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RESEARCH ARTICLE

Toward alpha-synuclein seed amplification assay in clinical practice

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Abstract

INTRODUCTION: Seed amplification assays (SAAs) demonstrate remarkable diagnostic performance in alpha-synucleinopathies. However, existing protocols lack accessibility in routine laboratories, mainly due to the requirement for in-house production of recombinant alpha-synuclein (aSyn). This study proposes a cerebrospinal fluid (CSF) aSyn-SAA protocol using solely commercial reagents to facilitate its clinical implementation.

METHODS: Routine clinical care CSF samples from 126 patients, comprising 47 with Lewy body diseases (LBD) (41 with dementia with Lewy bodies, six with Parkinson's disease), 37 without alpha-synucleinopathy, and 42 with Alzheimer's disease (AD), underwent assessment for aSyn-SAA activity.

RESULTS: CSF aSyn-SAA showed a sensitivity of 72.3% and a specificity of 100% when distinguishing clinically diagnosed LBD patients from those without alpha-synucleinopathy. In AD patients, 14.3% were tested positive for aSyn.

DISCUSSION: The commercial-only CSF aSyn-SAA protocol exhibited excellent specificity when applied to a real-life cohort, signaling progress toward the accessibility of an aSyn biomarker in clinical settings.

Mathieu Verdurand and Flora Kaczorowski contributed equally to this study.

KEYWORDS
alpha-synuclein (α-synuclein), aSyn, biomarker, brain homogenate (BH), cerebrospinal fluid (CSF), dementia with Lewy bodies (DLB), Lewy body disease (LBD), neurodegenerative diseases, Parkinson's disease (PD), proteinopathies, real-life cohort, real-time quaking-induced conversion (RT-QuIC), seed amplification assay (SAA), synucleinopathies

Highlights

- Diagnosis of LBD through aSyn-SAA lacks accessibility.
- This commercial-only aSyn-SAA has satisfactory performance in a real-life cohort.
- A negative aSyn-SAA does not completely exclude a synucleinopathy.
- Some technical points must be considered when developing aSyn-SAA.
- aSyn-SAA must be confined to expert laboratories due to prion-like risk management.

1 | INTRODUCTION

Alpha-synucleinopathies, encompassing dementia with Lewy bodies (DLB), Parkinson's disease (PD), and multiple system atrophy (MSA), are neuropathologically defined by the abnormal accumulation and deposition of alpha-synuclein (aSyn) protein aggregates within the brain. The definitive diagnosis of alpha-synucleinopathies relies on the identification of aSyn aggregates in *post mortem* brain tissue. However, the absence of a consensus biomarker for aSyn during the patient's lifetime represents a significant challenge.

The consequence in clinical practice is that the diagnosis of alpha-synucleinopathies predominantly relies on symptoms and presents difficulties given the clinical heterogeneity and phenotypic overlap with other neurodegenerative conditions. This is particularly the case when considering DLB, the second most prevalent neurodegenerative dementia following Alzheimer's disease (AD). Additionally, the well-documented frequent coexistence of AD-DLB co-pathology[1–3] underscores the need for caution, as positive biomarkers for AD do not necessarily exclude the presence of concurrent aSyn pathology, particularly in older individuals. The presence of these co-pathologies has been shown to negatively impact disease progression.[4] Establishing an in vivo aSyn biomarker is therefore essential to improve patient management, to include them in forthcoming clinical trials targeting specific proteinopathies, and to better manage therapy-related adverse effects.

While cerebrospinal fluid (CSF) biomarkers for AD have been available for years with excellent diagnostic accuracy,[5] recent studies highlight the potential of seeding amplification assay (SAA) in diagnosing alpha-synucleinopathies. This technology enables the detection and amplification of aggregated proteins such as prion protein and, more recently, aSyn.[6,7] Since the introduction of real-time quaking-induced conversion (RT-QuIC) for aSyn, numerous scientific investigations have demonstrated its remarkable performance in detecting alpha-synucleinopathies, even in prodromal stages, with high accuracy.[8]

However, the lack of standardization and limited accessibility of in-house recombinant aSyn described in the literature have limited its widespread adoption even among expert hospital facilities, making it inaccessible for routine clinical use. Moreover, studies typically involve well-defined cohorts that may not fully represent the everyday clinical reality of patients undergoing medical care (real-life cohorts).

In light of these observations, we propose an aSyn-SAA protocol using solely commercially available reagents to facilitate its implementation and accessibility in clinical practice at a large scale. Following successful validation of this protocol with human brain tissues, we assessed its potential using CSF samples from real-life clinically diagnosed patients (including DLB, PD, AD, and non-alpha-synucleinopathies) undergoing follow-up in memory clinics and who received lumbar puncture (LP) as part of routine care management. The objective of this study was to evaluate the diagnostic performance of this aSyn-SAA commercial-only protocol and to assess whether there were any differences in clinical and biological parameters between groups.

2 | METHODS

For the pilot study on brain homogenates (BHs), please see Supplemental File 1.

