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Head-to-head comparison of four cerebrospinal fluid and three plasma neurofilament light chain assays in Parkinsonism

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Neurofilament light chain protein (NfL) is a valuable biomarker for the differential diagnosis between Parkinson's disease (PD) and atypical parkinsonian disorders (APD). Here, we compared the performance of four cerebrospinal fluid (CSF) and three plasma NfL immunoassays in 253 PD and 265 APD. We measured NfL by ELISA in CSF and by SiMoA, CLEIA, and ELLA in both CSF and plasma. Additionally, we assessed Lewy body pathology by CSF α-synuclein real-time quaking-induced conversion assay (α-syn-RT-QuIC). In each biofluid, the tested assays showed comparable precision; however, CSF NfL showed higher diagnostic accuracy than plasma NfL for discriminating PD from APD (AUC range 0.966–0.974 vs 0.917–0.924). Combining CSF NfL and α-syn-RT-QuIC increased diagnostic accuracy. These results confirm the high diagnostic value of NfL in patients with parkinsonism, even when different assays are used. Combining CSF NfL and α-syn-RT-QuIC provides the highest accuracy, followed by CSF NfL and plasma NfL.

The neurofilament light chain protein (NfL), expressing neuroaxonal injury, is an established valuable biofluid marker supporting the differential diagnosis between Parkinson's disease (PD) and atypical parkinsonian disorders (APD), the latter including multiple system atrophy (MSA), progressive supranuclear palsy (PSP), and corticobasal syndrome (CBS). In contrast to PD, APD patients manifest a significant increase in NfL levels since the early disease stage, allowing their discrimination[1–5].

Following the application of enzyme-linked immunosorbent assays (ELISA) to measure NfL in cerebrospinal fluid (CSF), the development of the ultrasensitive technology single molecule array (SiMoA) detecting protein concentrations up to the femtomolar/sub picogram range allowed for the first time the accurate measurement of NfL in blood and showed a significant correlation of the biomarker levels in the two biofluids[6]. More recently, however, other automated platforms exploiting chemiluminescent enzyme immunoassay (CLEIA) or ELISA (ELLA) have recently become available to measure NfL in blood, creating the foundation for the widespread use of the biomarker in clinical practice.

However, to date, no study has systematically compared the performance of the available assays in both matrices in a cohort with homogeneous preanalytical sample handling and standardized clinical measures. Moreover, the initial results on the high diagnostic value of NfL across neurodegenerative Parkinsonism, and especially the comparison of the biomarker performance in CSF and blood, still need to be extended to sizeable cohorts before translating the test to the clinical setting.

Here, we performed a head-to-head comparison of four CSF and three plasma NfL assays in a large, monocentric cohort representative of the full spectrum of neurodegenerative Parkinsonism. We assessed each assay's ability to discriminate PD from APD. Additionally, we expanded initial findings on the added diagnostic value of combining CSF NfL dosage with the α-synuclein (α-syn) real-time quaking-induced conversion (RT-QuIC) assay, an established biomarker of Lewy body pathology.

Results

Participants

Of the 585 participants with Parkinsonism, 231 (39.3%) were women, and their mean age at biofluid sampling was 64.8 ± 9.7 years. Males were more frequent in the PD/PD cognitively impaired (PD-CI) cohort compared with

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the other groups ($P < 0.0001$), and patients with PD-CI, and PSP/CBS were significantly older than those with PD and MSA ($P < 0.001$ for all comparisons). The baseline demographic and clinical features of the study cohort and biomarker levels are summarized in Table 1. NfL was significantly associated with age in both CSF and plasma (β range, 0.120-0.385, $P < 0.01$ for all analyses), and with body mass index (BMI, β range, −0.153 to −0.184, $P < 0.01$ for all analyses), estimated Glomerular Filtration Rate (eGFR β range, −0.311 to −0.334, $P < 0.001$ for all analyses), and chronic kidney disease (CKD) (odd ratio range, 1.03–1.07, $P < 0.001$ for all analyses) in plasma. Additionally, CSF and plasma NfL levels were associated with disease duration (β range, 0.262-0.305, $P < 0.001$), Mini-Mental state examination score (MMSE: β range, −0.220 to −0.313, $P < 0.01$), Unified Parkinson's Disease Rating Scale-section III score (β range, 0.329–0.457, $P < 0.01$) and Hoen & Yahr stage (β range, 0.242–0.355, $P < 0.01$) in the PD and PD-CI groups, independently from used assay. Finally, there were no significant associations with diabetes, history of cerebrovascular and/or cardiovascular disease and biomarker levels for both biofluids.

Correlations of NfL values between assays and biofluids

We first compared the NfL values measured by the different assays in each biofluid. Then, we explored the correlation between CSF and plasma NFL values obtained by each assay (i.e., NfL^{CLEIA}, NfL^{SIMOA}, and NfL^{ELLA}) (Fig. 1).

There was a high concordance between NfL levels measured by different assays in CSF (ρ range, 0.965–0.982) and, to a lesser extent, in plasma (ρ range, 0.907–0.931). In contrast, the correlation between CSF and plasma NfL values (ρ range, 0.747–0.775) was moderate overall and weaker in MSA than in the other groups.

Diagnostic value of CSF and plasma NfL in the differential diagnosis of patients with parkinsonism

Independent of the assay used, patients with APD showed significantly higher CSF and plasma NfL levels than those with PD and PD-CI. Individuals with MSA showed higher mean CSF values than those with PSP/CBS, but the difference was no longer evident in plasma (Fig. 2).

ROC curve analysis demonstrated high accuracy for both CSF NfL and plasma NfL in distinguishing PD from APD with no significant differences between assays in CSF (AUC range, 0.966–0.974, $P > 0.05$ for all comparisons) and plasma (AUC range, 0.917–0.924, $P > 0.05$ for all comparisons) (Fig. 3, Tables 2 and 3).

Similarly, different CSF and plasma assays performed similarly in discriminating between PD and APD groups, and all reached the highest accuracy in distinguishing PD and MSA.

Compared to cognitively unimpaired PD, the diagnostic value of NfL, especially in plasma, was lower in discriminating PD-CI from the other groups (Tables 2 and 3). Consistently, merging the PD and PD-CI groups reduced the accuracy of all measures (PD/PD-CI vs APD: ΔAUC range, −0.010 to −0.014 in CSF; −0.026 to −0.031 in plasma).

Detailed diagnostic performances and cut-offs for each CSF and plasma NfL assay are reported in Table 4.

