Title

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

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Abstract

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

METHODS: CSF NFL levels were quantified in non-primarily neurodegenerative neurological and psychiatric diseases (NPND, n=122), 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), sporadic Creutzfeldt-Jakob disease (sCJD, n=132) and genetic prion diseases (n=182).

RESULTS: The highest NFL levels were detected in sCJD, followed by VaD, FTD, DLB/PDD, AD and MCI. In sCJD, NFL levels correlated with CSF tau and disease duration. NFL levels were able to differentiate sCJD from NPND (AUC=0.99, 95%CI:0.99-1) and from the other diagnostic groups showing cognitive impairment/dementia (CI/DEM) of a non-CJD etiology (AUC=0.90, 95%CI:0.87-0.92). Compared to NPND, NFL was also elevated in genetic prion diseases associated with the E200K, V210I, P102L and D178N prion protein gene mutations.

DISCUSSION: Increased NFL levels are a common feature in neurodegenerative dementias.

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, including mild cognitive impairment (MCI), Alzheimer's disease (AD), vascular dementia (VaD), sporadic Creutzfeldt-Jakob disease (sCJD) the spectrum of frontotemporal lobar degeneration-related syndromes and motor neuron diseases [1–7]. Beyond its potential role as a diagnostic biomarker, NFL may be a useful marker for predicting progression of disease in FTD [6], disease severity and survival in FTD, AD and amyotrophic lateral sclerosis (ALS) [2,7–9]. Further, NFL enables the differentiation between disease subtypes in FTD and ALS [10,11]. In the context of neuroinflammation-mediated axonal injury, CSF NFL is a proven marker of treatment response to effective disease-modifying drugs [12,13].

NFL is mainly expressed in large-caliber myelinated axons [14], therefore, increased CSF NFL levels are generally associated with white mater involvement and damage in subcortical brain regions [15,16]. Additionally, the recent characterization of NF proteins in the postsynaptic terminal associated with terminal dendritic branches, where they play a role in neurotransmission [17], suggests a role for NFL as a surrogate marker of synaptic degeneration. Therefore, differential analysis of NFL concentrations in the spectrum of dementias might shed light into the specific pathological singularities among these conditions.

Alterations of CSF NFL are disease-type dependent [2,18] 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 and hampers its potential introduction into the clinical routine.

The aim of this study was to thoroughly investigate the utility of CSF NFL for the discrimination of neurodegenerative dementias of 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 for prion diseases.

METHODS

Study population

The study included 732 patients from six different centers. They were classified as: non-primarily neurodegenerative neurological and psychiatric diseases (NPND), mild cognitive impairment (MCI), Alzheimer's disease (AD), dementia with Lewy bodies/Parkinson's disease dementia (DLB/PDD), vascular dementia (VaD), frontotemporal dementia (FTD), sporadic Creutzfeldt-Jakob disease (sCJD)

and genetic prion diseases. Lumbar punctures (LPs) were performed for diagnostic purposes at the time of diagnosis.

For the analysis of the role of CSF NFL in the differential diagnosis of neurodegenerative dementias, two cohorts comprising NPND, MCI, AD, DLB/PDD, VaD, FTD and sCJD cases were used: cohort 1 (training cohort) and cohort 2 (validation cohort).

In cohort 1, the NPND group included the following diagnostic groups: psychosis, paranoid psychosis, bipolar disorder, schizophrenia, ischemic stroke, multiple infarct, cerebral vasculitis, epilepsy, meningitis, alcohol abuse, vertigo, acute or chronic headache, pain syndromes, acute hypoxia, vascular encephalopathy, vasculitis, Graves' disease, cerebral lymphoma, astrocytoma and paraneoplasia. Six cases showed subjective cognitive complaint: vascular encephalopathy; n=3, ischemic stroke; n= 2 and alcohol abuse; n=1.

In cohort 2, the NPND group included the following diagnostic groups: depression, acute or chronic headache, peripheral polyneuropathy and benign intracranial hypertension. NPND cases in cohort 2 showed no subjective cognitive complaint. NPND cases were diagnosed according to acknowledged standard neurologic clinical and para-clinical findings based on the ICD 10 definitions. The presence of neurodegenerative diseases in the NPND cohort was excluded in follow-up evaluations.

AD was diagnosed according to Dubois criteria [19] in cohort 1 and the National Institute on Aging - Alzheimer's Association workgroups (NIA-AA) criteria [20] were used in cohort 2. As Dubois criteria include~~s~~ in the same set of criteria both prodromal and dementia patients, and the NIA-AA criteria separate~~s~~ AD patients in dementia due to AD and MCI due to AD, in this study, patients from both cohorts were divided for the analysis according to their clinical phase in two groups: AD dementia, when symptoms were sufficiently severe to meet currently accepted dementia criteria, and MCI.

FTLD was diagnosed according to the International Behavioural Variant FTD Criteria Consortium for bvFTD [21]. MCI was diagnosed based on international criteria [22]. The diagnosis of DLB was based on the criteria of McKeith [23]. PDD diagnosis was based on those cases that were initially diagnosed as PD [24] and later developed dementia following task force of the Movement Disorder Society (MDS) criteria [25]. PDD was differentiated from other Parkinson-plus syndromes through the use of established diagnostic criteria for corticobasal degeneration [26], DLB [23], progressive supranuclear palsy [27] and multiple system atrophy [28]. VaD diagnosis was based on clinical and radiological criteria as described by Roman (National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association - NINDS-AIREN) [29]. All patients with sCJD were classified as probable (n=57) or definite (n=75) cases according to diagnostic consensus criteria [30,31]. For the study of NFL levels in genetic prion disease cases, six cohorts were used.

Detailed information on the number of cases in each diagnostic group is provided in Supplementary Table 1.

CSF tests

CSF NFL was centrally quantified (Clinical Dementia Center-Göttingen) using a commercially available enzyme-linked immunosorbent assay (NF-light; Uman-Diagnostics). The kit has been previously validated in a multicenter study showing good assay sensitivity and intra- and inter-assay precision [32]. 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 [33] in each of the participants labs. Total-tau(tau) was quantified centrally (Clinical Dementia Center-Göttingen) using the enzyme-linked immunosorbent assay kit INNOTEST®hTAU-Ag(Fujirebio), with the exception of tau levels in cohort 2, which were locally measured using the same commercial kit and analytical procedures.