2.1 | Selection criteria of CSF study population

The study enrolled 126 patients, each of whom provided written informed consent for the collection and use of their biological fluid samples for research. Approved by the Lyon University Hospital ethics committee (N° 19-42), the study used human biological samples and associated data obtained from a biobank authorized by the French Ministry of Health (DC-2008-304) and the Hospices Civils de Lyon Biobank (CSR-HCL BB-0033-00046). All patients underwent LP for CSF biomarker, including neurofilament light chain (NFL) measurements following comprehensive neurological examinations as part of routine care. Four main etiological groups were analyzed. The

"non-ASYN" group ($N = 37$) included patients with amyotrophic lateral sclerosis (ALS; $N = 13$), vascular dementia (VASC; $N = 7$), frontotemporal dementia (FTD; $N = 4$), psychiatric symptoms (PSY; $N = 4$), inflammatory CSF (INFLAM; $N = 4$), infectious CSF (INFECT; $N = 3$), intracerebral hemorrhage ($N = 1$), and neoplasia ($N = 1$). The AD group consisted of patients meeting international working group (IWG) clinical-biological criteria for AD, with positive CSF biomarkers.[9] Due to frequent aSyn co-pathology in AD, this group was not included in "non-ASYN."[1–3] The last two groups were Lewy body disease (LBD) ($N = 47$): 41 patients with "probable DLB" per McKeith criteria[10] and six PD with cognitive complaints per Postuma criteria.[11] For the DLB group, clinical and paraclinical criteria were reviewed in detail and are presented in Table 1 with associated aSyn-SAA positivity rates. The CSF cohort of four main etiologies (non-ASYN, AD, DLB, and PD) was stratified according to their aSyn-SAA analysis status (positive vs negative) in the following analyses. As an example, "AD aSyn+" denotes AD patients exhibiting positive aSyn-SAA results. Patients with a mismatch between clinical diagnosis and aSyn-SAA response (AD aSyn+ and DLB aSyn−) were comprehensively reviewed by a multidisciplinary team using their medical records, including clinical, biological, and neuroimaging data.

RESEARCH IN CONTEXT

1. **Systematic review**: The authors reviewed the literature using conventional sources like PubMed, conference abstracts, and presentations. The diagnosis of alpha-synucleinopathies through the detection of pathological aSyn with SAA holds significant potential. However, existing techniques lack standardization and accessibility in routine laboratories due to the need for specialized expertise in recombinant aSyn production.
2. **Interpretation**: This original research introduces an aSyn-SAA protocol using only commercial reagents and CSF samples from 126 real-life patients, including LBD, AD, and control cases. The protocol demonstrated 100% specificity against controls and an overall sensitivity of 72.3% to LBD.
3. **Future directions**: These findings highlight the efficacy of a commercial-only CSF aSyn-SAA protocol, showing remarkable specificity in a real-life setting. This suggests a path toward standardization and accessibility of an aSyn biomarker in clinical practice beyond specialized research laboratories.

2.2 | CSF collection and measurements

CSF collection, sampling and storage were performed using a standard procedure according to the international consensus.[12] All CSF samples from LP were collected in a standardized polypropylene tube (Sarstedt ref. 62.610.201) and stored at −80°C until analysis. For cytochemistry measurements, CSF total protein concentrations were analyzed with a C16000 Abbot automated analyzer. The CSF concentrations of amyloid beta (Aβ) 1-42, t-Tau, pTau181, and Aβ1-40 were measured routinely using Lumipulse G 600II (Fujirebio). For each CSF sample, a positive AD CSF biomarker profile was considered if t-Tau ≥ 400 ng/L, pTau181 ≥ 60 ng/L, and Aβ1-42 ≤ 550 ng/L, and/or an Aβ1-42/Aβ1-40 ratio < 0.055.[13] For this study, CSF NFL protein measurements were performed using a NF-Light ELISA kit (Uman Diagnostics, Umea, Sweden). All the assays were performed in the same laboratory, according to the ISO 15189:2012 standard.

2.3 | Protocol for CSF aSyn-SAA

Alpha-synuclein (aSyn) SAA reactions were inspired by Groveman and colleagues[14] with minor modifications. For the detailed protocol with product references, see Supplemental File 2. Three quantitative

TABLE 1 Clinical and paraclinical features available in DLB patients and associated aSyn-SAA positivity response rates obtained.

McKeith's clinical criteria	Percentage (no./total)	Percentage in DLB aSyn−	Percentage in DLB aSyn+	*p* value
Fluctuating cognition	61 (25/41)	54 (7/13)	72 (18/25)	0.273
Recurrent visual hallucinations	76 (31/41)	69 (9/13)	81 (22/27)	0.404
REM sleep behavior disorders	46 (19/41)	54 (7/13)	48 (12/25)	0.729
One or more spontaneous cardinal features of Parkinsonism	78 (32/41)	62 (8/13)	86 (24/28)	0.086
1 out of 4	10 (4/41)	8 (1/13)	11 (3/28)	0.769
2 out of 4	34 (14/41)	54 (7/13)	25 (7/28)	0.720
3 out of 4	41 (17/41)	31 (4/13)	46 (13/28)	0.370
All 4 clinical criteria	15 (6/41)	8 (1/13)	18 (5/28)	0.408
Reduced DAT uptake in basal ganglia (123I-FP-CIT SPECT)*	65 (24/37)	73 (8/11)	100 (16/16)	**0.031**
Positive AD CSF biomarkers	24 (10/41)	23 (3/13)	25 (7/28)	0.891

Abbreviations: AD, Alzheimer's disease; aSyn, alpha-synuclein; CSF, cerebrospinal fluid; DAT, dopamine transporter; DLB, dementia with Lewy bodies; REM, rapid eye movement.
**Note* that not all patients had a DAT scan.