Diagnostic value of combining CSF NfL and α-syn RT-QuIC in parkinsonism

In line with previous studies[5,7], the α-syn RT-QuIC assay showed high sensitivity in PD and PD-CI patients (249 positives out of 272, 91.5%) while showing a negative response in most MSA and PSP/CBS participants (5 positives out of 241, 2.1%) (raw data of aggregation kinetics in Supplementary Figure 1 and Supplementary Table 1). Following a previously described approach,[53] we evaluated the diagnostic value of the combined CSF NfL and α-syn RT-QuIC analysis. We found significantly greater sensitivity and specificity of the combined test than CSF NfL alone in discriminating the merged PD/PD-CI from APD (Δaccuracy range, +7.2% to +10.7%). The added value of the combined test was most pronounced in the PD-CI group due to the higher median CSF NfL values and higher sensitivity of the α-syn RT-QuIC in PD-CI than in PD (Table 5).

Discussion

The NfL protein has emerged as the most valuable biomarker for detecting and measuring neuroaxonal injury in patients with neurological disorders. The availability of highly sensitive analytical platforms enabling reliable quantification of NfL in blood samples has laid the basis for using NfL as a diagnostic and prognostic biomarker in clinical practice. Using one of the largest cohorts studied to date, we sought to directly compare currently available assays for NfL determination in CSF and blood to establish their relative accuracy in the differential diagnosis between PD and APD.

All tested assays displayed relatively high and consistent diagnostic accuracy, with CSF NfL performing slightly better than plasma NfL. CSF and plasma biomarker values showed a good overall correlation, which, however, was weaker in MSA due to a lower relative increase in NfL levels in

Table 1 | Demographic and biomarker results in the diagnostic groups

	Median (IQR)				
Characteristic	PD	PD-CI	MSA	PSP/CBS	HC
N	253	67	141	124	40
Age, years	62 (55–68)[a,b]	72 (65–76)[c,d]	62 (57–68)[b]	71 (67–77)[d]	61 (59–63)
Female, n (%)	81 (32.0)[c,e]	17 (25.4)[c,e]	73 (51.8)	60 (48.4)[f]	12 (30.0)
Onset to collection (mos)	43 (22–98)[a]	77 (51–143)[b,c]	43 (29–66)	40 (24–59)	-
CSF α-syn RT-QuIC, positive/tested (%)	194/216 (89.8)[b,c]	55/56 (98.2)[b,c]	3/127 (2.4)	2/114 (1.8)	-
CSF NfL^{ELISA}, pg/ml	600 (457–761)[b,c,g]	938 (673-1153)[c,h]	2772 (2150–3862)[b]	1698 (1297–2337)	-
CSF NfL^{CLEIA}, pg/ml	527 (404–729)[b,c,g]	820 (637–1075)[c,h]	2774 (2101–3665)[b]	1765 (1387–2368)	-
CSF NfL^{SIMOA}, pg/ml	499 (377–651)[a-c]	797 (591–1012)[c]	2504 (1796–3414)[b]	1596 (1089–2195)	-
CSF NfL^{ELLA}, pg/ml	697 (539–915)[b,c,g]	1050 (858–1289)[c,h]	3083 (2338–4290)[b]	1994 (1558–2785)	-
Plasma NfL^{CLEIA}, pg/ml	16 (12–21)[b,c,i]	21 (18–33)[j,k]	39 (28.52)[d]	37 (25–49)[d]	13 (10–17)
Plasma NfL^{SIMOA}, pg/ml	10 (7–13)[b,c,g]	15 (11–21)[c,f]	27 (20–38)[d]	26 (18–33)[d]	10 (9–14)
Plasma NfL^{ELLA}, pg/ml	18 (13–26)[a-c]	30 (21–41)[c,d]	55 (40–76)[d]	54 (33–72)[d]	15 (11–19)

α-syn RT-QuIC α-synuclein real-time quaking-induced conversion, *CSF* cerebrospinal fluid, *HC* healthy controls, *mos* months, *MSA* multiple system atrophy, *PD* Parkinson's disease, *PD-CI* PD-cognitively impaired, *PSP/CBS* progressive supranuclear palsy/corticobasal syndrome.

Continuous data are expressed as median (interquartile range). Demographic factors and clinical characteristics were compared using chi-square and Kruskal-Wallis tests. Biomarkers (ln-transformed) were analyzed with univariable general linear models adjusting for age and sex (CSF and plasma), BMI and eGFR (only plasma).

[a] vs. PD-CI ≤ 0.001, [b] vs. PSP/CBS ≤ 0.001, [c] vs. MSA ≤ 0.001, [d] vs. HC ≤ 0.001, [e] vs. PSP/CBS ≤ 0.01, [f] vs. HC ≤ 0.05, [g] vs. PD-CI ≤ 0.01, [h] vs. PSP/CBS ≤ 0.05, [i] vs. PD-CI ≤ 0.05, [j] vs. MSA ≤ 0.01, [k] vs. HC ≤ 0.01

