Acta Neuropathologica (2025) 149:67
https://doi.org/10.1007/s00401-025-02904-4

ORIGINAL PAPER

Quantification of Lewy body pathology by cerebrospinal fluid endpoint dilution RT-QuIC in a neuropathological autopsy cohort of clinically heterogeneous participants

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Received: 27 March 2025 / Revised: 2 June 2025 / Accepted: 4 June 2025

Abstract

The identification of biomarkers predicting the burden of brain alpha-synuclein (α-syn) pathology in vivo represents a research priority in Lewy body disease (LBD). Recently, some kinetic parameters of seed amplification assays (SAAs) for α-syn showed associations with measures of clinical progression. However, preanalytical and analytical factors significantly affect these parameters, reducing reproducibility. The Endpoint Dilution (ED) SAA Real-time Quaking-induced Conversion (RT-QuIC) is emerging as an alternative, more accurate tool for seed quantification. Still, the approach needs validation in large patient cohorts. We applied the ED RT-QuIC to *postmortem* ventricular cerebrospinal fluid (CSF) samples from 357 brain donors, including 168 who showed LBD at neuropathologic examination. We estimated the seeding dose, yielding positive responses in 50% of replicate reactions (SD50), using the midSIN algorithm and correlated these values with *postmortem* synuclein pathology burden and clinical severity measures. LBD was staged through the Unified Staging System for Lewy Body Disorders and the Lewy pathology consensus criteria. The SD50 values (expressed in $\log_{10}$SD/ml) differed significantly among participants at different LBD stages ($p < 0.0001$), with those at a neocortical stage demonstrating higher values than those at a brainstem-predominant stage ($p < 0.0001$). The SD50 values were significantly associated with the LBD load evaluated through immunohistochemistry (Rho = 0.62, $p < 0.0001$). Participants showing higher SD50 values performed worse at the last available scores on clinical scales evaluating motor (Rho = 0.33, $p < 0.0001$) and olfactory functions (Rho = − 0.33, $p < 0.0001$). The SD50 scores accurately distinguished neocortical LBD participants from those at lower stages (area under the curve, 0.86; 95% confidence interval, 0.79–0.92). The CSF ED RT-QuIC measure of α-syn seeds correlated significantly with LBD burden and clinical severity scores. These findings validate the CSF ED RT-QuIC as a quantitative assay for misfolded brain α-syn in LBD. This novel approach may be clinically applied to identify individuals at different stages of LBD pathology in research settings.

Keywords Lewy body disease · Alpha-synuclein · Seed amplification assay · SD50 · Endpoint dilution · Staging · Autopsy

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Introduction

Lewy body disease (LBD), the second most common neurodegenerative disease, is characterized by the intraneuronal accumulation of misfolded alpha-synuclein (α-syn) in the nervous tissue, forming Lewy bodies and Lewy neurites. Besides constituting the hallmark pathology of Parkinson's disease (PD) and dementia with Lewy Bodies (DLB), LBD manifests as a frequent co-pathology in subjects with a neuropathological diagnosis of Alzheimer's disease (AD) [6, 16, 29].

Recently, the introduction of cerebrospinal fluid (CSF) α-syn seed amplification assays (SAA), like Real-Time Quaking-Induced Conversion (RT-QuIC), which detect misfolded α-syn based on an amplification strategy exploiting the prion-like properties of the protein on a recombinant native substrate, drastically improved the accuracy of in vivo diagnosis of LBD [1, 8, 22, 37–39]. The availability of a pathology-specific biomarker for LBD also paved the way for the proposal of biologically based research criteria for PD and a novel nomenclature for LBD [25, 41].

However, pathology-specific biomarkers in neurodegenerative diseases are helpful not only in improving diagnostic accuracy in vivo but also in predicting the neuropathological stage of the underlying proteinopathies. The latter also has diagnostic implications, as a more advanced neuropathological disease stage would increase the likelihood that it directly contributes to a clinical phenotype, such as the presence and severity of motor signs and cognitive impairment. Moreover, as neuroprotective therapies targeting protein misfolding processes are being tested for neurodegenerative diseases [34], the in vivo prediction of the underlying neuropathological stage may aid in stratifying patients and evaluating the efficacy of the drug.

In AD, tau positron emission tomography (PET) imaging is the only biomarker that can roughly predict the extent and distribution of neurofibrillary tau pathology—one of the hallmark pathologies of AD—in the central nervous system [18, 30]. Moreover, recent data provided evidence that a combination of plasma biomarkers can also aid in predicting the severity and distribution of pathological tau aggregates [27].

In contrast to AD, a reliable PET tracer that binds to pathological α-syn aggregates is lacking, posing a significant limitation for in vivo LBD staging. Although α-syn SAA mainly provides a binary positive vs. negative outcome, researchers have recently focused on the kinetic parameters of the CSF α-syn SAAs, such as the number of positive replicates and the time to the threshold (LAG), to search for potential quantitative parameters showing significant associations with LBD burden and stage in neuropathological cohorts [8]. Moreover, some studies have shown that parameters such as the number of positive replicates and the LAG correlate with measures of clinical severity [10, 11, 14, 32] and that their longitudinal changes are consistent with pathology progression over time [32]. However, these studies also showed that kinetic parameters, especially the LAG, can be affected by preanalytical and analytical factors, in addition to α-syn seed concentration, such as sample manipulation, matrix composition, and batch-to-batch variations of the recombinant protein substrate, which represents a potential limitation in their reproducibility [31, 43].

Recently, the estimation of pathological α-syn concentration through repeated RT-QuIC assays on serially diluted CSF samples (endpoint dilution RT-QuIC) was shown to be less dependent on the aggregation kinetic reaction [43]. Specifically, in analogy to endpoint dilution animal bioassays, this approach involves testing serial dilutions of samples and statistically estimating the seeding dose, which yields positive responses in 50% of replicate reactions (SD50). For this calculation, it was recently shown that data analysis using the midSIN (or Poisson) algorithms provides the most consistent and statistically significant discrimination of different seed concentrations [43].

In a recent study involving a relatively small patient cohort, an estimate of α-syn seeds based on the total number of positive replicates obtained by endpoint dilution RT-QuIC showed promising associations with measures of clinical severity [9]. However, this approach has not yet been validated in a large cohort against the gold standard of neuropathology.

In the present study, leveraging a neuropathological cohort of LBD participants at different disease stages with available *postmortem* collected CSF samples, we sought to quantify CSF levels of pathological α-syn and correlate them with the neuropathological burden and stage of the underlying proteinopathy. Moreover, we calculated the accuracy of pathological α-syn levels, alone or in combination with clinical variables, in identifying LBD participants with advanced pathology.

Materials and methods

Participants

Neuropathological samples were selected from the Arizona Study of Aging and Neurodegenerative Disorders (AZSAND)/Brain and Body Donation Program (BBDP) [4]. The analyzed cohort included autopsied BBDP participants with *postmortem* ventricular brain CSF of sufficient quality and an available total score at the University of Pennsylvania Smell Identification Test (UPSIT) ($N = 357$).