Genetic tests

For the detection of prion disease–associated mutations and assess codon 129 polymorphism in the prion protein gene (*PRNP*), genetic testing was performed as described before [34].

Statistical tests

Mann-Whitney U tests were used to compare two groups of samples. For multiple comparisons, Kruskal-Wallis tests followed by Dunn's post-hoc were applied. 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 (95%CI) were calculated using GraphPad-Prism6.01.

AUCs were calculated using a combination of the participants of the training and validation cohorts, to increase precision in case there was no heterogeneity of results between the respective cohorts. The best cut-off value was then estimated based on the Youden index derived from the training cohort; the diagnostic accuracy (sensitivity and specificity) of NFL in all cases was externally validated in the validation cohort. Spearman rank correlation coefficients were used to assess associations between continuous biomarker levels. 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 following Cox regression models 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-11/11/93 (+Amendments) and 9/6/08).

RESULTS

NFL in the differential diagnosis of neurodegenerative dementia

CSF NFL concentrations were initially assessed in the training cohort (cohort 1) comprising NPND and cases covering a broad spectrum of neurodegenerative disorders. Among the clinical diagnoses, highest NFL levels were detected in sCJD, followed by VaD, FTD, DLB/PDD, AD, MCI and NPND (Figure 1A and 1C). NFL concentration was significantly different in: NPND vs AD, AD vs FTD,

DLB/PDD vs VaD ($p<0.05$), in NPND vs DLB/PDD ($p<0.01$) and in NPND vs VaD, NPND vs FTD, NPND vs sCJD, MCI vs sCJD, AD vs VaD, AD vs sCJD, DLB/PDD vs sCJD ($p<0.001$) (Figure 1A). Data from training cohort were replicated in a validation cohort (cohort 2), showing a similar NFL levels pattern across clinical diagnoses compared to the training cohort (Figure 1B and 1C). Tau levels and 14-3-3 positive proportions for each diagnostic group (Figure 1C), were in agreement with those previously reported [35–37].

To determine the diagnostic accuracy of NFL quantification in the differential diagnostic context of neurodegenerative dementias, AUCs derived from both cohorts were calculated (Figure 1D). sCJD (AUC=0.99, 95%CI: 0.99-1), VaD (AUC=0.94, 95%CI: 0.90-0.98) and FTD (AUC=0.86, 95%CI: 0.79-0.93) cases were discriminated from NPND with high accuracy. In contrast, NFL levels showed worse performance in distinguishing DLB/PDD, AD and MCI from NPND (AUC<0.8 in all comparisons). Additionally, NFL displayed excellent accuracy in discriminating sCJD from MCI, AD and DLB/PDD (AUC>0.9 in all comparisons) and moderate accuracy in discriminating sCJD from FTD (AUC=0.83, 95%CI: 0.75-0.91) and VaD (AUC=0.76, 95%CI: 0.68-0.85). Finally, VaD could be discriminated with good accuracy, not only from MCI (AUC=0.83, 95%CI: 0.75-0.91), but also from AD (AUC=0.80, 95%CI: 0.73-0.87).

Diagnostic accuracy of NFL in the discrimination of sCJD cases

As highest NFL levels were detected in sCJD, we sought to determine the diagnostic performance of NFL quantification in the discrimination of sCJD from NPND and dementias of a non-prion etiology. For this purpose, different types of dementias (AD, DLB/PDD, VaD and FTD) and MCI cases from cohort 1 and 2 were grouped under the cognitive impairment/dementia (CI/DEM) label. Mean NFL levels were significantly higher in sCJD (31456±21243pg/mL) compared to CI/DEM (7971±10653pg/mL) and NPND (2138±1532pg/mL) ($p<0.001$). NFL levels were also significantly increased in the CI/DEM group compared to those detected in the NPND group ($p<0.001$). The diagnostic accuracy (AUC) of NFL quantification in the discrimination of sCJD cases was 0.99 (95%CI: 0.99-1) for the NPND vs sCJD comparison and 0.90 (95%CI: 0.87-0.93) for the CI/DEM vs sCJD comparison. A cut-off of 7000pg/mL determined in the training cohort revealed 100% sensitivity and 95% specificity in the discrimination of sCJD from NPND cases when applied to the validation cohort. Moreover, a cut-off of 10500pg/mL determined in the training cohort allowed discrimination of sCJD from non-CJD dementia cases with a sensitivity of 86% and a specificity of 80% when applied to the validation cohort (Figure 2B).

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

NFL levels in sCJD cases were neither affected by age at onset (ranging from 31 to 89 years old) ($p=0.71$) (Figure 3A) nor by the sex of the patients (76 females and 56 males) ($p=0.91$) (Figure 3B). To test whether patients genetic characteristics were associated with differential NFL levels, we stratified sCJD samples by *PRNP* codon 129 genotype, a well-known modifier of biomarkers' accuracy in prion diseases [38,39]. Information regarding codon 129 was available for 104 cases.

Mean NFL values were higher in sCJD valine/valine [VV] (42838±20192pg/mL) compared to methionine/methionine [MM] (29144±21141pg/mL) and methionine/valine [MV] (21402±14235pg/mL) cases ($p<0.01$) (Figure 3C). In contrast, CSF tau levels were lower in [MV] cases compared to [MM] and [VV] patients ($p<0.01$) in agreement with previous reports [39].

Correlation of NFL levels with surrogate prion biomarkers

The association of NFL levels with CSF 14-3-3 and tau, two surrogate markers of prion disease used in routine clinical practice [40], was studied. Elevated NFL levels were detected in sCJD cases tested positive for 14-3-3 (32228±21262pg/mL) compared to those that tested negative or inconclusive (17404±9099pg/mL) ($p=0.015$) (Figure 3D). Additionally, a positive but weak correlation was observed between CSF tau and NFL levels (Spearman's rho=0.39,$p<0.001$) (Figure 3E).