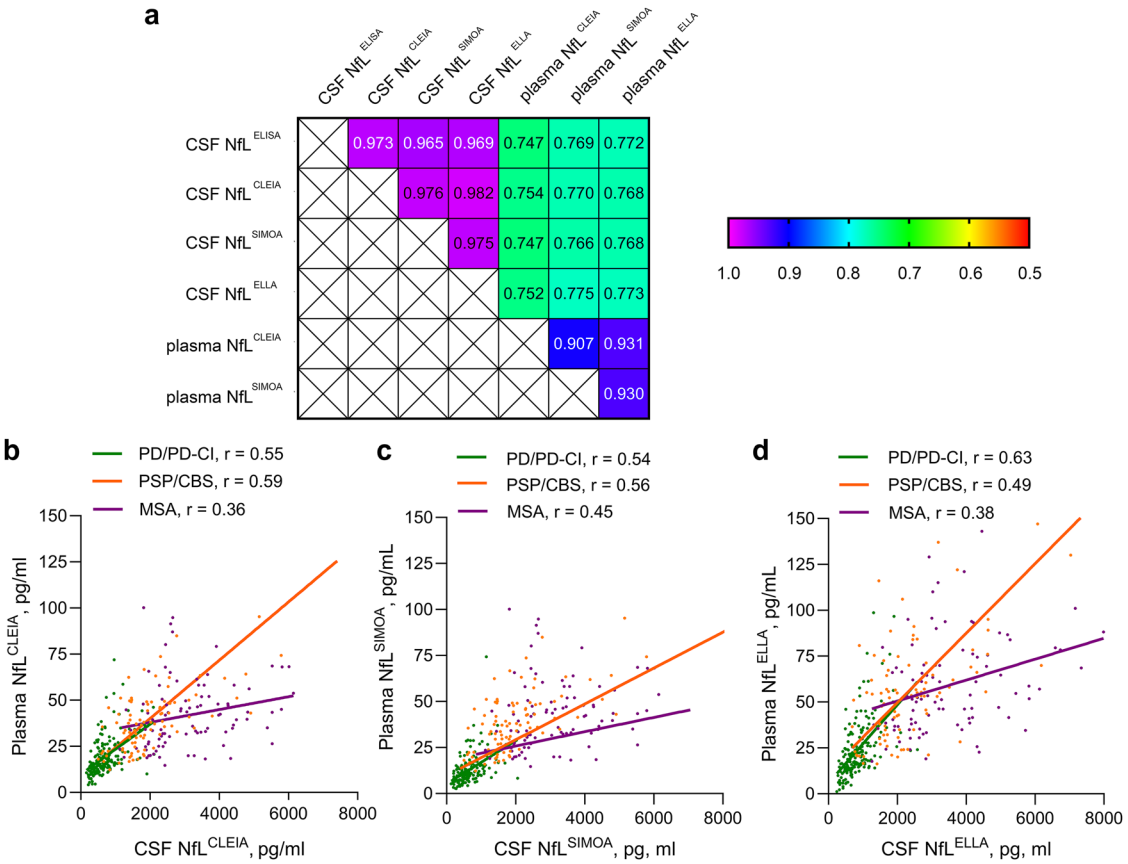

Fig. 1 | Correlations of NfL levels between assays and biofluids in the whole cohort and disease groups. a Heatmap of Spearman correlation between assays in the whole cohort. **b–d** CSF/plasma correlations, stratified for disease, of NfL^{CLEIA}, NfL^{SIMOA}, and NfL^{ELLA}. CSF cerebrospinal fluid, MSA multiple system atrophy, PD Parkinson's disease, PD-CI PD-cognitively impaired, PSP/CBS progressive supranuclear palsy/corticobasal syndrome.

plasma than in CSF compared to the other APD groups. Although there is no definitive explanation for the latter divergent behaviour of MSA, one possibility could be the lower leakage of proteins into the blood compared to CSF from brain regions (e.g., the cerebellum) that show significant degeneration only in MSA.

In addition to confirming previous findings on the diagnostic values of both CSF and blood NfL in patients with parkinsonism[3–5], our results support the use of all tested assays and provide potential reference cut-offs for each diagnostic platform and biofluid to be tested in the interlaboratory setting (Table 4). Of note, despite the comparable accuracy, we observed assay differences in absolute biomarker levels that are more pronounced in plasma and likely depend on the intrinsic analytic features of each platform. Accordingly, discriminative cut-offs slightly differ between the different immunoassays and are not interchangeable. Nonetheless, all NfL assays we tested in CSF and plasma showed a comparable ability to discriminate between neurodegenerative parkinsonism, supporting their use in clinical practice.

Besides the well-established association between NfL levels and increased age[8], we also confirmed that plasma NfL is negatively associated with BMI and eGFR, meaning that the biomarker concentrations are expected to be decreased in obese patients while increasing in those with CKD[9–11]. Moreover, disease duration[12], severity of motor symptoms[13], and MMSE[14] are all associated with NfL levels in PD, as we also confirmed in our cohort. Therefore, the diagnostic value of NfL decreases in older PD patients or those showing signs of advanced disease. Accordingly, we observed a drop in the diagnostic accuracy of NfL in discriminating PD-CI and APD (i.e., PD-CI participants were older, had longer disease duration and lower MMSE scores than cognitively unimpaired PD).

Our results also confirm in a larger cohort the added value of combining the assessment of CSF NfL with α-syn RT-QuIC[5]. The combined evaluation resulted in a noticeable increase in sensitivity and specificity and, above all, in a comparable accuracy in distinguishing between PD and PD-CI groups from APD.

The α-syn RT-QuIC assay we applied in this study is highly specific for the seeds of misfolded α-syn associated with Lewy body disease (LBD) but does not help in the distinction between MSA and PSP/CBS or other non-LBD parkinsonism since it does not recognize the misfolded α-syn targeting oligodendrocytes as glial cytoplasmic inclusions (GCIs) in MSA, which has a different structure[15,16]. Recently, a novel standardized fast α-syn seed amplification (SAA) for detecting and differentiating MSA from LBD synucleinopathies based on the amplification pattern has shown a good performance[17]. However, in this assay, the outcome must be determined according to two signal thresholds (negative vs MSA-like vs. LBD-like) rather than one (negative vs LBD positive), which might negatively affect specificity. Therefore, It would be interesting to explore whether the combined analysis with NfL we have applied here would have an added diagnostic value also using the α-syn SAA protocol, which discriminates between LBD and MSA.

The present study is not free of limitations. First, the diagnoses were based on clinical criteria. However, physicians specialized in movement disorders formulated the diagnoses at baseline and over time with reassessments at each follow-up visit. Moreover, the inclusion of patients only fulfilling the criteria for "probable" or "clinically established" disease to assure the highest likelihood of a correct diagnosis may limit the generalization of our findings to the clinical practice setting.

In conclusion, we show relatively high and consistent accuracy for several NfL immunoassays for both CSF and plasma. However, the plasma NfL assays' meaningful lower performance than CSF NfL, especially in MSA, must be considered. We also confirm the superior performance of combining NfL determination with α-syn RT-QuIC. The findings support