The operations of the Banner Sun Health Research Institute (SHRI) Brain and Body Donation Program have been approved by the WCG Institutional Review Board (IRB) of Puyallup, Washington, USA [4]. All enrolled subjects signed the IRB-approved informed consent.

Neuropathological examination

Brain harvesting, tissue preparation, and staining procedures have been described in detail previously [3, 4]. Here, we briefly report the staining protocol for α-syn and the criteria for LBD staging.

Immunohistochemical α-syn stainings were performed with a polyclonal antibody raised against a α-syn peptide fragment phosphorylated at serine 129 (pS129) [19, 44]. Ten standard brain regions (olfactory bulb and tract, anterior medulla, anterior and mid-pons, substantia nigra, mid-amygdala, transentorhinal area, anterior cingulate gyrus, middle temporal gyrus, middle frontal gyrus, and inferior parietal lobule) were sampled and graded for LB-density according to a semi-quantitative rating scale ranging from 0 to 4 (0 = none, 1 = mild, 2 = moderate, 3 = severe, and 4 = very severe pathology) [2, 33]. Participants obtaining a score of at least 1 in at least one examined brain region were deemed LB-positive. A final LB-density score was calculated for every LB-positive participant as the sum of the semi-quantitative scores at each of the ten sampled brain areas (maximum score of 40).

In LB-positive participants, LBD was staged according to both the Unified Staging System for Lewy Body Disorders (USSLB) [3] and the Lewy Pathology Consensus criteria (LPC) [2]. According to the USSLB, participants were classified as 0. No Lewy bodies; I. Olfactory bulb-only; IIa. Brainstem predominant; IIb. Limbic predominant; III. Brainstem/Limbic; and IV. Neocortical. According to the LPC, participants were classified into five categories: Olfactory only, Amygdala predominant, Brainstem predominant, Limbic, and Neocortical.

Details on the assessment of Alzheimer's disease pathology in included participants are reported in the Supplementary Materials.

Clinical assessment

For LB-positive participants, relevant clinical data were obtained, including the most recent neurological diagnosis and the last available scores on the Mini-Mental State Examination (MMSE), Unified Parkinson's Disease Rating Scale part III (UPDRS part III) in the OFF state, and the UPSIT. Neurological diagnoses were attributed after a consensus conference attended by movement disorders-specialized neurologists, cognitive/behavioral-specialized neurologists and neuropsychologists.

CSF collection and α-Synuclein RT-QuIC screening analysis

Postmortem CSF was collected from the lateral ventricles through the corpus callosum before brain removal, using 30 mL disposable polypropylene syringes fitted with 8 cm long, 18-gauge needles [4]. The CSF was ejected into 15 mL disposable polypropylene tubes for centrifugation at 2000 g for 10 min, and supernatants were aliquoted into 0.5 mL polypropylene microcentrifuge tubes and stored at −80 °C.

We performed the CSF α-syn SAA, including the purification of recombinant wild-type human αSyn, as previously described with minor analysis modifications [36, 38].

Briefly, for the SAA, we used Black 96-well plates with a clear bottom (Nalgene Nunc International) pre-loaded with six 0.8 mm silica beads (OPS Diagnostics) per well. After thawing CSF samples and vortexing them for 10 s, 15 µl of CSF were added to 85 µl of reaction mix, containing 40 mM PB pH 8.0, 170 mM NaCl, 10 µM thioflavin-T (ThT), 0.0015% sodium dodecyl sulfate (SDS), and 0.1 mg/ml of wild-type recombinant α-syn filtered using a 100 kDa MWCO filter (Amicon centrifugal filters, Merck Millipore). The plate was sealed with a plate sealer film and incubated in an FLUOstar Omega (BMG Labtech) plate reader at 42 °C with intermittent double orbital shaking at 400 rpm for one minute, followed by 1-min rest. ThT fluorescence measurements were taken every 45 min, using 450 nm excitation and 480 nm emission filters.

We loaded one negative and one positive control on each plate to verify the assay's correct functioning. As positive controls, we used brain homogenates (10% in PBS) from well-characterized LBD patients, who showed 4 out of 4 positive replicates at screening RT-QuIC. These were then diluted 10^{-5} in a pool of α-syn RT-QuIC negative CSF samples collected from patients clinically diagnosed with idiopathic normal pressure hydrocephalus (iNPH), which yielded a negative result in two RT-QuIC runs. The same iNPH samples were used as negative controls. Samples were deemed positive when at least three out of four replicates reached a threshold arbitrarily set at 30% of the median of the maximum fluorescence intensity (Imax) reached by the positive control replicates. To minimize the risk of false-positive results, the analysis was repeated three times for those samples whose seeding activity was detected in only one or two of four replicates in the first run. Ultimately, the result in the repeated samples was considered "positive" when at least 4 of the 12 total replicates reached the threshold.

Endpoint dilution (ED) α-Syn RT-QuIC and seed quantification

CSF samples showing positive results at screening were then analyzed in octuplicate by Endpoint Dilution RT-QuIC (ED

RT-QuIC) to quantify the misfolded α-syn seeds. A pool of selected iNPH cases showing no seeding activity (0/8 positive replicates) was used to dilute the samples. Specifically, twofold and threefold serial dilutions were performed, depending on the concentration of the sample seeds. We first tested all samples at 1:100 dilution as a reference. When the number of positive replicates at the '1:100' dilution was inferior or equal to 3/8, we applied a twofold dilution starting from the undiluted "neat" sample (e.g., 1:2, 1:4, 1:8, 1:16, etc.). All the other samples followed the threefold serial dilution starting from the 1:100 diluted sample (e.g., 1:300, 1:900, 1:2700, 1:8100, etc.). The serial dilution series were tested in both cases until the ED RT-QuIC yielded ≤ 2 out of 8 positive replicates. When the threefold dilution resulted in fewer than three "positive" outcomes (i.e., more than 2 out of 8 positive replicates), the samples were also diluted 1:2 to obtain at least four positive dilutions for each sample to estimate the α-syn seed concentration.

For the enhanced quantitation of pathological α-syn in patients' CSF, we used the midSIN mathematical algorithm (available open source at https://midsin.physics.ryerson.ca). It provides an estimate of the SD50, precisely the seeding dose that gives 50% of positive replicate wells, based on the total volume of the well (0.1 ml), the number of dilutions, the dilution factor, the number of the wells (8), and the specific positive replicates at each loaded dilution [15, 43]. Specifically, for the threefold dilution series, the 'starting dilution' was 1:100 (0.01), and the dilution factor was 0.33. In contrast, the neat run represented the 'starting dilution' (1) for the twofold dilution series, and 0.5 was indicated as the dilution factor.