NFL quantification as prognostic marker in sCJD

Subsequently, we assessed the influence of CSF sampling time on NFL concentration in sCJD patients. 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 for each patient by the total disease duration. Samples were classified 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 [41,42]. NFL levels were either non-altered or increased in the second LP (Figure 4A). As alterations in biomarkers profile may depend on the duration of the disease, reflecting the stage of 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, (the mean disease duration of our population study [43]), no significant differences on NFL concentrations were detected between serial LPs. In contrast, for cases showing disease duration longer than 6 months, NFL levels were statistically increased in the consecutive LP (Figure 4B). In addition we investigated the potential association between NFL levels at the time of the first LP and disease duration. Our data revealed a strong non-linear association between NFL values and survival time (NFL could be modelled as a linear combination of the terms 206*(survival time) and -7057*(survival time)$^{-2}$)(n=185) (Figure 4C). NFL showed a moderate ability as a prognostic marker, 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 levels in the discrimination of genetic prion diseases, which constitute about 10-15% of all human prion diseases [44]. Therefore, to provide statistical power to our study, we analyzed genetic prion diseases samples associated to the *PRNP* mutations E200K (n=83), V210I (n=35), P102L (n=10) and D178N (n=84) from six different unrelated cohorts. Highest NFL levels were detected in genetic CJD (gCJD) associated with the E200K mutation, followed by Fatal Familial Insomnia (FFI) - D178N mutation, Gerstmann-Sträussler-Scheinker syndrome (GSS-S) associated with the P102L mutation and gCJD associated with the V210I mutation

(Figure 5A). No significant differences among the four mutation groups were detected (Figure 5B). Tau and 14-3-3 levels followed the previously reported diagnostic parameters for these mutations [45] (Figure 5A).

In all types of genetic prion disease, increased NFL levels were detected compared to NPND ($p<0.001$) (Figure 5B). However, NFL concentrations in genetic prion diseases were lower than those detected in sCJD (Figure 5B). Although NPND cases presented a higher age at onset compared to P102L and D178N cases ($p<0.05$), no association between NFL levels and age at disease onset was detected, neither for NPND nor for any type of genetic prion diseases ($p>0.05$). Analysis of diagnostic parameters showed an excellent sensitivity and specificity of NFL in the discrimination of genetic prion diseases from NPND. AUC values were higher than 0.94 ($p<0.001$) for all mutations (Figure 5C). Disease-specific cut-offs calculated according to Youden index revealed sensitivities and specificities ranging from 87-100% and 86-96%, respectively (Figure 5C). Interestingly, NFL could discriminate D178N (FFI) and P102L (GSS-S) cases from NPND with high accuracy, in contrast to alternative prion biomarkers [35,45,46] (Figure 5C).

DISCUSSION

The clinical diagnosis of neurodegenerative dementias is often challenging due to 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 estimated.

In the present study, the spectrum of neurodegenerative dementias for which information on NFL levels is available was expanded, providing solid data on diagnostic accuracy values. Importantly, data from our training cohort were replicated in an independent validation cohort, underscoring their precision, robustness and reproducibility, which allowed 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. The highest NFL concentrations were found in the CSF of sCJD followed by FTD and VaD cases. AD and DLB/PDD cases also showed increased NFL levels compared to controls, but their discriminatory value was limited. Although elevated NFL concentrations in sCJD compared to controls [3] and AD cases [1] have been recently reported, no data were available comparing sCJD to other dementias groups with overlapping clinical symptoms.

While a strong correlation between CSF NFL and tau was reported in other dementias such as AD [7], we detected a weak one in sCJD. This observation, together with the differential alterations in tau and NFL levels in sCJD cases relative to the *PRNP* gene codon 129 genotype, suggests that tau and NFL may reflect partially overlapping outcomes associated to prion disease pathology.

In this regard, white matter degeneration in sCJD has been assumed [47] and recently, alterations in subcortical tracts have been identified not only in sCJD, but also in FFI, using functional magnetic resonance imaging (MRI), providing quantitative evidence of white matter involvement in prion diseases [48]. Importantly, the observation that sCJD VV2 cases show higher subcortical pathology compared to other subtypes [31], is in agreement with the elevated CSF NFL levels in sCJD VV observed in our study, including mainly sCJD VV2 cases (20 out of 21 sCJD VV cases with known protein type were VV2). On the other hand, the recent finding that NFL is also expressed in synapses [17] suggests that, in sCJD, CSF NFL might additionally reflect massive synaptic degeneration and neuronal damage, according to its positive association with CSF tau and 14-3-3. In contrast, and as broadly reported not only in sCJD but also in other neurodegenerative diseases such as AD, CSF tau probably reflects the degree of neuroaxonal injury or degeneration, which in turn correlates with disease severity [49,50].

Elevated NFL concentrations in VaD and FTD detected in our study are also in agreement with previous observations [2] potentially indicating extensive neuroaxonal damage in white matter and subcortical brain structures. Indeed, white matter involvement is one of the most common neuropathological features in VaD [51] and it is estimated that subcortical changes on MRI are detected in 82% of the cases [52]. Additionally, white mater pathology in frontotemporal lobar degeneration is widely described [53]. Since elevated NFL in FTD correlates with decreased gray and white matter volume [8], it has been recently suggested that NFL reflects corticospinal tract degeneration [54].

It is worth to mention that, based on neuropathological studies, the precise contribution of CSF NFL and tau reflecting neuronal and/or white matter alterations in different pathological conditions cannot be delineated. Indeed, no comparative studies reporting synaptic loss, neuronal and white matter degeneration in the spectrum of dementia syndromes are available to date.