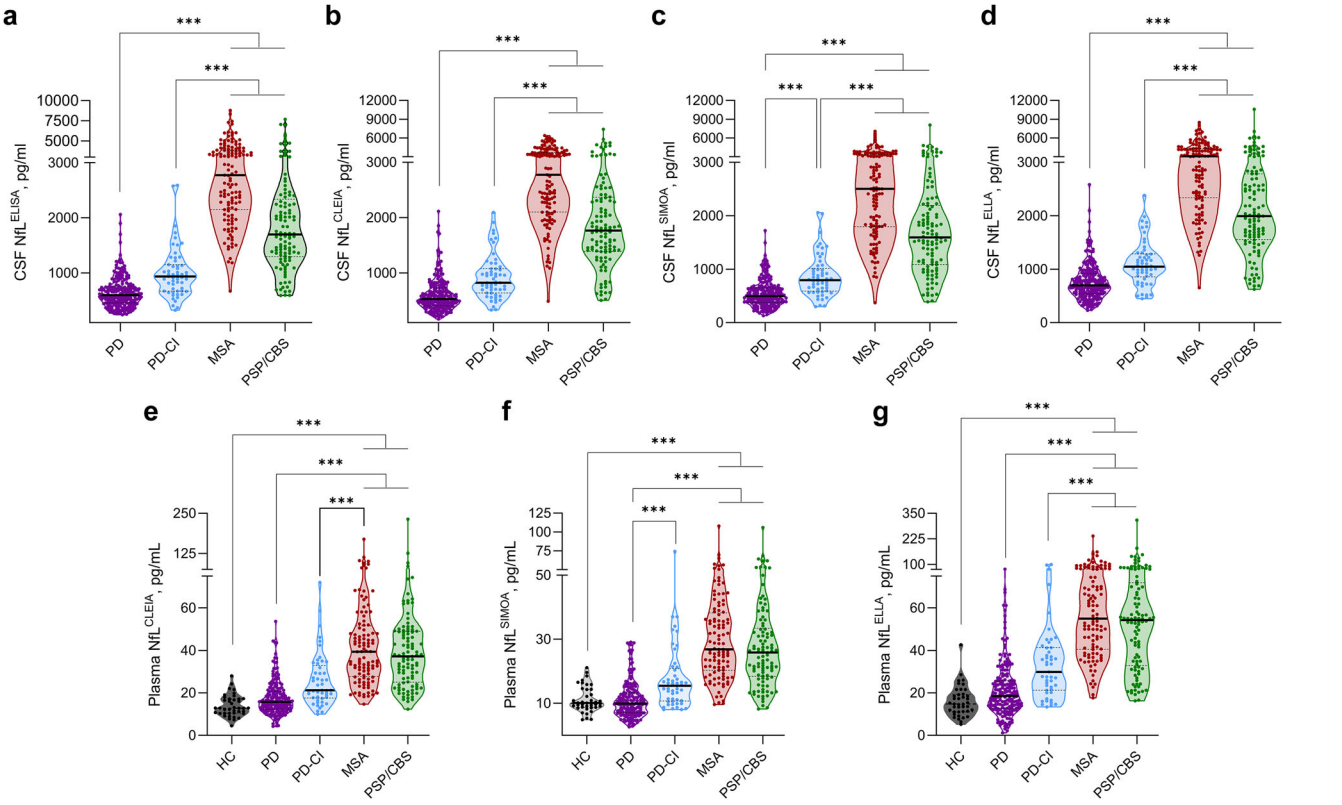

Fig. 2 | CSF and plasma NfL measures across diagnostic groups using different assays. a–d NfL analyses in CSF. **e–g** NfL analyses in plasma. Violin plots show value distributions; the horizontal bar indicates the median value. To improve readability, only statistically significant values reaching $p \leq 0.001$ (***) are shown. CSF cerebrospinal fluid, HC healthy controls, MSA multiple system atrophy, PD Parkinson's disease, PD-CI PD-cognitively impaired, PSP/CBS progressive supranuclear palsy/corticobasal syndrome.

using NfL assays as a biomarker for neurodegenerative parkinsonism in drug trials and clinical practice.

Methods

Participants

The study included 585 individuals with parkinsonism enrolled at the Istituto delle Scienze Neurologiche di Bologna between 2007 and 2023 (retrospective cohort from 2007 to 2020 and prospective cohort from 2021 to 2023), and 40 healthy controls. We included patients with at least one biofluid (CSF or plasma) available and final clinical diagnosis of "probable" or "clinically established" according to current internationally established criteria (PD[18], $n = 253$; PD-CI[19], $n = 67$; MSA[20], $n = 141$; PSP/CBS[21,22], $n = 124$). The PD-CI group included patients with dementia or mild cognitive impairment at the CSF collection. Among them, 514 participants had CSF and 444 blood (both biomatrices were available in 373). All healthy controls had blood available. A movement disorder specialist (GCB, GG, LS, SC, PC) formulated the clinical diagnosis at baseline and re-evaluated it at each follow-up visit (mean follow-up 2.0 ± 2.5 years).

In all cases, sex (assigned, not self-reported), age at disease onset, time from disease onset to lumbar puncture, follow-up duration, CKD defined by an eGFR <60 mL/minute/1.73 m^2), history of cerebrovascular disease (i.e., neuroradiologic assessment), cardiovascular disease (angina, heart attack) disease were collected. eGFR was calculated using the EPI-CKD equation[23]. Additionally, the scores of MMSE[24], Unified Parkinson's Disease Rating Scale-section III[25], and Hoehn & Yahr stage[26], were recorded, when available, in the PD and PD-CI groups.

Sample collection

CSF samples were obtained in the morning under fasting conditions by lumbar puncture at the L3/L4 or L4/L5 level, collected in 5 ml polypropylene tubes (Sarstedt, code number 63.504.027). Samples showing signs of blood contamination (even minimal) were centrifuged at room temperature at 2000 g for 10 min. Each sample (supernatant or non-centrifuged CSF) was divided into aliquots and stored in polypropylene tubes at − 80 °C until analysis.

EDTA plasma samples were collected by venous puncture, aliquoted, and stored at −80 °C according to standard procedures[27].

Biomarker analyses

All biomarker analyses were performed by personnel blinded to the clinical diagnostic group. The samples were analyzed randomly to avoid bias due to the effect of interassay variability on specific patient groups.

In blood and CSF, NfL analyses were performed by single molecule array (NfL^{SIMOA}) immunoassays, automated chemiluminescent enzyme immunoassay (NfL^{CLEIA}), and ELLA automated microfluidic-based ELISA (NfL^{ELLA}). In CSF, NfL was additionally measured by standard manual ELISA (NfL^{ELISA}).

CSF NfL^{ELISA} was quantified by a validated commercial enzyme-linked immunosorbent assay (NfL ELISA kit, IBL, Hamburg, Germany)[28]. The mean intra- and inter-assay coefficients of variation (CVs) were 4% and 10%.

NfL^{SIMOA} analyses were performed on a SiMOA SR-X analyzer platform (Quanterix, Billerica, MA, USA) using the SiMOA NF-light kit. The mean intra- and inter-assay CVs were 6% and 15% for CSF and 3% and 10% for plasma, respectively.

NfL^{CLEIA} was measured on the Lumipulse G600II platform (Fujirebio, Gent, Belgium) using the Lumipulse® G NfL CSF and Lumipulse® G NfL blood kits. The mean interassay CVs were 7% for CSF and 11% for plasma, respectively.

NfL^{ELLA} was analyzed using a commercial kit for the ELLA microfluidic system (BioTechne, Minneapolis, USA). The mean interassay CVs were 5% for CSF and 7% for plasma, respectively.