Statistical analyses

Statistical analyses were performed using GraphPad Prism V. 7 (GraphPad, La Jolla, CA, USA). SD50 values were $\log_{10}$-transformed in all statistical analyses. Since the data did not show a normal distribution, we used the Mann–Whitney or Kruskal–Wallis (followed by the Dunn–Bonferroni test) tests for group comparisons. Fisher's exact test was applied for categorical variables. The sensitivity of the α-syn RT-QuIC was calculated as the percentage of brain LB-positive participants who tested positive on the assay, while the assay's specificity was calculated based on the percentage of LB-negative participants testing negative on the assay. Spearman's rho coefficients were used to assess the association between $\log_{10}$SD50 values, brain LB-density scores, and clinical scale scores in LB-positive participants testing positive on the a-syn RT-QuIC (LB-positive/SAA-positive). Association analyses were also evaluated between $\log_{10}$SD50 and brain LB-density score values, as well as clinical scores, in subjects who did not exhibit significant (i.e., moderate or high) AD neuropathological changes (ADNC) (pure LBD).

To assess the performance of $\log_{10}$SD50 in predicting a high LBD burden, as well as the performance of $\log_{10}$SD50 alone or in combination with clinical parameters, in predicting an underlying USSLB and LPC neocortical LBD stage, we calculated diagnostic accuracy values using receiver-operating characteristic (ROC) curves. The cut-off values for continuous variables were determined using the Youden Index as the value that maximizes diagnostic accuracy. In the combination criteria, the included cut-off values for each variable were established through the Youden Index. Results are expressed as area under the curve (AUC) values and relative 95% confidence interval (CI). AUC values were compared through the DeLong test. Differences were considered statistically significant at $p < 0.05$.

Results

Prevalence of LB pathology in the whole cohort and performance of the CSF a-syn RT-QuIC

In the whole cohort, 168 (47.0%) participants showed LB pathology at autopsy neuropathological examination. Among them, according to the USSLB system, 62 (36.9%) were in the Neocortical stage (stage IV), 45 (26.8%) in the Brainstem/Limbic stage (stage III), 23 (13.7%) in a Limbic Predominant stage (stage IIb), and 30 (17.8%) in the Brainstem Predominant stage (stage IIa). Finally, eight participants (4.8%) showed focal LB pathology limited to the olfactory bulb and were staged as Olfactory Bulb-Only (stage I) (Table 1). The staging of LB-positive participants according to the LPC classification system is reported in Table 1. Additional neuropathological data are reported in Supplementary Table 1.

The CSF α-syn RT-QuIC tested positive in 149 LB-positive participants (LB-positive/SAA-positive), resulting in an overall sensitivity of 88.7%, with significant variability according to the LB stage. Specifically, the sensitivity was 100% in the neocortical-stage participants, 95.5% in those at the brainstem/limbic stage, 78.3% in the limbic-predominant participants, and 73.3% in those at the brainstem-predominant stage. Finally, only half (50%) of the eight participants at the olfactory bulb-only stage showed CSF α-syn seeding activity. Similar sensitivity values were obtained when LPC stages were considered instead of USSLB stages (Table 1).

Moreover, the assay yielded a positive result in six participants who did not exhibit LB pathology at neuropathological examination (LB-negative), resulting in an overall assay specificity against positive brain LB pathology of 96.8%. The clinical and neuropathological characteristics of participants who showed discordant results at CSF α-syn

Table 1 Demographic data and CSF a-syn SAA results in the whole cohort stratified by LB-positivity and neuropathological stage

	n	Age at death, years[a]	Females[b]	*Postmortem* interval, hours[a]	α-syn SAA positive[b]	α-syn SAA negative[b]
LB-negative	189	88.0 (83.0–93.0)	97 (51.3)	3.2 (2.6–4.1)	6 (3.2)	183 (96.8)
LB-positive	168	85.0 (79.0–90.7)	56 (33.3)	3.0 (2.5–3.8)	149 (88.7)	19 (11.3)
Unified Staging System for Lewy Body Disorders						
Olfactory bulb-only (Stage I)	8	90.5 (82.2–95.7)	3 (37.5)	2.9 (2.2–3.7)	4 (50.0)	4 (50.0)
Brainstem predominant (Stage IIa)	30	91.0 (85.5–96.0)	12 (40.0)	3.1 (2.5–4.0)	22 (73.3)	8 (26.7)
Limbic predominant (Stage IIb)	23	85.0 (80.0–89.0)	11 (47.8)	2.9 (2.4–4.2)	18 (78.3)	5 (21.7)
Brainstem/Limbic (Stage III)	45	84.0 (77.5–90.0)	11 (24.4)	3.2 (2.5–4.0)	43 (95.5)	2 (4.5)
Neocortical (Stage IV)	62	82.0 (78.0–87.0)	19 (30.6)	3.0 (2.4–3.7)	62 (100.0)	0 (0.0)
LP consensus criteria						
Olfactory only	7	91.0 (86.0–97.0)	3 (42.8)	3.2 (2.2–3.7)	3 (42.8)	4 (57.1)
Amygdala predominant	11	81.0 (78.0–85.0)	4 (36.4)	3.2 (2.5–4.5)	6 (54.5)	5 (45.4)
Brainstem predominant	24	91.5 (90.0–95.5)	12 (50.0)	3.2 (2.5–3.9)	15 (62.5)	9 (37.5)
Limbic	25	88.0 (84.0–92.0)	10 (40.0)	2.8 (2.5–4.2)	24 (96.0)	1 (4.0)
Neocortical	101	82.0 (78.0–87.0)	27 (26.7)	3.0 (2.4–3.7)	101 (100.0)	0 (0.0)

a: expressed as median (interquartile range); b: expressed as a fraction of total (percentage)

α-syn alpha-synuclein, *LB* Lewy Body, *LP* Lewy Pathology, *SAA* seed amplification assay

RT-QuIC and neuropathological examination are reported in Supplementary Tables 2–3.

LB-density scores and SD50 values according to LB stage and their associations

Median LB-density score values in all LB-positive participants, stratified by neuropathological stage, are reported in Table 2. Semiquantitative scores of LB pathology in the different sampled brain areas are reported in Supplementary Table 4. LB-density score values significantly differed among LB-positive participants at different neuropathological stages, as assessed by both staging criteria ($p < 0.0001$) (Table 2).

