calculated using GraphPad Prism 6.01. The best cut-off value was estimated based on the Youden index [27] derived from cohorts 1; the diagnostic accuracy (sensitivity and specificity) of NFL was then externally validated in cohort 2. Spearman rank correlation was used to assess associations between continuous biomarker levels.

In order to determine the association between NFL levels and total disease duration (time between disease onset and death of the patient) a fractional polynomial approach was used. Brier scores and Somers' *D* were calculated to assess the predictive power of NFL as a prognostic marker for survival in prion diseases.

Ethics

The study was conducted according to the revised Declaration of Helsinki and Good Clinical Practice guidelines and approved by local Ethics committees (University of Göttingen Number 11/11/93 (+Amendments) and Number 9/6/08). Informed consent was given by all participants or their legal next of kin.

RESULTS

NFL in the differential diagnosis of neurodegenerative dementia

CSF NFL concentrations were initially assessed in the study cohort (cohort 1) comprising NC and cases covering a broad spectrum of neurodegenerative dementias. Among the clinical diagnoses, highest levels were detected in sCJD, followed by VaD, FTD, DLB/PDD, AD, MCI and NC (Figure 1A and 1C). NFL concentrations were significantly different in: NC vs AD, AD vs FTD, DLB/PDD vs VaD ($p<0.05$), in NC vs DLB/PDD ($p<0.01$) and in NC vs DLB/PDD, NC vs VaD, NC vs FTD, NC vs sCJD, MCI vs sCJD, AD vs VaD, AD vs sCJD, DLB/PDD vs sCJD ($p<0.001$). Data from study cohort were replicated in a validation cohort (cohort 2), showing similar NFL levels pattern across clinical diagnoses than in

study cohort (Figure 1B and 1C). In validation cohort, NFL levels were significantly different in: NC vs MCI, NC vs VaD, NC vs FTD, AD vs sCJD ($p<0.05$) and in NC vs sCJD, MCI vs sCJD, DLB/PDD vs sCJD, FTD vs sCJD ($p<0.001$). Demographics and biomarkers information from study and validation cohorts are presented in Figure 1C. Tau levels and presence of 14-3-3 for each diagnostic group were in agreement with those previously reported [28–31]. In order to determine the diagnostic accuracy of NFL quantification in the differential diagnostic context of neurodegenerative dementias, AUCs derived from cohort 1 and 2 were calculated (Figure 1D). sCJD (AUC=0.9966), VaD (AUC=0.9383) and FTD (AUC=0.8615) cases were discriminated from NC with high accuracy. Instead, NFL levels showed lower performance in distinguishing DLB/PDD, AD and MCI from NC (AUC<0.8 in all comparisons). Additionally, NFL displayed excellent values discriminating sCJD from MCI, AD and DLB/PDD (AUC>0.9) and moderate values discriminating from FTD (AUC=0.8311) and VaD (AUC=0.7633). Finally, VaD could be discriminated with good accuracy, not only from MCI (AUC=0.8313), but also from AD (AUC=0.8013).

Diagnostic accuracy of NFL in the discrimination of sCJD

As higher NFL levels were detected in sCJD, we sought to determine the diagnostic performance of NFL quantification in the discrimination of sCJD from NC and dementias from a non-prion etiology. For this purpose, different types of dementias (AD, DLB/PDD, VaD and FTD) and cognitive impairment (MCI) cases from cohort 1 and 2 were grouped under the CI/DEM group. Mean NFL levels were significantly higher in sCJD (31456±21243 pg/mL) compared to CI/DEM (7971±10653 pg/mL) and NC (2138±1532 pg/mL) ($p<0.001$). Additionally, NFL levels were also significantly increased in the CI/DEM group compared to those detected in NC ($p<0.001$). Diagnostic accuracy of NFL quantification in the discrimination of sCJD cases was calculated from AUC derived from ROC analysis (Figure 2B). AUC was 0.9966 (95%CI: 0.9926-1) for the NC vs sCJD comparison and 0.9008 (95%CI: 0.8726-0.9289) for the CI/DEM vs sCJD comparison. A cut-off of 7000 pg/mL revealed 100% sensitivity and 95% specificity for the discrimination of sCJD from NC cases. In contrast, a cut-off of 10500 pg/mL allowed discrimination of sCJD from CI/DEM cases with a sensitivity of 86% and a specificity of 80% (Figure 2B). To avoid any bias on the selection of a specific diagnostic group in the group of CI/DEM cases, a cross-validation study using two independent cohorts was performed. Cohort 3 (Figure 3A) and cohort 4 (Figure 3B) included sCJD patients as well as cases diagnosed with cognitive impairment of dementia from unknown etiology. Tau levels and 14-3-3 protein presence were increased in sCJD compared to CI/DEM cases (Figure 3A and 3B). Similarly, NFL levels were increased in sCJD ($p<0.001$). Importantly, AUCs from cohort 3 (AUC=0.8967, 95%CI: 0.8208-0.9726) and cohort 4 (AUC = 0.8670, 95%CI: 0.7704-0.9636) (Figure 3) were in the range of those reported for cohort 1 and 2 (Figure 2B), and no statistical differences among AUCs from the four cohorts were detected ($p>0.05$).