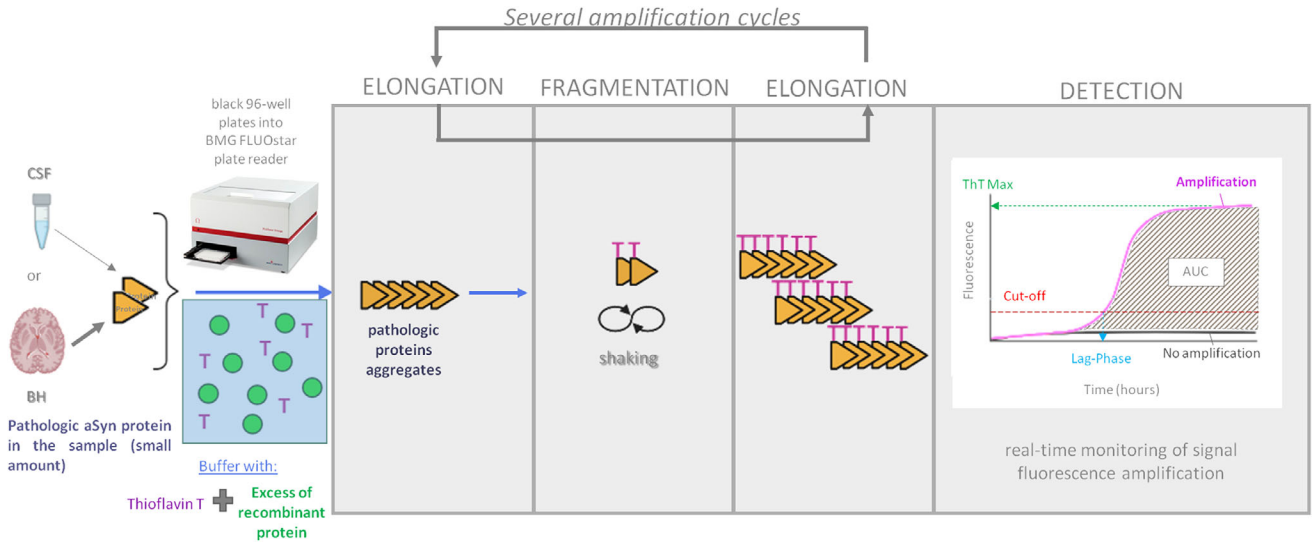

FIGURE 1 Alpha-synuclein-SAA's principle. The sample (here BH or CSF) containing a small amount of the misfolded aSyn protein is added to a buffer in an excess of recombinant aSyn protein and Thioflavin T (that intercalates into the newly formed aggregates and then emits fluorescence during the experiment). Several cycles of shaking and rest at a controlled temperature are performed to allow elongation and fragmentation of the newly formed aggregates. Fluorescence is regularly measured to follow the reaction. Quantitative parameters are calculated from the kinetics and include the lag phase (time to reach cutoff), the ThT Max (maximum fluorescence reached), and AUC (area under the curve). BH: brain homogenate; CSF, cerebrospinal fluid.

parameters were derived from the relative fluorescence responses of aSyn-SAA kinetics: (i) time to reach the threshold (lag phase), (ii) maximum fluorescence intensity (ThT Max), and (iii) area under the curve (AUC). For aSyn-SAA's principle, see Figure 1 (inspired by Kaczorowski et al.[15]).

2.4 | Intra-, inter-assay, and interbatch variability in CSF studies

We used two batches of recombinant protein and the results of the positive (i.e., CSF of the same known DLB patient) and negative (i.e., CSF of the same non-LBD patient) controls were exploited since they have been tested in all assays. The same aliquot was used for the positive and negative controls. The aSyn-SAA quantitative kinetic parameters assessed were ThT Max, AUC, and the lag phase (for the positive control only). See Supplemental File 3 for details on internal control variability results.

2.5 | Statistical analysis

Relative fluorescence responses of aSyn-SAA kinetics were analyzed using GraphPad Prism (version 8.0.1.) and the three quantitative parameters lag phase, ThT Max, and AUC were calculated. Since these parameters were not normally distributed, medians with interquartile ranges (IQRs) were used. Differences in aSyn-SAA parameters between groups stratified by aSyn-SAA status (Section 2.1) were assessed using non-parametric tests: Mann-Whitney *U* for two groups, Kruskal–Wallis *H* with Dunn's post hoc for multiple groups, and χ^2 for categorical variables. Spearman correlations examined associations between aSyn-SAA kinetics parameters and demographic (gender, age) and CSF biomarkers (Aβ42, t-Tau, pTau181, Aβ40, Aβ42/40 ratio, NFL, CSF total proteins, red and white blood cells). We also performed a comparative analysis of parameters between AD aSyn+ and AD aSyn− subgroups. Notably, parameters such as Aβ40 levels and Aβ42/40 ratio were "not applicable" (NA) due to data from only one AD aSyn+ patient. Statistical analyses were performed with SPSS (version 21), GraphPad Prism (version 8.0.1.), and MedCalc (version 22.013), with significance set at $p < 0.05$.

3 | RESULTS

3.1 | Pilot study with BH

For this pilot study using BH, we observed 100% sensitivity for PD and DLB patients and 100% specificity versus control patients (see Supplemental File 1 for details).