In each SAA-positive participant, CSF α-syn seeds were quantified through the midSIN algorithm, as described in

Table 2 LB-density score and SD50 values and their correlation in the whole LB-positive/SAA-positive group and stratified by neuropathological stage

	n	LB-density score[a]	$\log_{10}$SD50[a]	Rho[b]	*p* value
LB-positive/SAA-positive	**149**	**26.0 (14.0–32.5)**	**3.96 (2.93–4.56)**	**0.62 (0.50–0.71)**	**< 0.0001**
Unified Staging System for Lewy Body Disorders					
Olfactory bulb-only (Stage I)	4	2.5 (1.2–3.7)	1.90 (0.79–2.82)	0.80 (-)	0.33
Brainstem predominant (Stage IIa)	22	10.0 (6.7–15.7)	3.53 (1.98–3.96)	0.59 (0.21–0.81)	**0.004**
Limbic predominant (Stage IIb)	18	9.5 (6.7–13.0)	1.71 (1.48–2.24)	0.07 (−0.41–0.53)	0.77
Brainstem/Limbic (Stage III)	43	23.0 (21.0–27.0)	3.93 (3.31–4.51)	0.20 (−0.11–0.48)	0.19
Neocortical (Stage IV)	62	33.0 (30.2–35.0)	4.37 (3.98–4.84)	0.08 (−0.18–0.33)	0.53
LP consensus criteria					
Olfactory only	3	2.0 (1.0–3.0)	1.67 (0.50–3.05)	1.00 (-)	0.33
Amygdala predominant	6	5.5 (4.7–7.2)	2.01 (1.48–2.97)	−0.38 (-)	0.47
Brainstem predominant	15	9.0 (6.0–11.0)	2.17 (1.45–3.48)	0.44 (−0.11–0.78)	0.10
Limbic	24	13.0 (11.0–18.0)	2.85 (1.60–3.95)	0.47 (0.07–0.74)	**0.02**
Neocortical	101	30.0 (25.5–33.0)	4.23 (3.72–4.68)	0.29 (0.09–0.46)	**0.003**
LB-positive/SAA-negative	19	2.0 (1.0–4.0)	–	–	–
LB-negative/SAA-positive	6	–	1.35 (1.09–2.23)	–	–

a: expressed as median (interquartile range); b: expressed as main value (95% confidence interval)

P values of statistically significant associations are reported in bold. *LB* Lewy Body, *LP* Lewy Pathology, *SAA* seed amplification assay

the methods, and the corresponding SD50 values were calculated.

SD50 scores significantly differed among participants at different LBD neuropathological stages as defined by both staging criteria ($p < 0.0001$) (Fig. 1). Post hoc analyses for USSLB staging revealed higher values in neocortical participants compared to those staged as olfactory bulb-only ($p = 0.003$), brainstem predominant, and limbic predominant ($p < 0.0001$). Moreover, subjects at the brainstem/limbic stage had significantly higher SD50 values than those in the limbic-predominant stage ($p < 0.0001$). As for LPC staging, neocortical participants displayed higher SD50 than those in every other stage (p value: vs limbic and brainstem predominant $p < 0.0001$; vs amygdala-predominant $p = 0.002$; vs olfactory only $p = 0.04$). Considering that the analyses were conducted using the $\log_{10}$-transformed, it must be emphasized that a one-unit difference reflects a tenfold difference in the underlying seed concentration between groups.

In the whole LB-positive/SAA-positive subgroup, the SD50 showed a strong positive association with summary brain LB-density scores (Rho = 0.62, 95%CI 0.50–0.71), $p < 0.0001$) (Fig. 1). SD50 values were also positively associated with the semi-quantitative scoring of LB pathology severity in every sampled brain area, with the temporal neocortex (Rho = 0.60, $p < 0.0001$), the cingulate cortex (Rho = 0.59, $p < 0.0001$), and the locus coeruleus (Rho = 0.59, $p < 0.0001$) showing the strongest associations (Supplementary Table 5).

LB-density scores in the LB-positive/SAA-negative group and SD50 values in the LB-negative/SAA-positive group are reported in Table 2. LB-positive/SAA-negative participants displayed significantly lower LB-density scores than LB-positive/SAA-positive participants ($p < 0.0001$). SD50 values of LB-negative/SAA-positive individuals were lower than those of LB-positive/SAA-positive participants ($p < 0.0001$).

Kinetic curves of α-syn RT-QuIC at increasing dilutions from sample participants at different LBD neuropathological stages are shown in Fig. 2.

Accuracy of SD50 values in predicting an underlying high LBD burden

When stratifying LB-positive/SAA-positive participants in tertiles according to their LB-density scores, SD50 yielded a high value in identifying those with a high LB burden (i.e., the second and third tertiles) (AUC 0.85, 95%CI 0.79–0.92; sensitivity 76.0%, 95%CI 67.0–83.0; specificity 79.0%, 95%CI 67–0–88.0; cut-off > 3.7). The accuracy was also good when limiting the identification to those in the third tertile (AUC 0.77, 95%CI 0.70–0.85; sensitivity 87.0%, 95%CI 74.0–94.0; specificity 58.0%, 95%CI 49–0–67.0; cut-off > 3.8) or those above the median LB-density score (AUC

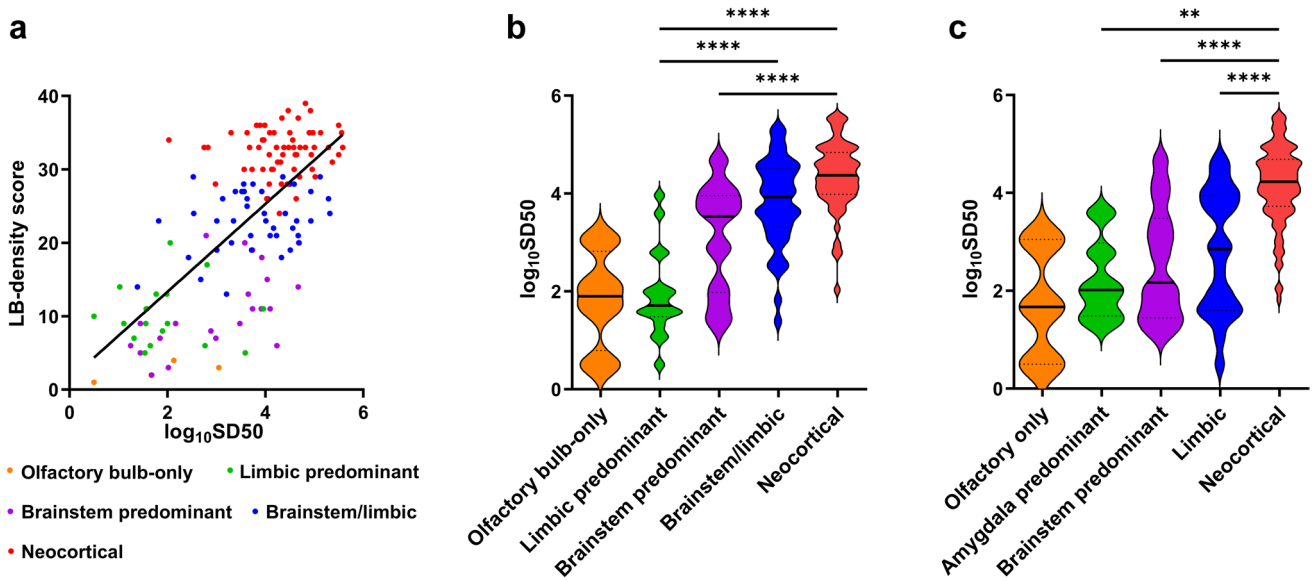

Fig. 1 $\log_{10}$SD50 values in the LB-positive/SAA-positive group stratified by LBD stage. **a** Association between $\log_{10}$SD50 and LB-density score values (Rho = 0.62, 95%CI 0.50–0.71); $p < 0.0001$); participants are shown in different colors according to their USSLB LBD stage: olfactory bulb-only (orange), brainstem predominant (purple), limbic predominant (green), brainstem/limbic (blue), and neocortical (red). **b** Distribution of $\log_{10}$SD50 values across participants at different USSLB LBD stages. **c** Distribution of $\log_{10}$SD50 values across participants at different LPC LBD stages; horizontal solid lines represent the median values, and horizontal dashed lines represent the 25th and the 75th percentiles. *LB* Lewy Body, *LBD* Lewy Body disease, *LPC* Lewy Pathology consensus criteria, *SAA* seed amplification assay, *USSLB* Unified Staging System for Lewy Body Disorders