Role of demographic and genetic parameters on NFL levels in sCJD patients.

NFL levels in sCJD were affected neither by age at onset (ranging from 30 to 89 years old) ($p>0.05$) (Figure 4A) nor by the sex of the patients ($p>0.05$) (Figure 4B). In order to test if genetic characteristics

of the patient cohort were associated to differential NFL levels, we stratified sCJD samples by the *PRNP* codon 129 polymorphism, a well-known modifier of biomarkers' accuracy in prion diseases [32,33]. Information regarding codon 129 usage was available for 140 sCJD cases. Mean NFL values were higher in sCJD valine/valine [VV] (38963±20640 pg/mL) than in methionine/methionine [MM] (24689±19823 pg/mL) and methionine/valine [MV] (22143±14955 pg/mL) cases ($p<0.01$) (Figure 4C).

Correlation of NFL levels with surrogate prion biomarkers

Association of NFL levels with CSF 14-3-3 and tau, two surrogate markers of prion disease used in routine clinical practice [34] was studied. Elevated NFL levels were detected in sCJD cases tested positive for 14-3-3 (17772±11468 pg/mL) compared to those that tested negative or inconclusive (29159±21496 pg/mL) ($p<0.05$) (Figure 4D). Additionally, a positive correlation was observed between CSF tau and NFL levels (Spearman's rho=0.37, $p<0.001$) (Figure 4E).

NFL quantification as prognostic marker in sCJD

Subsequently, we assessed the influence of time of CSF sampling on NFL concentration in sCJD patients. To this purpose we analyzed 18 sCJD patients with two LPs at different stages of the disease. In order to normalize time intervals between lumbar punctures (LPs), we divided the time of LP to disease onset in each patient by the total duration of the disease. Afterwards, samples were grouped in three categories according to whether they underwent LP in the first (time of LP to disease onset/total duration of the disease <0.33), second (0.33–0.66), or third (>0.66) stage of the disease, as previously reported [35,36]. NFL levels were either non-altered or increased in the second LP (Figure 3A). As alterations in biomarkers profile may depend on the duration of the disease, reflecting brain damage and neuronal degeneration, we stratified the data from serial LPs according to disease duration. When disease duration was equal or shorter than 6 months, reflecting the mean disease duration of our population study [37], no significant differences on NFL concentrations were detected between serial LPs. Instead, for cases showing disease duration longer than 6 months, NFL levels were statistically increased in the consecutive LP (Figure 5B). Next we aimed to investigate the potential association between NFL levels at time of first LP and disease duration. Our data revealed a strong non-linear association between NFL values and survival time (NFL can be modelled as a linear combination of the terms $206*(\text{survival time}) -7057*(\text{survival time})^{-2}$) (n=185) (Figure 5C). NFL showed a moderate ability as a prognostic marker being represented by a Brier score of 0.24 and a Somers' *D* value of 0.15.