3.2 | Study with CSF from real-life patients

3.2.1 | Clinical and paraclinical features

Relevant clinical and paraclinical features were available in patients diagnosed with DLB. These patients, even those presenting with only one of the four primary clinical features, demonstrated positive indicative biomarkers, such as reduced dopamine transporter uptake in the basal ganglia as detected by single photon emission computed tomog-

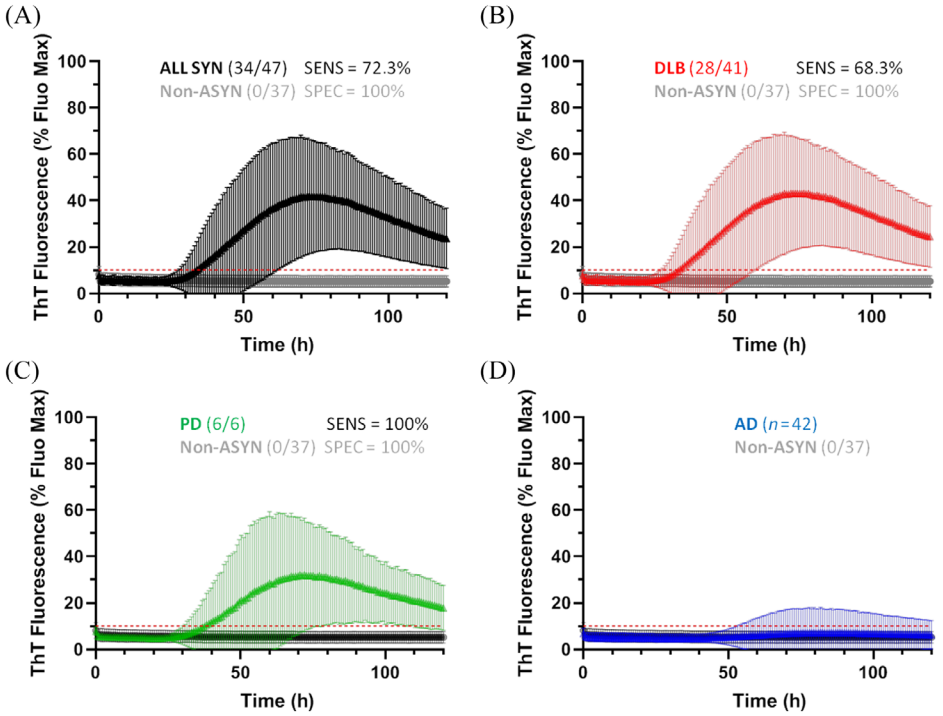

FIGURE 2 Alpha-synuclein SAA kinetics curves. (A) Overall aSyn seeding activity observed in all Lewy body diseases cases (ALL SYN, $N = 34/47$ aSyn+ patients, 72.3% sensitivity) versus non-ASYN patients ($N = 0/37$ aSyn+ patients, 100% specificity). (B) Seeding activity in the DLB cases ($N = 28/41$ aSyn+ patients, 68.3% sensitivity). (C) Seeding activity in PD cases ($N = 6/6$ aSyn+ patients, 100% sensitivity). (D) Seeding activity in the AD group overall ($N = 42$ cases) including positive ($N = 6$) and negative ($N = 36$) patients (see Figure S2 for details on the aSyn-SAA amplification kinetics in AD aSyn- and AD aSyn+ subgroups). AD, Alzheimer's disease; aSyn, alpha-synuclein; SAA, seed amplification assays.

raphy (SPECT) or positron emission tomography (PET). Consequently, each patient fulfilled the diagnostic criteria for "probable" DLB as per the consensus guidelines outlined by McKeith et al.[10] It is noteworthy that 24% of DLB patients exhibited concomitant AD co-pathology, representing 10 out of 41 cases. For a summary of clinical data, please see Table 1.

3.2.2 | aSyn-SAA CSF diagnostic performances

The diagnostic performance obtained with CSF was of an overall sensitivity of 72.3% for LBD ("ALL SYN") and an overall specificity of 100% against non-ASYN. In detail, sensitivity reached 68.3% (28/41) for DLB and 100% (6/6) for PD patients (Figure 2). We observed 14.3% (6/42) of AD aSyn+ patients (see Supplemental File 4 for details). For the "clinical-biological mismatch" cases, that is, for those where the clinical diagnosis differed from the results of the aSyn-SAA (DLB aSyn- and AD aSyn+), please refer to Supplemental File 5.

3.2.3 | Comparisons of clinical and biological variables between groups

The four main etiologies of our CSF's cohort (non-ASYN, AD, DLB, and PD) were stratified according to their aSyn-SAA analysis status (aSyn+ or aSyn−) to give six subgroups including non-ASYN, AD aSyn−, AD aSyn+, DLB aSyn−, DLB aSyn+, and PD aSyn+ (in the PD group, we only observed aSyn+ patients; see Table 2).

When comparing clinical characteristics, statistical analysis revealed that among the six subgroups, there were no differences in age ($p = 0.068$) or gender repartition ($p = 0.097$).

A comparison of the distribution of clinical and paraclinical signs within DLBs according to aSyn-SAA results revealed no significant differences between the two groups, apart from the result of the proportion of reduced dopamine transporter (DAT) uptake in basal ganglia (Table 1).

In comparisons of biological data concerning AD CSF biomarkers, statistically significant differences were observed among the six subgroups for Aβ42, pTau181, t-Tau, Aβ40, and the Aβ42/Ab40 ratio (all $p < 0.001$). However, in comparisons of these AD biomarkers between AD aSyn− and AD aSyn+ patients, no significant differences were found between the two subgroups (Aβ42: $p = 0.943$; pTau181: $p = 0.666$; t-Tau: $p = 0.900$). NFL concentrations appeared higher in the non-ASYN group, likely due to its composition more closely reflecting patients in clinical routine and which included patients with aggressive pathologies such as ALS, known for elevated NFL levels. While analysis across the six subgroups revealed a statistically significant difference ($p = 0.048$), specific post hoc comparisons did not demonstrate significant differences. Furthermore, evaluation of other biological variables showed no differences in total protein levels, whether considered as a

TABLE 2 Comparison of clinical and biological parameters between groups according to their aSyn-SAA response.