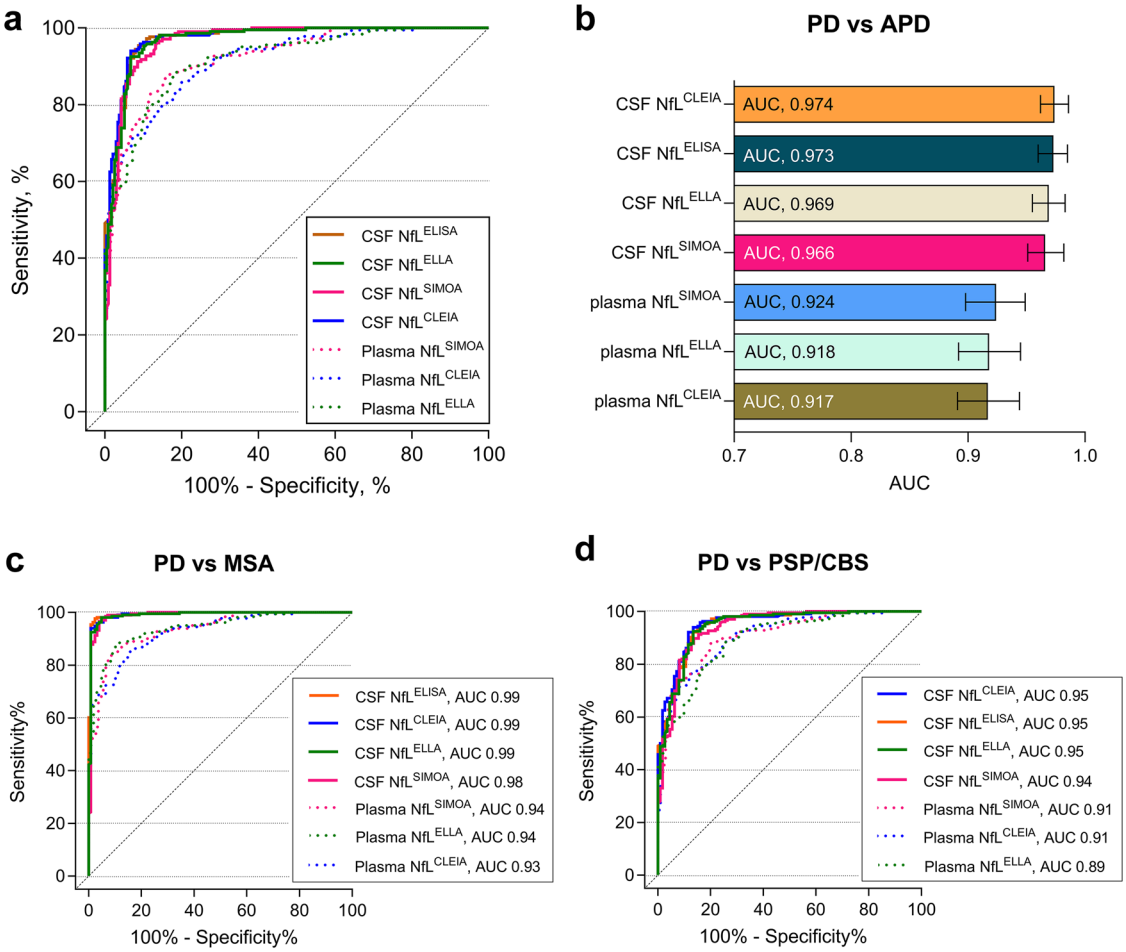

Fig. 3 | ROC curve analysis for the differential diagnosis of PD from APD. a ROC curves showing the discrimination between PD and APD (i.e., APD = MSA + PSP/CBS), and (**b**) AUC value for each assay. ROC curve analysis for the differential diagnosis of (**c**) PD vs MSA, and (**d**) PD vs PSP/CBS. APD atypical parkinsonian disorder, AUC area under the curve, CSF cerebrospinal fluid, MSA multiple system atrophy, PD Parkinson's disease, PSP/CBS progressive supranuclear palsy/corticobasal syndrome, ROC receiver operating characteristic.

Table 2 | Diagnostic accuracy of CSF NfL discriminating PD and PD-CI from APD

	AUC (95% CI)			
NfL assay	**CSF NfL^{ELISA}**	**CSF NfL^{CLEIA}**	**CSF NfL^{SIMOA}**	**CSF NfL^{ELLA}**
Entire cohort				
PD vs APD	0.973 (0.960–0.985)	0.974 (0.962–0.986)	0.966 (0.951–0.982)	0.969 (0.955-0.983)
PD-CI vs APD	0.904 (0.863–0.945)	0.924 (0.891–0.956)	0.898 (0.858–0.937)	0.918 (0.885–0.952)
Subcohorts				
PD vs MSA	0.994 (0.987–1.00)	0.991 (0.982–1.00)	0.989 (0.976–1.00)	0.990 (0.980–1.00)
PD-CI vs MSA	0.967 (0.940–0.993)	0.976 (0.956–0.995)	0.956 (0.928–0.985)	0.973 (0.952–0.994)
PD vs PSP/CBS	0.949 (0.925–0.973)	0.954 (0.932–0.976)	0.941 (0.913–0.969)	0.946 (0.920–0.971)
PD-CI vs PSP/CBS	0.833 (0.769–0.898)	0.865 (0.808–0.921)	0.831 (0.768–0.894)	0.857 (0.800–0.914)

APD atypical parkinsonian disorder, *MSA* multiple system atrophy, *PD* Parkinson's disease, *PD-CI* PD-cognitively impaired, *PSP/CBS* progressive supranuclear palsy/corticobasal syndrome.

Approximately half of the patients (n = 355) were included in a previous study reporting results of NfL analyzed by standard ELISA (NfL ELISA) in the CSF and by SiMoA (NfL SIMOA) in plasma[5]. CSF and plasma samples generating the NfL^{ELISA} and plasma NfL^{SIMOA} data included in our previous study[5] were not re-analyzed. To evaluate the effect of different batches and sample storage duration on the reproducibility of NfL levels, we included the same internal quality control samples (i.e., three samples loaded in duplicate) in all plates. We did not observe significant variations of the intra- and inter-assay CVs between the two study periods [CSF NfL^{ELISA} interassay CVs 2% (2021) vs. 4% (2024), interassay CVs 10% (2021) vs. 10% (2024); plasma NfL^{SIMOA} interassay CVs 4% (2021) vs. 3% (2024), interassay CVs 11% (2021) vs. 10% (2024)].