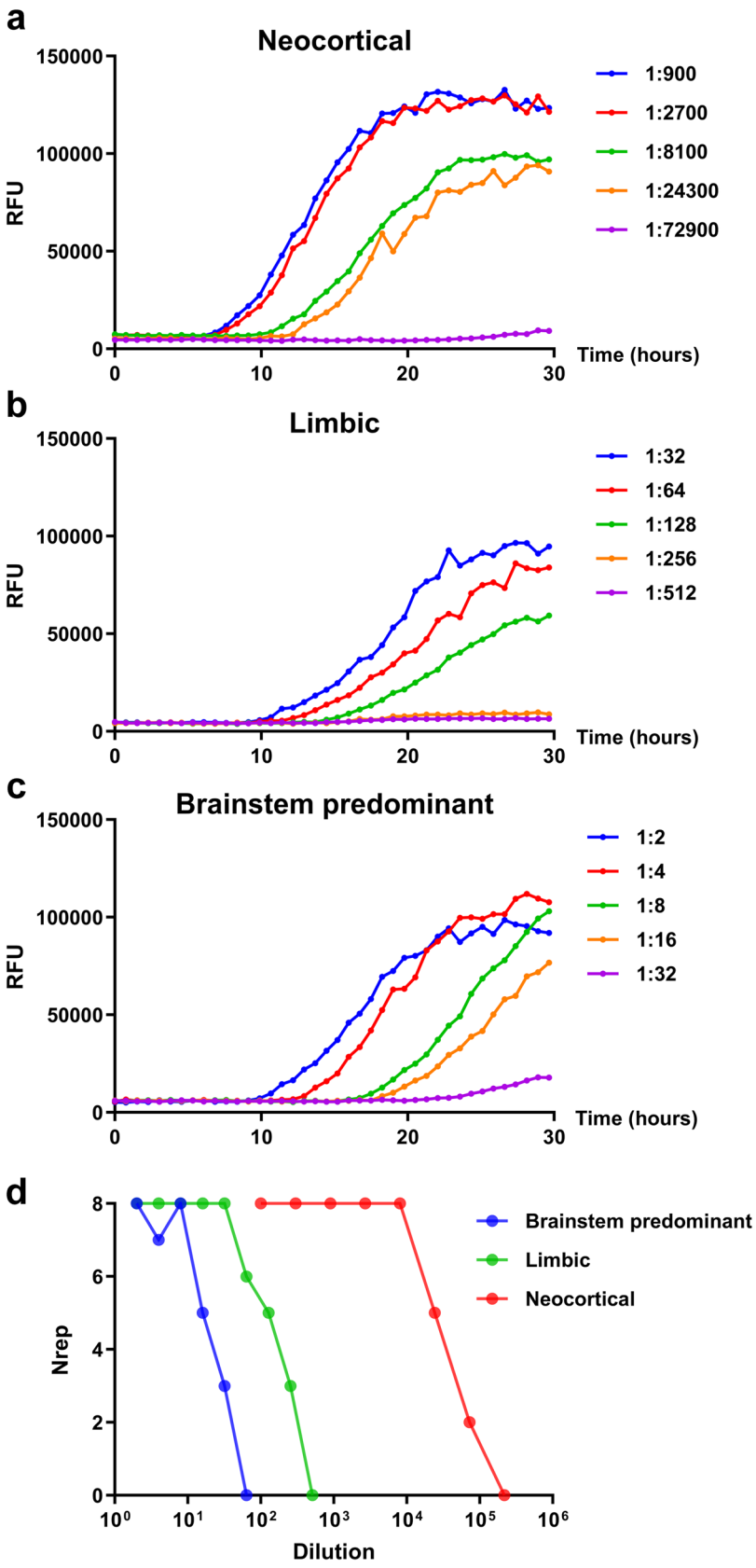

Fig. 2 CSF seeding activity at increasing dilutions in LBD participants at different LPC stages. A-syn RT-QuIC kinetic curves at increasing dilutions in a participant at neocortical stage (**a**: $\log_{10}$SD50 5.50, LB-density score 36), limbic stage (**b**: $\log_{10}$SD50 3.01, LB-density score 23) and brainstem-predominant stage (**c**: $\log_{10}$SD50 2.17, LB-density score 9). For each dilution, the median value of fluorescent intensity of eight replicates is shown, and interquartile range values are hidden for graphical reasons; only the kinetic curves with the five highest dilution factors are shown. **d** Number of positive replicates at increasing dilutions in the three above-described participants; samples from the neocortical participant were diluted by a threefold factor, while those from the limbic and brainstem-predominant participants by a twofold factor. Dilution factors are plotted as powers of ten. *a-syn* alpha-synuclein, *CSF* cerebrospinal fluid, LB Lewy Bodies, *LBD* Lewy Body Disease, *LPC* Lewy Pathology consensus criteria, *Nrep* number of positive replicates, *RFU* relative fluorescent units

0.79, 95%CI 0.72–0.86; sensitivity 82.0%, 95%CI 71.0–89.0; specificity 63.0%, 95%CI 52–0–73.0; cut-off > 3.7).

Association of SD50 values with measures of clinical severity

Clinical and neuropathological data for the LB-positive/SAA-positive participants are reported in Table 3 and Supplementary Tables 6–9. Associations between LB-density scores and the last available scores on the MMSE, UPDRS Part III, and UPSIT scales are reported in Supplementary Tables 10 and 11. Details on the median time between the last available clinical scores and death are reported in Supplementary Table 12.

In the whole LB-positive/SAA-positive group, SD50 values were positively associated with the last available scores at UPDRS part III (Rho = 0.33, $p < 0.0001$) and UPSIT (Rho = − 0.33, $p < 0.0001$). In contrast, we found no significant association between SD50 and MMSE scores in the LB-positive/SAA-positive group ($p = 0.91$). The exclusion of participants with a significant AD co-pathology did not significantly affect the results of the associations (Table 4).