		Non-ASYN - (N = 37/37)	AD aSyn- (N = 36/42)	AD aSyn+ (N = 6/42)	DLB aSyn- (N = 13/41)	DLB aSyn+ (N = 28/41)	PD aSyn+ (N = 6/6)	p value
Age (years)		68.0 (IQR = 58.3, 72.0)	69.0 (IQR = 68.0, 73.5)	69.5 (IQR = 69.0, 73.0)	71.0 (IQR = 65.8, 74.0)	73.0 (IQR = 68.5, 75.5)	75.5 (IQR = 66.0, 80.0)	0.068
Gender, *n* females (%)		20/37 (54.1%)	19/36 (52.8%)	4/6 (66.7%)	4/13 (30.8%)	8/28 (28.6%)	1/6 (16.7%)	0.097
aSyn-SAA parameters								
Lag phase (h)	median (IQR)	NA	NA	73.9 (IQR = 56.3, 95.6)	NA	47.3 (IQR = 39.4, 60.4)	58.2 (IQR = 43.1, 64.1)	**0.031***
ThT Max (%RFU)	median (IQR)	6.0 (IQR = 5.8, 6.5)	6.4 (IQR = 5.5, 7.6)	39.1 (IQR = 17.4, 47.5)	6.2 (IQR = 5.9, 7.8)	50.3 (IQR = 42.1, 64.9)	32.1 (IQR = 16.6, 54.8)	**<0.001*****
AUC (RFU)	median (IQR)	507.0 (IQR = 469.5, 602.4)	498.7 (IQR = 459.6, 664.1)	1921.4 (IQR = 685.0, 2210.0)	521.1 (IQR = 474.3, 634.7)	2538.0 (IQR = 2036.3, 3373.3)	1616.0 (IQR = 1256.0, 3212.0)	**<0.001*****
AD biomarkers								
CSF Aβ42 (ng/L)	median (IQR)	845.0 (IQR = 694.0, 1102.5)	475.5 (IQR = 417.0, 548.5)	477.5 (IQR = 418.0, 552.0)	788.0 (IQR = 606.5, 967.0)	567.5 (IQR = 422.5, 873.0)	499.0 (IQR = 380.0, 738.0)	**<0.001*****
	N (%)	24/37 (67.9%)	36/36 (100%)	6/6 (100%)	13/13 (100%)	28/28 (100%)	6/6 (100%)	
CSF t-Tau (ng/L)	median (IQR)	281.0 (IQR = 241.5, 434.0)	731.5 (IQR = 615.5, 1056.0)	745.5 (IQR = 623.0, 827.0)	386.0 (IQR = 317.0, 1053.5)	344.5 (IQR = 274.0, 482.5)	233.5 (IQR = 153.0, 276.0)	**<0.001*****
	N (%)	24/37 (67.9%)	36/36 (100%)	6/6 (100%)	13/13 (100%)	28/28 (100%)	6/6 (100%)	
CSF p-Tau181 (ng/L)	median (IQR)	32.5 (IQR = 30.0, 41.0)	135.0 (IQR = 106.0, 175.0)	116.5 (IQR = 98.0, 182.0)	46.0 (IQR = 30.8, 88.3)	45.0 (IQR = 34.0, 66.5)	23.5 (IQR = 21.0, 35.0)	**<0.001*****
	N (%)	24/37 (67.9%)	36/36 (100%)	6/6 (100%)	13/13 (100%)	28/28 (100%)	6/6 (100%)	
CSF Aβ40 (ng/L)	median (IQR)	6737.0 (IQR = 5102.5, 15971.5)	16044.0 (IQR = 13226.0, 17880.0)	15531.0 (IQR = 15531.0, 15531.0)	17674.0 (IQR = 10474.3, 20104.5)	8850.0 (IQR = 5419.3, 11355.0)	6038.0 (IQR = 4391.8, 7511.0)	**<0.001*****
	N (%)	7/37 (18.9%)	14/36 (38.9%)	1/6 (16.7%)	5/13 (38.5%)	19/28 (67.9%)	5/6 (83.3%)	
CSF RatioAβ42/40	median (IQR)	0.068 (IQR = 0.055, 0.078)	0.043 (IQR = 0.037, 0.046)	0.048 (IQR = 0.048, 0.048)	0.042 (IQR = 0.037, 0.050)	0.061 (IQR = 0.052, 0.067)	0.092 (IQR = 0.077, 0.093)	**<0.001*****
	N (%)	6/37 (16.2%)	12/36 (33.3%)	1/6 (16.7%)	4/13 (30.8%)	19/28 (67.9%)	5/6 (83.3%)	

(Continues)

TABLE 2 (Continued)