Diagnostic accuracy of NFL in the discrimination of genetic prion diseases

We sought to determine the usefulness of NFL level in the discrimination of prion disease from genetic etiology. Genetic prion diseases are characterized by a low prevalence consisting in about 10-15% of all type of human prion diseases [38]. Therefore, to provide statistical power to our study, we collected genetic prion diseases samples associated to the Prion protein gene (*PRNP*) mutations E200K (n=83), V210I (n=35), P102L (n=10) and D178N (n=84) from six different unrelated cohorts. Tau and 14-3-3 levels followed the previously reported diagnostic parameters for these mutations [39]. Increased sensitivity was detected in genetic CJD (E200K and V210I mutations), followed by P102L mutation

(associated to the Gerstmann-Sträussler-Scheinker (GSS) syndrome) and D178N mutation (associated to Fatal Familial Insomnia) (Figure 6A).

In all types of genetic prion diseases, increased NFL levels were detected compared to NC ($p<0.001$) (Figure 6B). Although NC cases presented a higher age at onset compared to P102L and D178N cases ($p<0.05$), no association between NFL levels and age at onset was detected, neither for NC nor for any type of genetic prion diseases ($p>0.05$). Among genetic prion diseases, higher levels were detected for E200K mutation, but no significant differences among the four mutation groups were achieved (Figure 6B). Analysis of diagnostic parameters showed an excellent sensitivity and specificity of NFL in the discrimination of genetic prion diseases from NC. AUC values were superior to 0.94 ($p<0.001$) for all mutations (Figure 6C). Disease-specific cut-offs calculated according to Youden index revealed sensitivities and specificities ranged of 87-100% and 86-96%, respectively (Figure 6C). Interestingly, NFL was able to discriminate D178 (FFI) and P102L (GSS) cases with high accuracy, in contrast to alternative prion biomarkers [28,39–41] (Figure 6C).

DISCUSSION

The clinical diagnosis of neurodegenerative dementias is often challenging in view of their overlapping clinical features, thus appropriate biomarker tools able to discriminate among different conditions are urgently needed. In this regard, NFL quantification has emerged as a potential CSF biomarker of different types of neurodegenerative dementias. However, its accuracy in the differential diagnostic context has not been fully addressed.

In the present study, we determined for the first time to the best of our knowledge, the CSF NFL signatures in the broad spectrum of neurodegenerative dementias. Importantly, data from our study cohort were replicated in an independent validation cohort, underscoring the precision, robustness and reproducibility, which allow us to provide external validation of diagnostic parameters. Additionally, by means of multi-comparative analysis of the AUC from ROC curves, the precise diagnostic accuracy for each dementia type was evaluated.

Highest NFL concentrations were found in the CSF of sCJD followed by FTD and VaD cases. While AD and DLB/PDD cases also showed increased NFL levels compared to controls, their discriminatory value was limited.

Elevated NFL concentrations in sCJD compared to controls [3] and AD cases [1] have been were previously reported, but no data were available comparing sCJD to other dementias groups. NFL in sCJD would reflect massive synaptic degeneration and neuronal damage in agreement which is with its positive association with CSF tau and 14-3-3. A strong correlation between CSF NFL and tau was also reported in other dementias such as AD [8]. In sCJD, a primary involvement of the white matter is assumed [42] and recently, alterations in subcortical tracts have been identified in sCJD using functional magnetic resonance imaging (MRI), providing quantitative evidence of white matter involvement in prion diseases [43].

Elevated NFL concentrations in VaD and FTD were also in agreement with previous observations [2] and would be indicative of the presence of extensive neuroaxonal damage in white matter and subcortical brain structures. Indeed, white matter involvement is one of the most common neuropathological features in VaD [44] and it is estimated that subcortical changes on MRI can be found in 82% of the cases [45]. Additionally, white mater pathology in frontotemporal lobar degeneration is also widely described [46]. Since elevated NFL concentrations in FTD correlate with decreased gray and white matter volume [7], it has been recently suggested NFL reflect corticospinal tract degeneration [47].

Overall, the observation that all types of neurodegenerative dementias displayed increased NFL concentrations suggests their levels mirror the disease-specific pathological processes occurring in the brain, and thus, it can be considered as a direct surrogate marker of neurodegeneration. Our data indicate that, although NFL could be useful in the discrimination of some specific types of dementia from a non-prion etiology (MCI and AD from VaD and FTD and VaD from DLB/PDD), the highest diagnostic accuracy lies in the discrimination of sCJD cases.