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

Although the NPND group includes various conditions in which axonal damage and white matter involvement is quite common, we observed that NFL quantification is 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 its capability to discriminate sCJD from control cases [40,46,55,56]. Indeed, while NFL measurement successfully detect sCJD cases with full sensitivity and a specificity of 95%, 14-3-3 protein, one of the gold-standard biomarkers in prion diseases, reached a sensitivity of 93% and a specificity of 87%. A lower, yet significant, diagnostic accuracy was achieved in the discrimination of

sCJD from the combined group of dementias (CI/DEM) utilizing CSF NFL. Therefore, due to the partial overlap of CSF NFL levels between the different diagnostic groups evaluated in our study, the role of NFL as a diagnostic marker in the differential context of dementia should be considered with caution.

In recent studies, CSF NFL levels have been associated with cognitive deterioration and disease severity in AD and FTD [4,6,7]. However, no correlations could be established in our cohort as cognitive status was not available. This limitation is common in studies with sCJD cases, where the rapid course of the disease, often impedes the quantification of cognitive decline rates. However, a relevant finding from our study is the potential prognostic value displayed by NFL in sCJD cases, since its levels were negatively correlated with disease duration, as previously shown in other dementia-types, especially AD [2]. Other prion biomarkers such as tau and α-synuclein also show a prognostic value for in sCJD cases [42,57,58], but data for 14-3-3 [59,60] and RT-QuIC [56,61] are inconsistent. It should be noted that the prognostic accuracy of NFL as demonstrated here by the Brier score and Somers'*D* is only in a modest range. Thus, further studies are needed to confirm the usefulness of NFL as prognostic marker in prion diseases.

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 in typical sCJD cases [62]. Consequently, the diagnostic value of classical CSF prion biomarkers in gCJD is in range with that reported for sCJD. On the contrary, the diagnostic value of tau, 14-3-3, α-synuclein and RT-QuIC in discriminating FFI and GSS-S cases is poor [45,46,55], likely due to a more restricted pathology and/or to a longer disease duration compared to sporadic cases. Furthermore, the low prevalence of these diseases 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 elevated NFL levels in the four types of mutations analyzed. The most salient finding is the increased NFL concentration in FFI and GSS-S (P102L), for which no biochemical or imaging biomarker is currently able to discriminate these cases with high diagnostic accuracy [45,63].

GSS-S and FFI present a different clinical outcome than that generally observed in sCJD and in the dementia syndromes included in our study. Characteristic clinical symptoms in FFI are insomnia, sleep fragmentation, altered arousal and a state of dreamlike automatism during the day, together with dysautonomia [63,64]. This is followed by cognitive impairment and motor disorders leading to terminal dementia. Additionally, disease duration in these disorders is prolonged compared to that of sCJD, and signs typical of CJD are either absent or seen only late in the disease course. Similarly, the clinical spectrum of GSS-S is more diverse than that of sCJD, including slowly progressive cerebellar ataxia and late cognitive decline [65]. Therefore, CSF NFL quantification could be an appropriate test in the differential diagnosis of GSS-S and FFI cases from other alternative neurological conditions presenting a clinical overlap with these two types of genetic prion diseases (i.e: sleep disturbances

with cognitive complaints in FFI and dysarthria, ataxia and/or gait disturbance with muscle atrophy in GSS-S (P102L)).

In conclusion, our data unequivocally demonstrates the presence of increased CSF NFL levels in the spectrum of neurodegenerative dementias analyzed in this study, with highest values in sCJD, and a considerable overlap among diagnostic groups. Additionally, we report a potential diagnostic role of CSF NFL for genetic prion diseases, such as FFI and GSS-S (P102L), for which no biomarkers are currently available.

Acknowledgements

We thank Silja Koechy for excellent technical assistance. This study was funded by the Spanish Ministry of Health - Instituto Carlos III (Miguel Servet programme- CP16/00041) to FL, by the IKYDA Greek-German collaboration project 57260006 to FL, IZ and TS, by the Robert Koch Institute through funds from the Federal Ministry of Health (grant no. 1369–341) to IZ, by the Spanish Ministry of Health, Instituto Carlos III (Fondo de Investigación Sanitaria - FIS PI11/00968, FIS PI14/00757) to IF, by FIS PI16/00235 to RSV, by CIBERNED project BESAD-P to IF, and by the Red Nacional de priones (AGL2015-71764-REDT- MINECO) to FL, IZ, IF and RS-V. AV-P is funded by a Dorothea Schlözer Scholarship (Georg August University – Göttingen).

Authors' contributions

IZ and FL designed the study. IZ, MS, AK, AV-P, IB, and FL performed experiments, analyzed data and interpreted the results. EK, EG, DD-L and KK performed experiments. IZ, EK, EG, PH, TK, SG, DV, TS, BS, PPL, IS, IF, HZ, KB, OC, MC, AL, RS-V and IB 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 related to the present study.

FIGURE LEGENDS

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

NFL levels in non-primarily neurodegenerative neurological and psychiatric diseases (NPND), mild cognitive impairment (MCI), Alzheimer's disease (AD), dementia with Lewy bodies/Parkinson's disease dementia (DLB/PDD), vascular dementia (VaD), frontotemporal dementia (FTD) and sporadic Creutzfeldt-Jakob disease (sCJD) in training cohort (cohort 1) (A) and validation cohort (cohort2) (B). In the training cohort NFL levels were significantly different between: NPND vs AD, AD vs FTD, DLB/PDD vs VaD ($p<0.05$), in NPND vs DLB/PDD ($p<0.01$) and in NPND vs VaD, NPND vs FTD, NPND vs sCJD, MCI vs sCJD, AD vs VaD, AD vs sCJD, DLB/PDD vs sCJD ($p<0.001$). In the

validation cohort, NFL levels were significantly different between: NPND vs MCI, NPND vs VaD, NPND vs FTD, AD vs sCJD ($p<0.05$) and in NPND vs sCJD, MCI vs sCJD, DLB/PDD vs sCJD, FTD vs sCJD ($p<0.001$). Kruskal-Wallis test and Dunn's post-hoc test (correction for multiple testing) 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), 14-3-3 presence 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) Area Under the Curve (AUC) derived from Receiver Operating Characteristic curves (shown below diagonal line) and 95% CI (shown above diagonal line) for all comparisons between pairs of diagnostic groups derived from the study and validation cohorts.