Prediction of an underlying LBD neocortical stage in participants with different clinical diagnoses

When considering all LB-positive/SAA-positive participants, SD50 yielded an AUC of 0.80 in distinguishing subjects harboring LB pathology at a USSLB neocortical stage from those at lower stages (sensitivity 85.0%, 95%CI 75.0–92.0; specificity 66.0%, 95%CI 55.0–75.0; cut-off > 3.8). This was significantly higher than that obtained in the presence of dementia alone (AUC 0.65, 95%CI 0.56–0.74; $p = 0.006$). Combining SD50 values and UPSIT provided the highest prediction power (AUC 0.81, 95%CI 0.74–0.89; sensitivity 81.0%, 95%CI 69.0–89.0; specificity 82.0%, 95%CI 72.0–88.0) among the examined parameters (Table 5). The accuracy of SD50 alone was even higher when identifying LPC neocortical participants (AUC 0.86, 95%CI 0.79–0.92; sensitivity 89.0%, 95%CI 82.0–94.0; specificity 69.0%, 95%CI 55.0–80.0; cut-off > 3.3) (Supplementary Table 13).

In the subgroup of participants with a clinical diagnosis of PD, SD50 obtained an AUC of 0.70 in predicting an underlying USSLB neocortical LBD stage (sensitivity 93.0%, 95%CI 80.0–97.0; specificity 41.0%, 95%CI 26.0–59.0; cut-off > 3.8), which was higher than that of any clinical predictor. The combination of SD50 values and UPSIT scores slightly improved the prediction power (AUC 0.74, 95%CI 0.62–0.87; sensitivity 80.0%, 95%CI 65.0–90.0; specificity 69.0%, 95%CI 51.0–83.0), with the comparison with the AUC of the UPSIT score alone being statistically significant ($p = 0.01$) (Table 5).

In the participants with a clinical diagnosis of AD dementia, SD50 values distinguished those with an underlying USSLB neocortical LBD from subjects at lower stages with high accuracy (AUC 0.87, 95%CI 0.73–1.00); sensitivity 100%, 95%CI 68.0–100; specificity 67.0%, 95%CI 45.0–83.0; cut-off > 2.0). The combination of SD50 values and UPSIT scores slightly improved the diagnostic accuracy (AUC 0.88, 95%CI 0.76–1.0; sensitivity 100%, 95%CI

Table 3 Clinical and neuropathological characteristics of the whole LB-positive/SAA-positive group, stratified by clinical diagnosis

	Whole LB-positive/SAA-positive ($n = 149$)	PD ($n = 69$)	AD dementia ($n = 29$)	DLB ($n = 13$)
Age at death[a]	85.0 (79.0–90.0)	81.0 (76.5–85.5)	89.0 (79.0–93.5)	85.0 (78.0–88.0)
MMSE[a]	24.0 (17.0–27.0), 147	25.0 (19.0–27.0), 69	12.5 (7.0–21.7), 28	18.0 (12.2–21.7), 12
UPDRS part III[a]	23.5 (7.0–42.0), 127	40.0 (28.0–51.2), 49	11.2 (6.2–25.0), 28	34.5 (14.5–50.2), 12
UPSIT[a]	14.0 (10.0–19.0), 149	12.0 (10.0–16.0), 69	13.0 (10.5–21.5), 29	11.0 (9.0–14.0), 13
Dementia, *n* (%)	94 (63.1)	42 (60.9)	29 (100)	13 (100)
AD staging, *n* (%)				
Non-AD	27 (18.1)	16 (23.2)	1 (3.4)	1 (7.7)
Low ADNC	16 (10.7)	14 (20.3)	0 (0.0)	0 (0)
Intermediate ADNC	68 (45.6)	31 (44.9)	7 (24.1)	10 (76.9)
High ADNC	30 (20.1)	1 (1.4)	21 (72.4)	2 (15.4)
Not available	8 (5.4)	7 (10.1)	0 (0)	0 (0)

a: expressed as median (interquartile range), participants with available data

AD Alzheimer's disease, *ADNC* Alzheimer's disease neuropathologic changes, *LBD* Lewy Body disease, *MMSE*, Mini-Mental State Examination, *PD* Parkinson's disease, *UPDRS* Unified Parkinson's Disease Rating Scale; *UPSIT* University of Pennsylvania Smell Identification Test

Table 4 Associations between SD50 values and scores at clinical scales in the whole LB-positive/SAA-positive group and stratified by clinical diagnoses and AD co-pathology

	MMSE			UPDRS part III			UPSIT		
	n	Rho[a]	*p* value	*n*	Rho[a]	*p* value	*n*	Rho[a]	*p* value
LB-positive/SAA-positive									
Whole (*n* = 149)	147	− 0.01 (− 0.17–0.16)	0.91	127	0.33 (0.17–0.48)	**< 0.0001**	149	− 0.33 (− 0.47 to − 0.17)	**< 0.0001**
Pure LBD (*n* = 43)	42	− 0.15 (− 0.44–0.17)	0.35	32	0.36 (0.002–0.64)	**0.04**	43	− 0.39 (− 0.62 to − 0.09)	**0.009**
Parkinson's disease									
Whole (*n* = 69)	69	− 0.11 (− 0.34–0.13)	0.36	49	0.09 (− 0.21–0.37)	0.55	69	− 0.11 (− 0.34–0.14)	0.38
Pure LBD (*n* = 30)	30	− 0.02 (− 0.39–0.35)	0.90	19	− 0.10 (− 0.54–0.38)	0.68	30	− 0.21 (− 0.54–0.17)	0.26
Dementia with Lewy Bodies									
Whole (*n* = 13)	12	0.15 (− 0.48–0.68)	0.64	12	0.31 (− 0.33–0.76)	0.32	13	0.15 (− 0.45–0.66)	0.63
Pure LBD (*n* = 1)	0	–	–	1	–	–	1	–	–

Bold highlight the statistically significant associations

a: expressed as main value (95% confidence interval)

AD Alzheimer's disease, *LB* Lewy Bodies, *LBD* Lewy Body disease, *MMSE* Mini-Mental State Examination, *SAA* seed amplification assay, *UPDRS* Unified Parkinson's Disease Rating Scale, *UPSIT* University of Pennsylvania Smell Identification Test

68.0–100); specificity 76.0%, 95%CI 55.0–89.0) (Table 5). High AUC, and sensitivity and specificity values were also obtained when defining neocortical participants according to the LPC staging (Supplementary Table 13).

Discussion

The search and validation of biomarkers that predict the stage and burden of the underlying neuropathological process are research priorities in neurodegenerative diseases. In a previous study, we demonstrated that α-syn RT-QuIC, the only pathology-specific in vivo marker available for synucleinopathies, executed on serially diluted brain homogenates, provides an estimate of the α-syn seed concentration that positively correlates with the LBD load evaluated through immunohistochemistry [8]. In this work, we tested whether a "quantitative" approach is feasible and reliable when using CSF. The results demonstrate that seed quantification based on endpoint dilution RT-QuIC applied to CSF samples enables a reliable prediction of the burden and extent of the underlying LBD. Specifically, SD50 values showed a strong positive association with the brain LB-density score, a measure of LBD burden detected by immunohistochemistry, and with LBD stages, with more advanced LBD pathology corresponding to higher SD50 values (i.e., higher seeding activity). This suggests that, in patients with LBD, α-syn seeds are effectively released in the CSF to an extent proportionate to the burden of the underlying proteinopathy. Notably, LBD patients with more extensive pathology, as expected, displayed a more severe pathology in each different brain region, as expressed by LB pathology semi-quantitative severity scores, and these were also significantly associated with SD50 values.