Importantly, we observed that NFL quantification would be in range of the best surrogate and direct prion biomarkers (tau, 14-3-3, α-synuclein and real-time quaking-induced conversion (RT-QuIC)) in their capability to discriminate sCJD from control cases [34,40,48,49]. Instead, lower diagnostic accuracy was achieved in the discrimination of sCJD from the combined group of dementias (CI/DEM). The validation of the later findings in two independent cohorts with unknown diagnosis for the CI/DEM group demonstrated the absence of bias in the selection of dementia cases in cohort 1 and 2.

Another relevant finding from our study is the potential prognostic value displayed by NFL in sCJD cases, since their levels were negatively correlated with disease duration, as it was previously shown in other dementia-types, especially in AD [2]. Other prion biomarkers such as tau and α-synuclein also show a prognostic value for sCJD cases [36,50,51], while data for 14-3-3 [52,53] and RT-QuIC [49,54] are inconsistent.

Genetic prion diseases constitute a separate layer of prion diseases with heterogenic presentation depending on the mutation in the *PRNP* gene. The clinicopathological features in gCJD cases (E200K and V210I mutations) are similar to those reported for typical sCJD cases [55]. Consequently diagnostic value of classical CSF prion biomarkers in gCJD are in range with those reported for sCJD. On the contrary, the diagnostic value of tau, 14-3-3, α-synuclein and RT-QuIC in discriminating FFI and GSS cases is poor [39,40,48], likely due to a more restricted pathology and/or to a longer disease duration compared to sporadic cases. Furthermore, the low prevalence of these cases hinders the establishment of the precise biomarkers' diagnostic parameters. Here, with the use of the largest cohort of prion genetic diseases studied so far, we unequivocally demonstrate the presence of elevated NFL levels in the four types of mutations analyzed. The most salient finding is the increased NFL concentrations in FFI, for which no biochemical or imaging biomarker is currently able to discriminate these cases with high diagnostic accuracy [56].

In conclusion, our data strongly support the use of CSF NFL analysis for the differential diagnosis of sCJD and genetic prion diseases, especially for those forms for which no biomarkers are currently available such as FFI and GSS (P102L).

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Authors contributions

IZ and FL designed the study. MS, AK, AV-P, IB, and FL performed experiments, analyzed data and interpreted the results. EK, EG and DD-L performed experiments. IZ, EK, EG, TS, BS, PPL, DV, PH, TK, SG, IF, HZ, KB, MC, IB and RS-V contributed to clinical data acquisition, interpretation and sampling. FL wrote the manuscript draft. All authors critically revised the manuscript and approved its contents before submission.

Conflict of interest:

The authors report no conflicts of interest.

FIGURE LEGENDS

Figure 1. Analysis of CSF NFL levels in the differential diagnosis of neurologic diseases and neurodegenerative dementias.

Total NFL levels in NC, MCI, AD, DLB/PDD, VaD, FTD and sCJD in study cohort (cohort 1) (A) and validation cohort (cohort2) (B). In study cohort NFL levels were significantly different in: NC vs AD, AD vs FTD, DLB/PDD vs VaD ($p<0.05$), in NC vs DLB/PDD ($p<0.01$) and in NC vs DLB/PDD, NC vs VaD, NC vs FTD, NC vs sCJD, MCI vs sCJD, AD vs VaD, AD vs sCJD, DLB/PDD vs sCJD ($p<0.001$). In validation cohort, NFL levels were significantly different in: NC vs MCI, NC vs VaD, NC vs FTD, AD vs sCJD ($p<0.05$) and in NC vs sCJD, MCI vs sCJD, DLB/PDD vs sCJD, FTD vs sCJD

($p<0.001$). Kruskal-Wallis test and Dunn's post-hoc test was applied. (C) Demographic and biomarkers data from study and validation cohorts. Number of cases (n), age in years (mean values ± standard deviation), sex (female (f)/males (m)), CSF tau levels (mean values ± standard deviation in pg/mL), presence of 14-3-3 in the CSF (positive (P), trace (T) and negative (N)) and NFL levels (mean values ± standard deviation in pg/mL) are indicated. NA = not-analyzed. (D) AUC derived from ROC curves and 95% CI for all comparisons between pairs of diagnostic groups derived from study and validation cohorts. Two decimal places are shown for simplified data visualization.