		Non-ASYN - (N = 37/37)	AD aSyn- (N = 36/42)	AD aSyn+ (N = 6/42)	DLB aSyn- (N = 13/41)	DLB aSyn+ (N = 28/41)	PD aSyn+ (N = 6/6)	p value
Other biological parameters								
CSF NFL (pg/mL)	median (IQR)	3267.0 (IQR = 942.0, 8764.3)	1353.5 (IQR = 1039.5, 1944.0)	1188.5 (IQR = 1078.0, 2967.0)	1203.0 (IQR = 668.0, 1569.3)	1305.0 (IQR = 915.0, 1634.0)	1260.5 (IQR = 973.0, 1462.0)	**0.048***
	N (%)	37/37 (100%)	36/36 (100%)	6/6 (100%)	13/13 (100%)	28/28 (100%)	6/6 (100%)	
CSF Total protein (g/L)	median (IQR)	0.42 (IQR = 0.34, 0.51)	0.43 (IQR = 0.32, 0.50)	0.38 (IQR = 0.32, 0.46)	0.38 (IQR = 0.34, 0.51)	0.43 (IQR = 0.33, 0.56)	0.59 (IQR = 0.45, 0.72)	0.463
	N (%)	37/37 (100%)	36/36 (100%)	6/6 (100%)	13/13 (100%)	28/28 (100%)	6/6 (100%)	
	<0,5	27 (73.0%)	28 (77.8%)	5 (83.3%)	9 (69.2%)	19 (67.9%)	2 (33.3%)	0.343
	>0,5	10 (27.0%)	8 (22.2%)	1 (16.7%)	4 (30.8%)	9 (32.1%)	4 (66.7%)	
CSF RBC (/mm^3)	N (%)	36/37 (97.3%)	35/36 (97.2%)	6/6 (100%)	12/13 (92.3%)	25/28 (89.3%)	5/6 (83.3%)	
	0-100	29 (80.6%)	29 (82.9%)	6 (100.0%)	10 (83.3%)	23 (92.0%)	5 (100.0%)	0.839
	100-3000	6 (16.7%)	5 (14.3%)	0 (0%)	1 (8.3%)	2 (8.0%)	0 (0%)	
	>3000	1 (2.8%)	1 (2.9%)	0 (0%)	1 (8.3%)	0 (0%)	0 (0%)	
CSF WBC (/mm^3)	N (%)	36/37 (97.3%)	35/36 (97.2%)	6/6 (100%)	12/13 (92.3%)	25/28 (89.3%)	5/6 (83.3%)	
	0-10	33 (91.7%)	34 (97.1%)	6 (100.0%)	12 (100.0%)	25 (100.0%)	4 (80,0%)	0.281
	>10 (and RBC >1000 × WBC)	0 (0%)	1 (2.9%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
	>10 (and RBC <1000 × WBC)	3 (8.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (20.0%)	

Note: Kruskall–Wallis tests were used to compare the medians between groups of continuous variables, and the chi-squared test was used to compare the category proportions between groups of non-continuous (or categorical) variables. For CSF WBC, we categorized them into traumatic LP (WBC >10/mm^3 and RBC >1000 times WBC) and atraumatic LP (WBC >10/mm^3 and RBC <1000 times WBC). *P* values of these tests are represented in the last column (significances were set as follows: $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

Abbreviations: AD, Alzheimer's disease; aSyn, alpha-synuclein; CSF, cerebrospinal fluid; DLB, dementia with Lewy bodies; NFL, neurofilament light chain; PD, Parkinson's disease; RBC, red blood cells; SAA, seed amplification assay; WBC, white blood cells.

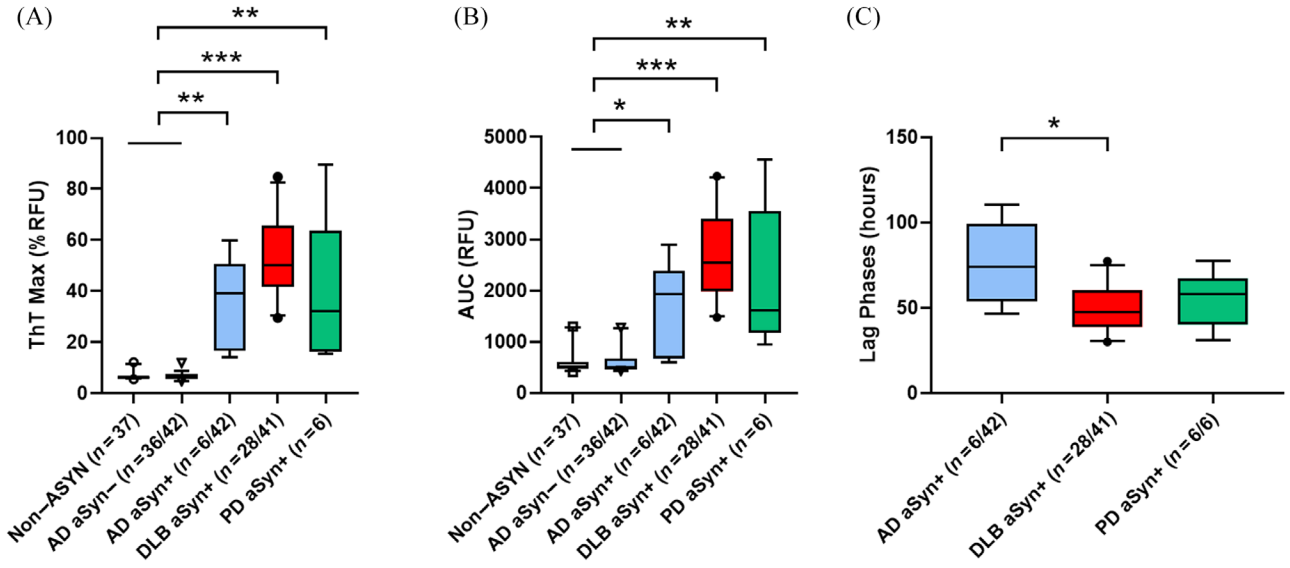

FIGURE 3 Parameters characterizing kinetics of aSyn aggregation in aSyn-SAA assay. (A) Comparative analysis of ThT Max values across each experimental group. (B) Evaluation of AUC for each diagnostic group examined. (C) Assessments of lag-phase duration within aSyn-SAA positive groups. The colors of the box plots correspond to those depicted in the kinetics curves illustrated in Figure 2. Each box plot displays the median and the 5th to 95th percentiles of the data distributions. AUC, area under the curve; aSyn, alpha-synuclein; SAA, seed amplification assays.

continuous variable ($p = 0.463$) or as a categorical variable ($p = 0.343$), and no differences in red blood cell concentration ($p = 0.839$) or white blood cell concentration ($p = 0.281$) (Table 2).