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

(A) NFL levels in non-primarily neurodegenerative neurological and psychiatric diseases (NPND), cases with a diagnosis of cognitive impairment/dementia (CI/DEM) of a non-prion etiology 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 between: sCJD vs NPND, sCJD vs CI/DEM and NPND vs CI/DEM cases (*** $p<0.001$). Kruskal-Wallis test and Dunn's post-hoc test (correction for multiple testing) was applied. (B) ROC curve for NFL in the comparative analysis for ND vs sCJD cases and CI/DEM vs sCJD comparisons. Area Under the Curve (AUC) derived from Receiver Operating Characteristic curves, 95% CI and p value for NPND vs sCJD and CI/DEM vs sCJD comparisons. Optimal cut-offs (based on Youden Index), derived from the study cohort, sensitivity and specificity as calculated in the validation cohort.

Figure 3. 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. Spearman rank correlation was used. (B) NFL levels in sCJD stratified by sex. Mann-Whitney U test was used. (C) NFL levels in sCJD stratified by prion protein gene (*PRNP)* codon 129 polymorphism (M = Methionine, V = Valine). Kruskal-Wallis test followed by Dunn's post-test (correction for multiple testing) 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 testing outcomes. Mann-Whitney U test was used (* $p<0.01$). (E) Correlation analysis between CSF NFL and tau levels in sCJD cases (Spearman's rho=0.39; $p<0.001$).

Figure 4. Association between CSF NFL levels and disease duration in sCJD patients.

(A) NFL levels in serial lumbar punctures (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 5. Diagnostic accuracy of CSF NFL as a biomarker for genetic prion diseases.

(A) 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), 14-3-3 presence in the CSF (positive (P), trace (T) and negative (N)) and NFL levels (mean values ± standard deviation in pg/mL) are indicated. (B) NFL levels in non-primarily neurodegenerative neurological and psychiatric diseases (NPND), sCJD and genetic prion diseases including genetic CJD associated to E200K and V210I mutations, Gerstmann-Straussler-Scheinker syndrome (GSS-S) (P102L mutation) and fatal familial insomnia (FFI) (D178N mutation) cases. Statistically significant differences were observed between NPND and all types of genetic prion diseases (***$p<0.001$) as well as between sCJD and genetic CJD associated to E200K (*$p<0.05$) and V210I (***$p<0.001$) mutations, Gerstmann-Straussler-Scheinker syndrome (GSS-S) (P102L mutation) (***$p<0.001$) and fatal familial insomnia (FFI) (D178N mutation) (***$p<0.001$) cases. Kruskal-Wallis test followed by Dunn's post-test (correction for multiple testing) was applied. (C) AUC derived from ROC curves, 95% CI and p value for NPND 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. NPND: non-primarily neurodegenerative neurological and psychiatric diseases, MCI: mild cognitive impairment, DLB/PDD: dementia with Lewy bodies/Parkinson's disease dementia, VaD: vascular dementia, FTD: frontotemporal dementia, sCJD: sporadic CJD.

For the analysis of the role of CSF NFL in the differential diagnosis of neurodegenerative dementias, two cohorts comprising NPND, MCI, AD, DLB/PDD, VaD, FTD and sCJD cases were used. Cohort 1 (training cohort) cases were collected at the Clinical Dementia Center and the National Reference Center for CJD Surveillance at the University Medical Center of Göttingen (Germany). Cohort 2 (validation cohort) cases were collected at the Dementia Clinic, Neurology Department of Coimbra-University Hospital Portugal and at the National Referral Center for CJD (Portugal).

For the study of NFL levels in genetic prion disease cases, six cohorts were used: the previously mentioned cohorts 1 and 2; cohort 1 (Clinical Dementia Center and the National Reference Center for CJD Surveillance at the University Medical Center of Gottingen-Germany) and cohort 2 (National Referral Center for CJD, Coimbra-Portugal), plus four additional cohorts: cohort 3 (Medical University of Lodz-Poland), cohort 4 (Alzheimer's Disease and Other Cognitive Disorders Unit, Hospital Clínic, Barcelona, Spain), cohort 5 (National Centre of Microbiology-Carlos III Institute of Health, Madrid-Spain) and cohort 6 (Istituto Superiore di Sanità, Rome-Italy).

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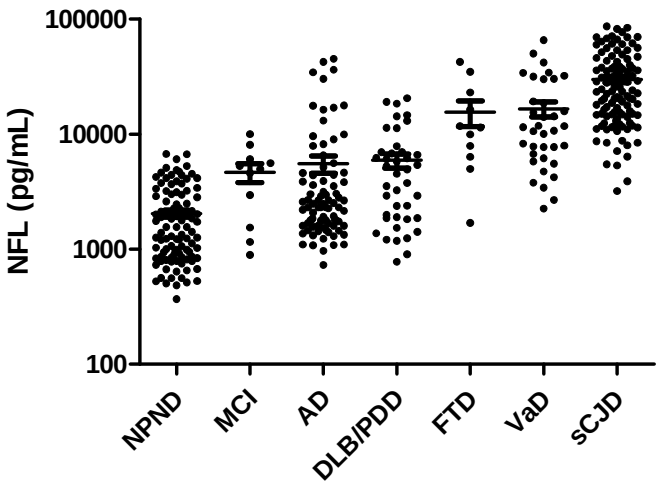
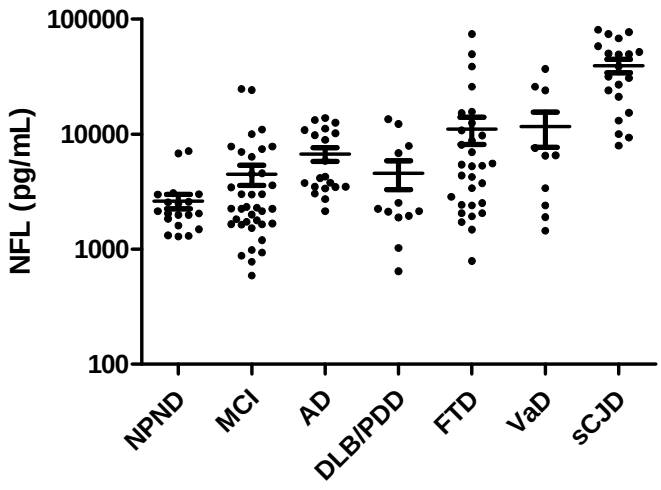
Figure 1