When examined in more detail in participants stratified by the LBD stage, SD50 values and LB-density scores showed a significant positive association only in those at the USSLB brainstem-predominant stage and those at the LPC limbic and neocortical stages. The small sample size in some groups must be considered when interpreting these results. However, the suboptimal correlations likely also reflect the heterogeneity of LB scores across groups defined by the two classification systems, affecting the discriminative power of SD50 values.

Besides the association with pathology severity, we demonstrated significant correlations between the SD50 values and the last available clinical scale scores, such as the UPDRS part III for motor impairment and the UPSIT for olfactory dysfunction. Notably, these associations remained significant in the whole LB-positive/SAA-positive group, even after excluding subjects with significant AD co-pathology, but not after stratification by clinical diagnoses. This is probably due to the limited number of participants in each subgroup. Moreover, it is relevant that even the associations between clinical scale scores and LB-density scores almost failed to reach statistical significance. Unlike motor and olfactory impairment, which are more specifically linked to LBD, cognitive impairment (as measured by the MMSE

Table 5 Performance of SD50 and clinical variables, alone or in combination, in the identification of participants at a USSLB neocortical stage in the whole LB-positive/SAA-positive group and stratified by clinical diagnoses

	Cut-off	AUC (95%CI)	Sensitivity (95%CI)	Specificity (95%CI)
Whole LB-positive/SAA-positive group ($n = 149$)				
log_{10}SD50 alone	> 3.8	0.80 (0.73–0.87)	85.0 (75.0–92.0)	66.0 (55.0–75.0)
Dementia	–	0.65 (0.56–0.74)[a]	81.0 (69.0–89.0)	49.0 (39.0–60.0)
UPSIT score	< 17	0.73 (0.65–0.81)	94.0 (85.0–97.0)	52.0 (41.0–62.0)
log_{10}SD50 > 3.8 and dementia	–	0.74 (0.65–0.82)	66.0 (54.0–77.0)	82.0 (72.0–88.0)
log_{10}SD50 > 3.8 and UPSIT < 17	–	0.81 (0.74–0.89)[b]	81.0 (69.0–89.0)	82.0 (72.0–88.0)
Dementia and UPSIT < 17	–	0.74 (0.66–0.82)	79.0 (67.0–87.0)	69.0 (59.0–78.0)
Parkinson's disease ($n = 69$)				
log_{10}SD50 alone	> 3.8	0.70 (0.57–0.82)	93.0 (80.0–97.0)	41.0 (26.0–59.0)
Dementia	–	0.61 (0.47–0.75)	70.0 (55.0–82.0)	52.0 (34.0–69.0)
UPSIT score	< 16	0.60 (0.45–0.75)[c]	85.0 (71.0–93.0)	48.0 (31.0–66.0)
log_{10}SD50 > 3.8 and dementia	–	0.64 (0.51–0.77)	63.0 (47.0–76.0)	66.0 (47.0–80.0)
log_{10}SD50 > 3.8 and UPSIT < 16	–	0.74 (0.62–0.87)	80.0 (65.0–90.0)	69.0 (51.0–83.0)
Dementia and UPSIT < 16	–	0.67 (0.55–0.80)	63.0 (47.0–76.0)	72.0 (54.0–85.0)
Alzheimer's disease dementia (n = 29)				
log_{10}SD50 alone	> 2.0	0.87 (0.73–1.0)	100 (68.0–100)	67.0 (45.0–83.0)
Dementia	–	–	–	–
UPSIT score	< 15	0.79 (0.63–0.96)	100 (68.0–100)	52.0 (32.0–72.0)
log_{10}SD50 > 2.0 and dementia	–	0.83 (0.69–0.98)	100 (68.0–100)	67.0 (45.0–83.0)
log_{10}SD50 > 2.0 and UPSIT < 15	–	0.88 (0.76–1.0)[d]	100 (68.0–100)	76.0 (55.0–89.0)
Dementia and UPSIT < 15	–	0.76 (0.59–0.93)	100 (68.0–100)	52.0 (32.0–72.0)

a: Statistically significant comparison (DeLong Test): vs log_{10}SD50 alone, log_{10}SD50 and UPSIT, dementia and UPSIT ($p < 0.001$); vs log_{10}SD50 and dementia ($p = 0.009$). b: Statistically significant comparison (DeLong Test): vs UPSIT score ($p = 0.03$); vs log_{10}SD50 and dementia ($p = 0.02$). c: Statistically significant comparison (DeLong Test): vs log_{10}SD50 and UPSIT ($p = 0.01$). d: Statistically significant comparison (DeLong Test): vs dementia and UPSIT ($p = 0.01$)

Only statistically significant comparisons are shown

AUC area under the curve, *CI* confidence interval, *LB* Lewy Bodies, *SAA* seed amplification assay, *UPSIT*, University of Pennsylvania Smell Identification Test, *USSLB* Unified Staging System for Lewy Body disorders

scale) was not associated with SD50 values in any analysis. This result may be explained by the multifactorial nature of cognitive impairment in patients with LBD [13, 23, 26, 42], where co-pathologies, such as AD and vascular disease, also play a significant role. In this scenario, the significant negative association between LB-density score and MMSE we obtained in the whole LB-positive/SAA-positive group may reflect the high prevalence of high-grade AD co-pathology, which may be a key driver of lower MMSE scores, although a recent study from the AZSAND cohort, but with a larger sample size, found a statistically significant association between the two variables, independent of AD pathology [7]. Other cognitive measures might show stronger associations, as the previous studies have suggested that the MMSE may not be the most appropriate tool for assessing cognitive impairment related to LBD. Further studies should investigate the associations between SD50 and other scales of cognitive dysfunction, with a focus on executive and visuospatial abilities, such as the Montreal Cognitive Assessment (MoCA).