All CSFs were tested by the α-syn RT-QuIC assay. The purification of recombinant wild-type α-syn was performed according to a previously published protocol[29]. For analyses, we followed a previously published protocol[30]. Briefly, six 0.8-mm silica beads (OPS Diagnostics) per well were pre-loaded into black 96-well plates with a clear bottom (Nalgene Nunc International). CSF samples were thawed and vortexed for 10 seconds

Table 3 | Diagnostic accuracy of plasma NfL discriminating PD and PD-CI from APD

NfL assay	AUC (95% CI) Plasma NfL^{CLEIA}	Plasma NfL^{SIMOA}	Plasma NfL^{ELLA}
Entire cohort			
PD vs APD	0.917 (0.891–0.943)	0.924 (0.898–0.949)	0.918 (0.892–0.945)
PD-CI vs APD	0.771 (0.696–0.846)	0.783 (0.708–0.858)	0.788 (0.715–0.861)
Subcohorts			
PD vs MSA	0.926 (0.898–0.955)	0.936 (0.909–0.963)	0.940 (0.914–0.966)
PD-CI vs MSA	0.783 (0.705–0.862)	0.809 (0.732–0.886)	0.821 (0.746–0.897)
PD vs PSP/CBS	0.907 (0.873–0.941)	0.910 (0.876–0.943)	0.896 (0.859–0.932)
PD-CI vs PSP/CBS	0.758 (0.675–0.841)	0.753 (0.668–0.839)	0.752 (0.669–0.835)

APD atypical parkinsonian disorder, *MSA* multiple system atrophy, *PD* Parkinson's disease, *PD-CI* PD-cognitively impaired, *PSP/CBS* progressive supranuclear palsy/corticobasal syndrome.

Table 4 | Diagnostic performance of different CSF and plasma NfL assays for the differential diagnosis of PD and PD-CI vs APD, MSA, and PSP/CBS

Diagnostic group	Biomarker	NfL cut-off, pg/ml	Sensitivity (%) 95% CI	Specificity (%) 95% CI	Accuracy (%) 95% CI
PD vs APD	CSF NfL^{ELISA}	< 1149	95.4 (91.7–97.8)	91.3 (86.9–94.5)	93.2 (90.5–95.3)
	CSF NfL^{CLEIA}	< 1082	93.9 (89.9–96.8)	93.3 (89.4–96.1)	93.6 (90.9–95.7)
	CSF NfL^{SIMOA}	< 908	91.3 (86.5–94.7)	91.5 (87.2–94.7)	91.4 (88.4–93.8)
	CSF NfL^{ELLA}	< 1244	92.6 (88.2–95.7)	93.3 (89.3–96.1)	92.9 (90.2–95.1)
	plasma NfL^{CLEIA}	< 24.8	86.3 (80.4–90.9)	78.5 (72.2–83.9)	82.2 (77.9–85.9)
	plasma NfL^{SIMOA}	< 16.1	85.8 (79.9–90.5)	84.8 (79.2–89.3)	85.2 (81.3–88.6)
	plasma NfL^{ELLA}	< 34.7	88.5 (82.9–92.7)	81.8 (75.9–86.8)	84.9 (80.9–88.3)
PD-CI vs APD	CSF NfL^{ELISA}	< 1301	83.6 (71.2–92.2)	77.1 (69.3–83.8)	78.9 (72.6–84.5)
	CSF NfL^{CLEIA}	< 1246	85.5 (73.3–93.5)	89.1 (84.5–92.8)	88.4 (84.2–91.8)
	CSF NfL^{SIMOA}	< 1444	92.6 (82.1–97.9)	74.9 (68.8–80.3)	78.2 (72.9–82.8)
	CSF NfL^{ELLA}	< 1428	83.3 (70.7–92.1)	89.5 (84.8–93.1)	88.3 (84.1–91.8)
	plasma NfL^{CLEIA}	< 29.5	75.0 (60.4–86.4)	70.9 (64.3–76.9)	71.7 (65.8–77.1)
	plasma NfL^{SIMOA}	< 17.6	72.9 (58.2–84.7)	81.4 (75.5–86.5)	79.8 (74.4–84.6)
	plasma NfL^{ELLA}	< 46.2	87.2 (74.3–95.2)	65.1 (58.2–71.5)	69.1 (63.1-74.7)
PD vs MSA	CSF NfL^{ELISA}	< 1253	97.7 (94.7–99.2)	97.6 (93.3–99.5)	97.7 (95.5-98.9)
	CSF NfL^{CLEIA}	< 1082	93.9 (89.9–96.8)	99.2 (95.7–99.9)	95.9 (93.3-97.8)
	CSF NfL^{SIMOA}	< 1105	96.6 (93.1–98.6)	96.0 (90.9–98.7)	96.4 (93.8-98.1)
	CSF NfL^{ELLA}	< 1559	98.1 (95.3–99.5)	95.2 (89.9–98.2)	97.1 (94.7-98.6)
	plasma NfL^{CLEIA}	< 24.8	86.3 (80.4–90.9)	83.3 (74.9–89.8)	85.2 (80.5-89.1)
	plasma NfL^{SIMOA}	< 15.6	83.1 (76.8–88.2)	92.6 (85.9–96.8)	86.6 (82.1-90.3)
	plasma NfL^{ELLA}	< 34.7	88.5 (82.9–92.7)	88.9 (81.4–94.1)	88.6 (84.4-92.0)
PD-CI vs MSA	CSF NfL^{ELISA}	< 1741	94.5 (84.9–98.9)	88.9 (82.2–93.8)	90.7 (85.5-94.5)
	CSF NfL^{CLEIA}	< 1786	96.4 (87.5–99.6)	88.9 (82.2–93.84	91.2 (86.1-94.9)
	CSF NfL^{SIMOA}	< 1446	92.6 (82.1–97.9)	86.4 (79.1–91.9)	88.3 (82.6-92.6)
	CSF NfL^{ELLA}	< 1907	96.3 (87.3–99.5)	87.2 (80.1–92.5)	89.9 (84.6-93.9)
	plasma NfL^{CLEIA}	< 29.8	75.0 (60.4–86.4)	72.2 (62.8–80.4)	73.1 (65.4-79.9)
	plasma NfL^{SIMOA}	< 17.6	72.9 (58.2–84.7)	84.3 (76.0–90.5)	80.8 (73.7-86.6)
	plasma NfL^{ELLA}	< 46.2	87.2 (74.3–95.2)	68.5 (58.9–77.1)	74.2 (66.6–80.9)
PD vs PSP/CBS	CSF NfL^{ELISA}	< 1057	91.7 (87.2–94.9)	86.7 (79.1–92.4)	89.9 (86.2-92.9)
	CSF NfL^{CLEIA}	< 1089	93.9 (89.9–96.8)	86.6 (78.9–92.3)	91.5 (87.9–94.3)
	CSF NfL^{SIMOA}	<881	89.8 (84.8–93.6)	86.4 (78.5–92.2)	88.6 (84.6–91.9)
	CSF NfL^{ELLA}	< 1244	92.6 (88.2–95.7)	86.6 (78.9–92.3)	90.5 (86.8–93.5)
	plasma NfL^{CLEIA}	< 21.2	76.4 (69.5-82.3)	87.3 (79.2–93.0)	80.3 (75.2–84.8)
	plasma NfL^{SIMOA}	< 16.7	86.9 (81.1–91.4)	80.4 (71.4–87.6)	84.6 (79.8–88.5)
	plasma NfL^{ELLA}	< 34.7	88.5 (82.9–92.7)	74.3 (64.6–82.4)	83.4 (78.5–87.5)