3.2.4 | Comparisons of aSyn-SAA parameters between groups

The maximum ThT fluorescence intensity (ThT Max) demonstrated an overall significant difference between the non-ASYN, AD aSyn–, AD aSyn+, DLB aSyn+, and PD aSyn+ groups ($p < 0.001$). Dunn's post hoc analysis revealed that the non-ASYN and AD aSyn– groups had significantly lower ThT Max in comparison to the AD aSyn+ ($p < 0.01$), DLB aSyn+ ($p < 0.001$), and PD aSyn+ groups ($p < 0.01$). However, there were no significant differences in ThT Max between the synucleinopathies (Figure 3 and Table 2).

Examination of the area under the curve (AUC) of the aSyn-SAA amplification kinetics, revealed there was also an overall significant difference between the non-ASYN, AD aSyn–, AD aSyn+, DLB aSyn+, and PD aSyn+ groups ($p < 0.0001$). Post hoc analysis demonstrated that the non-ASYN and AD aSyn– groups had significantly lower AUC in comparison to AD aSyn+ ($p < 0.05$), DLB aSyn+ ($p < 0.001$), and PD aSyn+ groups ($p < 0.001$). However, there were no significant differences in AUC between the synucleinopathies (Figure 3 and Table 2).

Investigation of the lag-phase parameter (in the positive amplifications) revealed an overall significant difference between the AD aSyn+, DLB aSyn+, and PD aSyn+ groups ($p = 0.031$). Dunn's post hoc analysis demonstrated a significantly longer lag phase (+56.2%; $p < 0.05$) in the AD aSyn+ patients in comparison to the DLB aSyn+ group (Figure 3 and Table 2).

3.2.5 | Correlations between aSyn-SAA parameters and other continuous variables

We first looked at possible associations within aSyn-SAA parameters (lag phase, ThT Max, AUC) in patients who had positive amplifications with aSyn-SAA ($N = 40$, including 28 DLB, six PD, and six AD). We found strong negative significant correlations between lag phase and ThT Max (Rho = –0.722, $p < 0.001$) and between lag phase and AUC (Rho = –0.784, $p < 0.001$). ThT Max and AUC presented a strong significant positive correlation (Rho = 0.974, $p < 0.001$).

We then looked at possible associations between aSyn-SAA parameters and other continuous variables considering only the aSyn-SAA positive patients ($N = 40$), we found that age was moderately associated with the lag phase (Rho = –0.365, $p = 0.039$) and associated with the ThT Max (Rho = 0.290, $p = 0.001$) and the AUC (Rho = 0.288, $p = 0.001$). In addition, total CSF protein concentration was positively associated with the lag phase (Rho = 0.374, $p = 0.017$).

4 | DISCUSSION

This study aimed to develop a reproducible CSF aSyn-SAA protocol using commercially available reagents and validate its use in a real-life clinical cohort. Our goal was to facilitate integration into routine expert laboratories, increasing accessibility for patient care.

A pilot study on a limited neuropathologically confirmed cohort using BHs showed 100% sensitivity in DLB and PD and 100% specificity against controls. In vivo CSF samples from clinically diagnosed patients showed 100% sensitivity in PD, 68.3% in DLB, and 100% specificity against non-alpha-synucleinopathies.

Several factors may explain the discrepancies in sensitivity compared to the existing literature. First, the study's relatively small sample size, in contrast to large and well-defined cohorts,[4,16,17] did not allow us to draw firm conclusions about the diagnostic performance of this protocol. Nonetheless, this cohort's major advantage lies in its reflection of daily clinical practice in real-life patients, emphasizing the need for clinicians in routine settings to be aware of the relatively lower sensitivity of aSyn-SAA compared to standardized research cohorts. Another factor could be the recruitment strategy based on McKeith's clinical criteria, which have a sensitivity of 60.2%.[10,18] In contrast, recruitment strategies predominantly employed by large and standardized cohorts rely on patients with confirmed neuropathological diagnoses, thereby achieving sensitivities of approximately 90% to 95%.[19] Indeed, a recent meta-analysis highlighted the influence of diagnostic recruitment on aSyn-SAA performance, with enhanced diagnostic accuracy observed when *post mortem* confirmation was available compared to clinically based diagnoses.[20] Variation in CSF aSyn concentrations, influenced by disease stage and affected brain regions, may also impact the likelihood of amplifying aggregates through aSyn-SAA.[21,22]

We further explored potential associations between the McKeith clinical criteria and aSyn-SAA responses. Our data revealed only a significantly higher number of positive reduced DAT uptake in basal ganglia in DLB aSyn+ patients compared to DLB aSyn−. Moreover, DLB aSyn+ patients tend to meet higher clinical criteria than DLB aSyn−, although it was not significant. This suggests that patients with more clinical and paraclinical criteria might have a higher synucleinopathy burden. However, small sample sizes limited the strength of these findings. Further studies with larger cohorts are necessary to clarify this relationship and its potential implications for understanding disease progression in DLB.