A



B



C

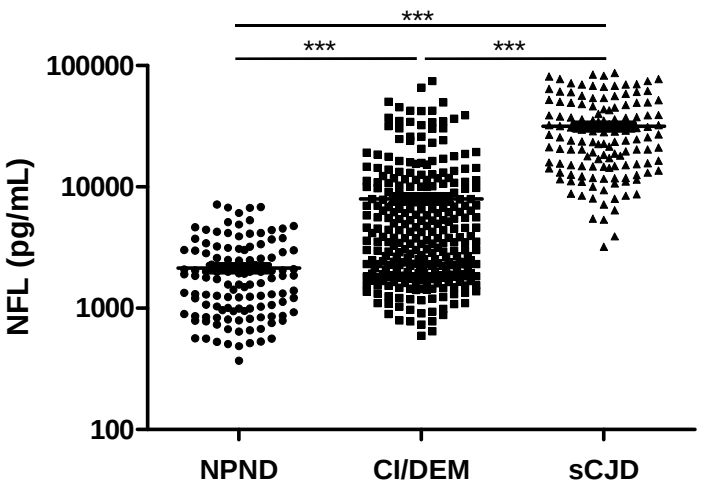
	n	age (years)	gender (f/m)	tau (pg/mL)	14-3-3 (P/T/N)	NFL (pg/mL)
Study cohort						
NPND	103	67 ± 11	58/45	221 ± 145	7/7/89	2049 ± 1512
MCI	11	68 ± 8	5/6	308 ± 192	1/0/10	4663 ± 2885
AD	88	71 ± 11	50/37	577 ± 522	7/6/75	5538 ± 8887
DLB/PDD	41	72 ± 10	24/17	385 ± 343	4/2/35	5926 ± 5323
FTD	11	59 ± 12	7/4	297 ± 201	0/9/2	15576 ± 12951
VaD	36	71 ± 10	21/15	423 ± 412	4/3/29	16664 ± 14848
sCJD	112	66 ± 10	68/45	9145 ± 8075	101/4/7	30016 ± 20495
Validation cohort						
NPND	19	67 ± 10	10/9	151 ± 56	NA	2402 ± 1789
MCI	37	68 ± 9	17/20	494 ± 219	NA	4483 ± 5517
AD	20	70 ± 6	8/12	604 ± 306	NA	7298 ± 5409
DLB/PDD	12	68 ± 9	5/7	263 ± 204	NA	4595 ± 4463
FTD	30	67 ± 8	13/17	328 ± 193	NA	10832 ± 15215
VaD	10	67 ± 9	3/7	330 ± 235	NA	11672 ± 12565
sCJD	20	69 ± 7	8/12	11806 ± 9876	15/5/0	42404 ± 23690

D

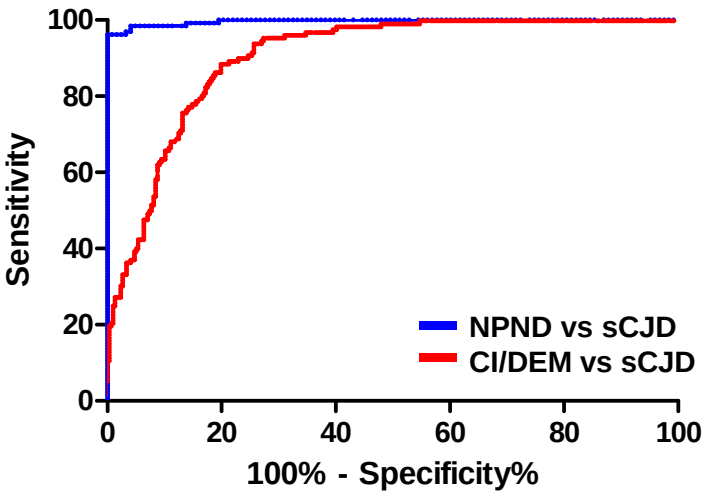
	NPND	MCI	AD	DLB/PDD	FTD	VaD	sCJD	95% CI
NPND		0.59 - 0.77	0.64 - 0.78	0.66 - 0.82	0.79 -0.93	0.90 - 0.98	0.99 - 1	
MCI	0.68 ± 0.05		0.42 - 0.63	0.46 - 0.68	0.61 - 0.83	0.75 - 0.91	0.93 - 0.99	
AD	0.71 ± 0.03	0.53 ± 0.05		0.45 - 0.65	0.61 - 0.79	0.73 - 0.87	0.90 - 0.97	
DLB/PDD	0.74 ± 0.04	0.57 ± 0.06	0.55 ± 0.05		0.54 - 0.76	0.68 - 0.86	0.91 - 0.98	
FTD	0.86 ± 0.03	0.72 ± 0.05	0.70 ± 0.05	0.65 ± 0.06		0.49 - 0.73	0.75 - 0.91	
VaD	0.94 ± 0.02	0.83 ± 0.04	0.80 ± 0.04	0.77 ± 0.05	0.61 ± 0.06		0.68 - 0.85	
sCJD	0.99 ± 0.002	0.96 ± 0.01	0.94 ± 0.02	0.94 ± 0.02	0.83 ± 0.04	0.76 ± 0.04		
AUC								

Figure 2

A



B



	NPND vs sCJD	CI/DEM vs sCJD
AUC (Area ± SE)	0.9966 ± 0.0020	0.9008 ± 0.0143
95% CI	0.9925 to 1	0.8726 to 0.9289
p value	< 0.0001	< 0.0001
Cut-off (pg/mL)	> 7000	> 10500
Specificity (%)	95	80
Sensitivity (%)	100	86