Table 4 (continued) | Diagnostic performance of different CSF and plasma NfL assays for the differential diagnosis of PD and PD-CI vs APD, MSA, and PSP/CBS

Diagnostic group	Biomarker	NfL cut-off, pg/ml	Sensitivity (%) 95% CI	Specificity (%) 95% CI	Accuracy (%) 95% CI
PD-CI vs PSP/CBS	CSF NfL^{ELISA}	< 1301	83.6 (71.2–92.2)	75.2 (66.2–82.9)	77.9 (70.9–83.9)
	CSF NfL^{CLEIA}	< 1246	85.5 (73.3–93.5)	81.3 (72.8–88.0)	82.6 (76.0–88.1)
	CSF NfL^{SIMOA}	< 1081	79.6 (66.5–89.4)	76.4 (67.3–83.9)	77.4 (70.3–83.6)
	CSF NfL^{ELLA}	< 1440	83.3 (70.7–92.1)	81.3 (72.8–88.0)	81.9 (75.2-87.5)
	plasma NfL^{CLEIA}	< 29.5	75.0 (60.4–86.4)	69.6 (59.7–78.3)	71.3 (63.4–78.4)
	plasma NfL^{SIMOA}	< 17.6	72.9 (58.2–84.7)	78.4 (69.2–85.9)	76.7 (69.1–83.2)
	plasma NfL^{ELLA}	< 46.9	87.2 (74.3–95.2)	61.4 (51.2–70.9)	69.6 (61.5–76.9)

APD atypical parkinsonian disorder, *CI* confidence interval, *CSF* cerebrospinal fluid, *MSA* multiple system atrophy, *PD* Parkinson's disease, *PD-CI* PD-cognitively impaired, *PSP/CBS* progressive supranuclear palsy/corticobasal syndrome.

Table 5 | Diagnostic performance of the CSF NfL and α-syn RT-QuIC assays in the differential diagnosis of patients with PD and PD-CI vs APD, MSA and PSP/CBS

Diagnostic group	Biomarker	NfL cut off, pg/ml	+/−	Sensitivity (%) 95% CI	Specificity (%) 95% CI	Accuracy (%)95% CI
PD	α-syn RT-QuIC	-	194/22	89.8	-	-
PD-CI	α-syn RT-QuIC	-	55/1	98.2	-	-
MSA	α-syn RT-QuIC	-	3/124	-	97.6	-
PSP/CBS	α-syn RT-QuIC	-	2/112	-	98.2	-
APD	α-syn RT-QuIC	-	5/236	-	97.9	-
PD vs APD	CSF NfL^{ELISA} + α-syn RT-QuIC	< 700	212/4 vs 8/232	98.2 (95.3–99.5)	96.7 (93.5–98.5)	97.4 (94.5–98.6)
	CSF NfL^{CLEIA} + α-syn RT-QuIC	< 700	212/4 vs 13/225	98.2 (95.3–99.5)	94.5 (90.8–97.1)	96.3 (94.1–97.8)
	CSF NfL^{SIMOA} + α-syn RT-QuIC	< 650	205/1 vs 10/225	99.5 (97.3–99.9)	95.7 (92.3–97.9)	97.5 (95.6-98.8)
	CSF NfL^{ELLA} + α-syn RT-QuIC	< 750	210/6 vs 5/232	97.2 (94.0-98.9)	97.9 (95.2–99.3)	97.6 (95.7–98.8)
PD-CI vs APD	CSF NfL^{ELISA} + α-syn RT-QuIC	< 700	53/1 vs 8/232	98.2 (90.1–99.9)	96.7 (93.5–98.5)	96.9 (94.3-98.6)
	CSF NfL^{CLEIA} + α-syn RT-QuIC	< 700	53/1 vs 13/225	98.2 (90.1–99.9)	94.5 (90.8–97.1)	95.2 (92.1–97.4)
	CSF NfL^{SIMOA} + α-syn RT-QuIC	< 650	53/1 vs 10/225	98.2 (90.1–99.9)	95.7 (90.1–99.9)	95.7 (92.3–97.9)
	CSF NfL^{ELLA} + α-syn RT-QuIC	< 750	52/1 vs 5/232	98.1 (89.9-99.9)	97.9 (95.2–99.3)	97.9 (95.6–99.2)
PD vs MSA	CSF NfL^{ELISA} + α-syn RT-QuIC	< 700	212/4 vs 1/126	98.2 (95.3–99.5)	99.2 (95.7–99.9)	98.5 (96.6-99.5)
	CSF NfL^{CLEIA} + α-syn RT-QuIC	< 700	212/4 vs 1/126	98.2 (95.3–99.5)	99.2 (95.7–99.9)	98.5 (96.6–99.5)
	CSF NfL^{SIMOA} + α-syn RT-QuIC	< 650	205/1 vs 1/124	99.5 (97.3–99.9)	99.2 (95.6–99.9)	99.4 (97.8–99.9)
	CSF NfL^{ELLA} + α-syn RT-QuIC	< 750	210/6 vs 1/124	97.2 (94.0–98.9)	99.2 (95.6–99.9)	97.9 (95.8–99.2)
PD-CI vs MSA	CSF NfL^{ELISA} + α-syn RT-QuIC	< 700	53/1 vs 1/126	98.2 (90.1–99.9)	99.2 (95.7–99.9)	98.9 (96.1–99.9)
	CSF NfL^{CLEIA} + α-syn RT-QuIC	< 700	53/1 vs 1/126	98.2 (90.1–99.9)	99.2 (95.7–99.9)	98.9 (96.1–99.9)
	CSF NfL^{SIMOA} + α-syn RT-QuIC	< 650	53/1 vs 1/124	98.2 (90.1–99.9)	99.2 (95.6–99.9)	98.9 (96.0–99.9)
	CSF NfL^{ELLA} + α-syn RT-QuIC	< 750	52/1 vs 1/124	98.1 (89.9–99.9)	99.2 (95.6–99.9)	98.9 (96.0–99.9)
PD vs PSP/CBS	CSF NfL^{ELISA} + α-syn RT-QuIC	< 700	212/4 vs 7/106	98.2 (95.3–99.5)	93.8 (87.7–97.5)	96.7 (94.1–98.3)
	CSF NfL^{CLEIA} + α-syn RT-QuIC	< 700	212/4 vs 7/105	98.2 (95.3–99.5)	93.8 (87.6–97.5)	96.7 (94.1–98.3)
	CSF NfL^{SIMOA} + α-syn RT-QuIC	< 650	205/1 vs 9/101	99.5 (97.3–99.9)	91.8 (85.0–96.2)	96.8 (94.3–98.5)
	CSF NfL^{ELLA} + α-syn RT-QuIC	< 750	210/6 vs 4/108	97.2 (94.0–98.9)	96.4 (91.1–99.0)	96.9 (94.5–98.5)
PD-CI vs PSP/CBS	CSF NfL^{ELISA} + α-syn RT-QuIC	< 600	53/1 vs 3/110	98.2 (90.1–99.9)	97.4 (92.4–99.5)	97.6 (93.9–99.3)
	CSF NfL^{CLEIA} + α-syn RT-QuIC	< 600	53/1 vs 2/110	98.2 (90.1–99.9)	98.2 (93.7–99.8)	98.2 (94.8–99.6)
	CSF NfL^{SIMOA} + α-syn RT-QuIC	< 550	53/1 vs 6/104	98.2 (90.1–99.9)	94.6 (88.5–97.9)	95.7 (91.4–98.3)
	CSF NfL^{ELLA} + α-syn RT-QuIC	< 650	52/1 vs 1/111	98.1 (89.9–99.9)	99.1 (95.1–99.9)	98.8 (95.7–99.9)