Figure 2. Diagnostic accuracy of CSF NFL as sCJD biomarker.

(A) NFL levels in neurological controls (NC), all the cases with a diagnosis of cognitive impairment or dementia (CI/DEM) and sCJD cases from cohort 1 and 2. The CI/DEM group included MCI, AD, DLB/PDD, VaD and FTD cases. NFL levels were significantly different in: sCJD vs NC, sCJD vs CI/DEM and NC vs CI/DEM cases ($p<0.001$). Kruskal-Wallis test and Dunn's post-hoc test was applied. (B) ROC curve for NFL in the comparative analysis for NC vs sCJD cases and CI/DEM vs sCJD comparisons. AUC derived from ROC curves, 95% CI and p value for NC vs sCJD and CI/DEM vs sCJD comparisons. Optimal cut-offs (based on Youden Index), sensitivity and specificity were calculated.

Figure 3. Validation of CSF NFL as a discriminatory biomarker in the differentiation of sCJD from dementias of a non-prion etiology.

NFL levels in cognitive impairment/dementia (CI/DEM) and sCJD cases in two independent cohorts of cases (A, cohort 3 and B, cohort 4). The CI/DEM group was composed of subjects with cognitive impairment or dementia (of unknown eiology) suspected of CJD at a preliminary stage of diagnosis. Mann-Whitney U tests were used. NFL levels were significantly different between sCJD and CI/DEM ($p<0.001$) in both cohorts. Demographics and biomarker levels as well as AUC values are indicated.

Figure 4. Influence of demographic and genetic factors on CSF NFL levels and correlation with prion disease biomarkers. (A) Relationship analysis between NFL levels and age at disease onset in sCJD cases ($p>0.05$). Spearman rank correlation was used. (B) NFL levels in sCJD stratified by sex. Mann-Whitney U test was used ($p>0.05$). (C) NFL levels in sCJD stratified by *PRNP* codon 129 polymorphism (M = Methionine, V = Valine). Kruskal-Wallis test followed by Dunn's post-test was applied ($p<0.01$ for MM vs VV and MV vs VV comparisons). (D) NFL levels in sCJD stratified by 14-3-3 protein test. Mann-Whitney U test was used ($p<0.05$). (E) Correlation analysis between CSF NFL and tau levels in sCJD cases (Spearman's rho=0.37; $p<0.001$).

Figure 5. Association between CSF NFL levels and disease duration in sCJD patients.

(A) NFL levels in serial LPs in sCJD cases at different stages of the disease. Samples were grouped in three categories according to whether they underwent LP in the first (<0.33), second (0.33–0.66) or third (>0.66) stage of the disease. (B) Fold change in NFL levels (baseline 100%) in serial LPs according to disease duration (shorter or longer than 6 months). Mann-Whitney U test was used ($p<0.05$). (C) Association between CSF NFL levels and disease duration (months) in sCJD patients analyzed by a

fractional polynomial approach.

Figure 6. Diagnostic accuracy of CSF NFL as a biomarker for genetic prion diseases.

(A) NFL levels in neurological controls (NC) and genetic prion diseases including genetic CJD associated to E200K and V210I mutations, GSS cases (P102L mutation) and FFI cases (D178N mutation). Statistically significant differences were observed between NC and all types of genetic prion diseases ($p<0.001$). Kruskal-Wallis test followed by Dunn's post-test was applied. (B) Demographic and biomarkers data from genetic prion disease cases. Number of cases (n), age in years (mean values ± standard deviation), sex (female (f)/males (m)), CSF tau levels (mean values ± standard deviation in pg/mL), presence of 14-3-3 in the CSF (positive (P), trace (T) and negative (N)) and NFL levels (mean values ± standard deviation in pg/mL) are indicated. (C) AUC derived from ROC curves, 95% CI and p value for NC vs genetic prion diseases. Optimal cut-offs (based on Youden Index), sensitivity and specificity were calculated.

Supplementary Table 1.

Number of cases from each diagnostic group and cohort used in the present study.

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