Our analysis of the performance of the SD50 values, alone or in combination with clinical parameters, for distinguishing participants at neocortical-stage LBD or with high LBD burden from those with less-advanced pathology warrants discussion. The spread of LBD to the neocortex represents the final stage of this proteinopathy, is associated with the development of cognitive impairment, and has, therefore, independent prognostic implications [17, 20, 28, 42]. However, clinicopathological studies demonstrate that the presence of dementia alone in patients with clinical LBD is neither sensitive nor specific to neocortical-stage pathology [12, 26, 28, 35]. Our results support this finding, but also show that SD50 values, either alone or in combination with scores of olfactory impairment, are highly effective in identifying participants at a neocortical stage, outperforming the presence of dementia or UPSIT scores. This was true in the whole LB-positive/SAA-positive group, including clinically unimpaired participants, representative of the general population, as well as in participants with a clinical diagnosis of PD or AD dementia, albeit not reaching

statistical significance. Notably, in participants with a clinical diagnosis of AD dementia, both SD50 and the combination SD50/UPSIT yielded full sensitivity (100%). Moreover, SD50 values accurately identified participants with a high burden of proteinopathy. The utility of olfactory function scores in identifying participants with LBD is well known from clinicopathological studies [5, 45]. Moreover, a recent study suggested that worsening olfactory function may be a surrogate biomarker of LBD progression [32]. Our data expand on these results and demonstrate that, in a cohort which is virtually representative of the general population, quantifying α-syn seeds in CSF, alone or in combination with olfactory function assessment, may help identify LBD participants at a neocortical stage in research settings. This would be relevant in clinical trials of disease-modifying agents for PD, with participants showing LBD at a higher stage or burden possibly benefiting the least from therapies, and in AD dementia, where subjects with advanced LBD co-pathology may warrant exclusion from initial recruitment.

As an ancillary result, we confirmed here the high specificity of our RT-QuIC assay in another neuropathological cohort by showing that only six participants (3.2%) without LB pathology tested positive on the assay. Notably, these subjects had low SD50 values, comparable to those obtained in LB-positive/SAA-positive participants at LBD stage I, primarily exhibiting focal pathology restricted to the olfactory bulb. Therefore, it is possible that our "false positive" subjects may indeed show focal LB pathology in some central nervous system regions due to the sparse LB-density.

Moreover, this study confirms that a low LBD burden and/or stage is the primary determinant of incomplete α-syn RT-QuIC sensitivity [8, 22]. Indeed, all our LB-positive/SAA-negative participants exhibited a low LBD burden, with LB-density scores comparable to those of LB-positive/SAA-positive individuals at USSLB stage I, and most were classified as USSLB stage I, IIa, or IIb. Regarding the diagnostic performance of α-syn CSF RT-QuIC in patients in the latter group, also known as amygdala-predominant LBD, previous studies involving both *antemortem* and *postmortem* CSF samples reported a reduced sensitivity, with values ranging from 14.3 to 63.6% [1, 8, 22]. In our cohort, α-syn RT-QuIC yielded a sensitivity of 78.3%, comparable to that of a previous study using the same staging approach for LBD [22]. However, when considering only participants with LBD strictly confined to the limbic system (i.e., an amygdala-predominant stage according to the LPC), the test's sensitivity in our cohort dropped to 54.5%, which is similar to that reported in the other studies [1, 8]. Further studies are required to define other factors, besides the low LBD burden, that influence the limited assay sensitivity in LBD participants with focal limbic pathology.

A major strength of this study is the inclusion of a large, clinically well-characterized cohort with *postmortem* neuropathological analysis across different stages of LBD, including clinically unimpaired subjects. Another key strength is the robustness of our α-syn RT-QuIC assay, as shown here and in the previous works, along with the meticulous dilution process performed on over 150 CSF samples.

The use of *postmortem* ventricular CSF instead of in vivo collected samples represents a potential limitation of this work. Although there is currently no convincing evidence that the composition of lumbar CSF significantly differs from that at the ventricular level, postmortem CSF may undergo death-related modifications, potentially altering protein concentrations [4, 24, 40]. This may also affect α-syn seed concentrations, which are likely lower in *antemortem* sampled CSF, as recently suggested [21, 43]. Finally, we cannot fully exclude the possibility of contamination of CSF samples through the needle passing through the corpus callosum. However, based on our extensive experience with LBD staging using α-syn immunohistochemistry, LB pathology is not significantly present in the deep white matter, including the corpus callosum. Further studies based on in vivo sampled CSF should replicate the results obtained in the present study. Another technical issue that potentially limits the applicability of the present SAA protocol concerns the need for large amounts of α-syn-negative CSF for the endpoint dilutions. In this work, we used CSF samples from NPH patients who typically undergo evacuative lumbar puncture, but we recognize that the availability of such samples may be limited. Further studies should investigate the impact of other types of diluting matrices, such as artificial CSF, on the assay kinetics and the accuracy of seed quantification.

In conclusion, we describe the first pathology-specific approach for LBD staging based on a biological fluid which can be obtained in vivo. Endpoint dilution RT-QuIC from periventricular CSF provided an accurate quantification of α-syn seeds, strongly correlating with the LBD stage and burden, as evaluated through immunohistochemistry, and was associated with clinical severity scores. The SD50 values, alone or in combination with clinical scales of olfactory impairment, showed a high value in identifying LBD participants at a neocortical stage and may be used in research settings for distinguishing subjects with advanced pathology from those at earlier LBD stages.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00401-025-02904-4.

Author contributions Conceptualisation and design of the study: PP and OH. Drafting/revising the manuscript for content, including medical writing for content: AMas, SC, and PP. Performance of neuropathological analyses and data preparation: GES and TB. Performance of clinical assessments: HS, AA, ED-D, SM, and CHA. αSyn RT-QuIC SAA experiments: SC, EV, AMam, and MR. Major role in the acquisition, analysis, or interpretation of data: AMas, SC, SEM, EV, SJ, MS,

SB, GES, TB, OH, and PP. Statistical analysis: AMas and SC. Critical review of the manuscript and approval of the final version: all authors.

Funding The study was supported by the grants Ricerca Finalizzata-2021–12374386, PNRR-MCNT2-2023–12378190, and "Ricerca Corrente" funded by the Ministero della Salute, and the #NextGenerationEU (NGEU), funded by the Ministero dell'Università e della Ricerca (MUR), National Recovery and Resilience Plan (NRRP), project MNESYS (PE0000006). The Arizona Study of Aging and Neurodegenerative Disorders/Brain and Body Donation Program at Banner Sun Health Research Institute, Sun City, Arizona has been supported by the National Institute of Neurological Disorders and Stroke (U24 NS072026 National Brain and Tissue Resource for Parkinson's Disease and Related Disorders), the National Institute on Aging (P30 AG019610 and P30AG072980, Arizona Alzheimer's Disease Center), the Arizona Department of Health Services (contract 211002, Arizona Alzheimer's Research Center), the Arizona Biomedical Research Commission (contracts 4001, 0011, 05–901, and 1001 to the Arizona Parkinson's Disease Consortium), and the Michael J. Fox Foundation for Parkinson's Research. The funding sources had no role in the design and conduct of the study.

Data availability The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interests OH is employed by Lund University and Eli Lilly.

Ethical approval The operations of the Banner Sun Health Research Institute (SHRI) Brain and Body Donation Program have been approved by the WCG Institutional Review Board (IRB) of Puyallup, Washington, USA [4]. All enrolled subjects signed the IRB-approved informed consent.

Consent for publication Not applicable.

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