APD atypical parkinsonian disorder, *α-syn RT-QuIC* α-synuclein real-time quaking-induced conversion, *CI* confidence interval, *CSF* cerebrospinal fluid, *MSA* multiple system atrophy, *PD* Parkinson's disease, *PSP/CBS* progressive supranuclear palsy/corticobasal syndrome, *PD-CI* PD-cognitively impaired, *SAA* seed amplification assay, +/− positive/negative

before use. Then, 15 µl CSF was added to 85 µl of reaction mix composed of 40 mM PB, pH 8.0, 170 mM NaCl, 10 µM thioflavin-T (Sigma), 0.0015% SDS (Bio-Rad) and 0.1 g l − 1 of filtered recombinant α-syn (100-kDa Amicon centrifugal filters, Merck Millipore). Plates were closed with a plate sealer film (Nalgene Nunc International) and incubated into a FLUOstar Omega plate reader (BMG Labtech) at 42 °C with intermittent double orbital shaking at 400 r.p.m. for 1 min, followed by 1 min rest. Fluorescence was measured every 45 min with 450 nm excitation and 480 nm emission filters during the 30-hour test run. We ran each CSF sample in quadruplicate. Each plate was designed to include internal positive (min, $n = 1$, to max, $n = 3$) and negative ($n = 1$) controls. Controls were the same in each set of experiments. The test run duration was set at 30 h. The threshold to define a positive replicate among tested samples was arbitrarily set at 30% of the median of the maximum fluorescence intensity of each plate's positive replicates of control samples and evaluated within the first 25 h. CSF samples were deemed "positive" when at least 2 out of 4 replicates reached the

threshold, "negative" when none reached the threshold, and "unclear" when only 1 out of 4 replicates reached the threshold. "Unclear" cases were re-analysed up to three times (12 replicates); then, cases showing ≥ 4 positive replicates out of 12 were classified "positive", otherwise they were deemed "negative"[29].

Statistical analyses

SPSS version 22 (IBM) and GraphPad were used for statistical analyses. Outliers (n = 6) with NfL values above 5 standard deviations of the mean were excluded. Correlations between biomarkers were assessed with the Spearman test (p). Univariate linear regression analyses were used to test the association of clinical/demographic variables with NfL: the results are presented as Beta coefficients (β) and 95% CI. Binomial logistic regressions were used to test the association with comorbidities: results are presented as odd ratios and 95% CI. Differences between diagnostic groups were tested using the Kruskal-Wallis test (followed by Dunn's post hoc test) or the chi-square test as appropriate. NfL concentrations in CSF and plasma were transformed by natural log (ln) to fulfil the normal distribution. All statistical analyses were performed on transformed data: NfL differences between diagnostic groups were investigated with univariate general linear models adjusting for age and sex. Unadjusted 2-sided P values < 0.05 were considered statistically significant. Discrimination accuracies of biomarkers were determined with receiver operating characteristic (ROC) curve analysis. The optimal cut-off value for NfL in both CSF and plasma was chosen using Youden's Index. The two tests were also analysed in parallel to investigate the performance of the CSF NfL and α-syn RT-QuIC assays in combination. The rationale behind this approach relies on the different nature of the two markers. While NfL is a marker of neurodegeneration not specific to a given pathology, which can increase in conditions unrelated to parkinsonism or disease severity (e.g., increased NfL levels in "true" PD patients might prompt an incorrect APD diagnosis), the α-syn RT-QuIC is a highly sensitive and specific marker of LBD that is expected to be positive in most PD patients. Therefore, the combined approach aims to increase NfL specificity, using α-syn RT-QuIC to maintain high sensitivity. To this aim, we first chose a new cut-off for CSF NfL that maximizes its specificity (see also Table 5). Then, we formulated a diagnosis of PD when at least one of the two tests was positive (i.e., CSF NfL lower than the cut-off value and/or positive α-syn RT-QuIC seeding activity) and a diagnosis of APDs when both tests were negative (for PD). De Long test was used to compare the AUC between the tests performed individually and the combined test.

Ethics approval

This study involved human participants and was approved by the local ethics committee (approval numbers AVEC: 09070, 17093, 18025, and 18027). Written informed consent was given by study participants or by the next of kin for patients unable to communicate.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Author contributions

S.B. and P.P. contributed to the conception and design of the study. S.B., M.R., G.G., A.M., B.P., L.S., F.M., A.M., P.C., S.C., G.C.B., and P.P. contributed to the acquisition and analysis of the data. S.B. and P.P. contributed to drafting the text and preparing the figures. All authors read and approved the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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