Figure 3

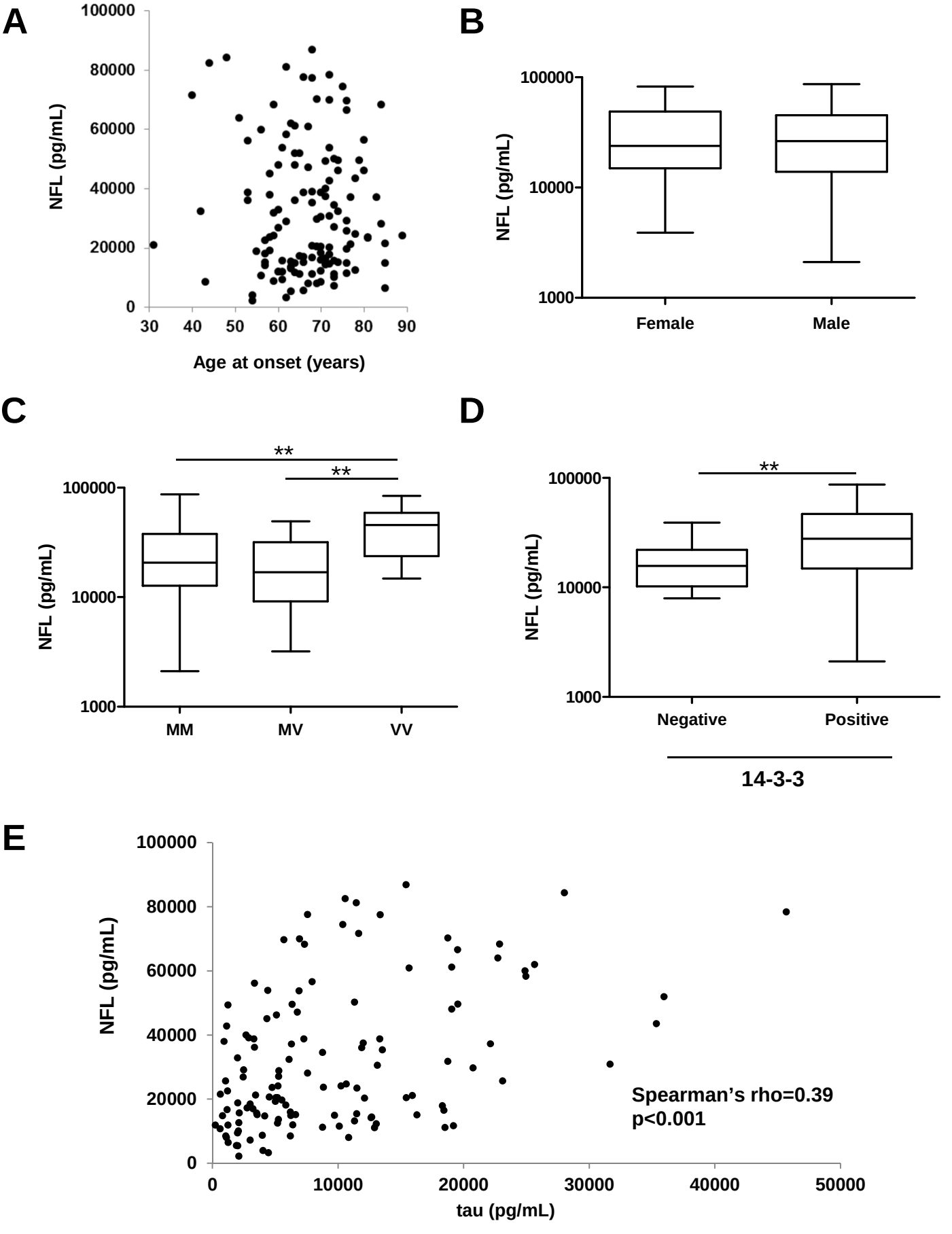

A
100000
80000
60000
40000
20000
0
NFL (pg/mL)
30
40
50
60
70
80
90
Age at onset (years)
B
100000
10000
1000
NFL (pg/mL)
Female
Male
C
**
**
100000
10000
1000
NFL (pg/mL)
MM
MV
VV
D
**
100000
10000
1000
NFL (pg/mL)
Negative
Positive
14-3-3
E
100000
80000
60000
40000
20000
0
NFL (pg/mL)
0
10000
20000
30000
40000
50000
tau (pg/mL)
Spearman's rho=0.39
p<0.001

Figure 4

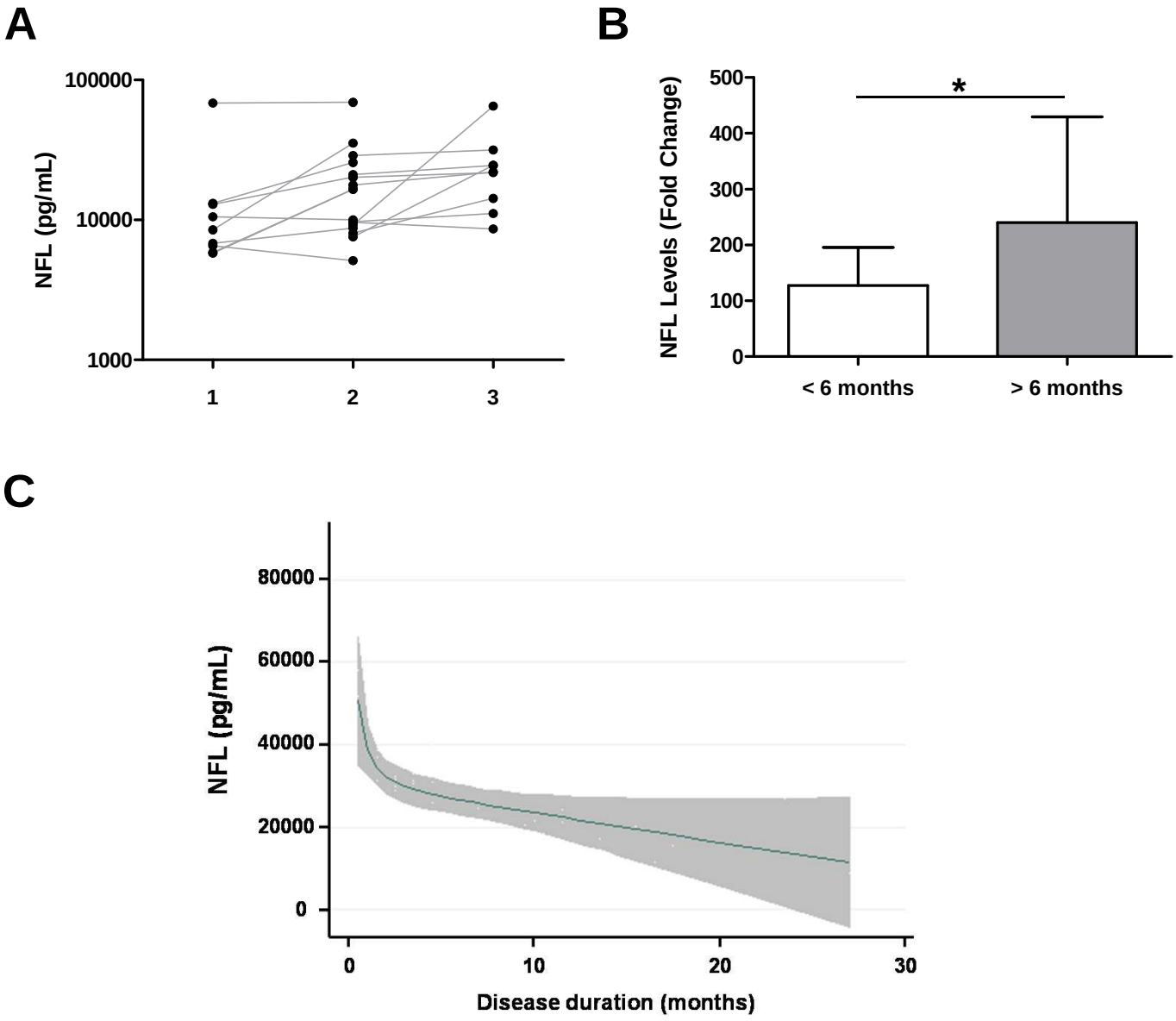
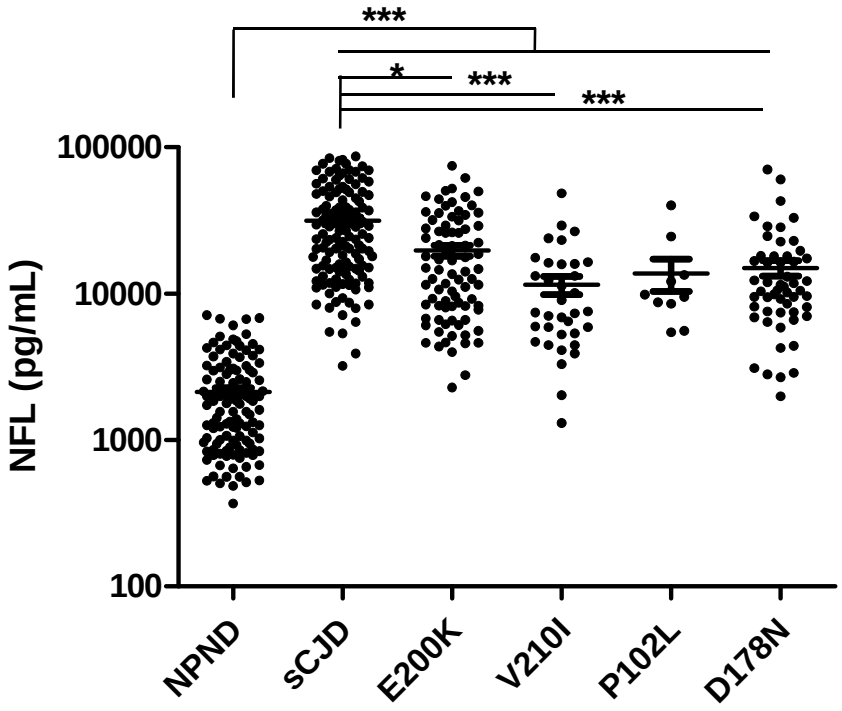

A
100000
10000
1000
NFL (pg/mL)
1
2
3
B
500
400
300
200
100
0
NFL Levels (Fold Change)
*
< 6 months
> 6 months
C
80000
60000
40000
20000
0
NFL (pg/mL)
0
10
20
30
Disease duration (months)

Figure 5

A

	n	age (years)	gender (f/m)	tau (pg/mL)	14-3-3 (P/T/N)	NFL (pg/mL)
E200K -gCJD	83	60 ± 10	50/33	6012 ± 5855	68/6/9	19760 ± 15485
V210I - gCJD	35	65 ± 10	20/15	8966 ± 7908	28/5/2	11494 ± 9615
P102L - GSS-S	10	53 ± 12	5/5	2865 ± 2018	2/0/8	13798 ± 10729
D178N - FFI	54	52 ± 11	30/24	833 ± 978	5/1/48	15028 ± 13220

B



C

	NPND vs E200K	NPND vs V210I	NPND vs P102L	NPND vs D178N
AUC (Area ± SE)	0.9814 ± 0.0070	0.9438 ± 0.0221	0.9919 ± 0.0665	0.9673 ± 0.0128
95% CI	0.9676 to 0.9951	0.9005 to 0.9871	0.9788 to 1	0.9420 to 0.9925
p value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Cut-off (pg/mL)	> 4560	> 3900	> 5380	> 5580
Specificity (%)	92	86	96	96
Sensitivity (%)	95	91	100	87

Supplementary Table 1

	Cohort 1 Germany	**Cohort 2** Portugal	**Cohort 3** Poland	**Cohort 4** Spain (Barcelona)	**Cohort 5** Spain (Madrid)	**Cohort 6** Italy
NPND	103	19				
MCI	11	37				
AD	88	20				
DLB/PDD	41	12				
VaD	36	10				
FTD	11	30				
sCJD	112	20				
genetic Prion Disease	87	4	6	15	25	45