AD patients with confirmed CSF biomarkers were excluded from the non-alpha-synucleinopathy cohort due to the significant presence of aSyn co-pathologies.[1–3] These co-lesions, often overlooked in clinical assessments,[23,24] were confirmed by the aSyn-SAA results within the AD group. Indeed, among 42 biologically confirmed AD patients, six tested positive for aSyn (14.3%), closely reaching consistency with recent studies reporting aSyn-SAA positivity rate ranging from 21% to 45% among AD patients with confirmed biomarkers.[4,19,22,25,26] Given the overlapping clinical presentations of AD, DLB, and AD-DLB patients, distinguishing between these conditions can be challenging. Comparative analysis of AD diagnostic biomarkers and NFL between AD aSyn+ and AD aSyn− groups showed no significant differences, underscoring the importance of aSyn-SAA in detecting aSyn co-pathology. Moreover, when a LP is performed for AD diagnosis, this offers the advantage of using the same biological sample for aSyn-SAA without the need for additional invasive procedures. Interestingly, upon careful review of clinical records, five out of six AD aSyn+ patients finally met diagnostic criteria for possible or probable DLB, leading to their reclassification into AD-DLB. This demonstrated that the mismatch observed between the initial suspicion and the result in aSyn-SAA enabled more informed discussions in multidisciplinary consultation meetings and occasionally facilitated valuable reclassification of patients.[27] For clinically diagnosed probable AD patients without evident signs of DLB, a positive aSyn-SAA response could also be significantly beneficial for future patients' care. Indeed, it is well documented that AD patients with DLB co-pathology experience more severe cognitive and neuropsychiatric symptoms and progress more rapidly compared to those without DLB co-pathology.[1,4,19,25,28] This technique could identify AD-DLB co-pathology, enabling more tailored treatments and providing insights into the efficacy of AD therapies in these subgroups. AD aSyn+ patients exhibited a longer lag phase compared to DLB aSyn+ patients, a novel finding.[22,25] This observation may suggest a possible lower concentration of misfolded aSyn in the CSF of AD aSyn+ patients, potentially linked to the burden of Lewy body pathology.[21] While the longer lag phase may indicate a lower seed concentration, we did not perform SD50 measurements in this study, which would provide a more quantitative assessment of seeding activity. However, the limited volume of CSF available in clinical settings makes SD50 determination difficult. Although SD50 remains a valuable parameter for comparing seeding capacity across different cohorts, larger studies with sufficient CSF volumes are warranted to validate this hypothesis and further explore the potential role of SD50 in clinical practice.

Furthermore, recent evidence indicates that preclinical LB pathology with a positive aSyn-SAA result progressed to clinical LBD over a decade.[16] Consequently, the development of an accessible and reliable aSyn biomarker represents a significant advancement in the field to improve personalized diagnosis and precision medicine. From another perspective, the role of aSyn-SAA in future DLB therapeutic trials is noteworthy, as it could facilitate the accurate inclusion of DLB patients and enable the evaluation of biomarker changes as outcomes of disease-modifying effects.

This protocol, developed in a routine expert laboratory using commercial reagents on in vivo CSF samples, includes important technical points rarely addressed in the literature. First, we observed fluctuations in diagnostic performance depending on the matrix used, either BH or CSF, when testing different commercial recombinant proteins. This is why we chose not to use the same recombinant protein across both matrices (*data not shown*). Another pivotal aspect concerned the selection of the kinetic duration at which the cutoff was determined, as it directly affected the kinetics parameters of the aSyn-SAA, thereby influencing result interpretation and diagnostic performances. Notably, the literature highlights the lack of standardization in aSyn-SAA protocols across laboratories, with each institution often adapting its experimental conditions.[20,29] Our protocol therefore needs more interlaboratory comparisons and collaborative efforts among expert laboratories to harmonize experimental conditions and interpretation criteria. Although recombinant proteins are typically stable, "batch effects" resulting in non-specific amplifications were exceedingly rare in our observations. This highlights the key role of the recombinant protein in aSyn-SAA protocol reaction. This critical aspect, often understated in the literature, warrants further scrutiny through additional evaluation studies. Researchers are advised to rigorously test each new batch before implementation, testing that should involve comparisons with previous batches through multiple parallel runs, in accordance

with good laboratory practices under expert routine conditions. Pre-analytical factors are important, as certain CSF proteins can inhibit SAA amplifications,[30] supported by our observation of a negative correlation between lag phase and total protein concentration. Lastly, we underscore the indispensable nature of laboratory expertise, given the substantial time investment required for protocol development, implementation, and the inherent biological risks associated with aSyn-SAA technology.[31] The stringent health and safety conditions demanded by aSyn-SAA technology calls for a thorough understanding of the "prion-like" risk associated with biological sample handling, stressing the importance of proper waste management protocols.[32–34]

Considering the diagnostic performance of aSyn-SAA, it is imperative for clinicians and patients to recognize that a negative result does not definitively exclude LBD but rather warrants further investigations and diligent follow-up to confirm or refute the diagnosis. Conversely, a positive result from aSyn-SAA corroborates the suspicion of LBD and represents the initial reliable biological marker for LBD. It is crucial to emphasize that, presently, the diagnosis of LBD relies on a combination of clinical and biological assessments, notwithstanding recent literature advocating for a more biologically oriented definition of LBD predicated on aSyn-SAA findings.[35,36]

In conclusion, we have established a robust and readily implementable protocol for aSyn-SAA in CSF using exclusively commercial reagents, achieving an overall sensitivity of 72.3% and 100% specificity against non-alpha-synucleinopathies. This protocol should facilitate its dissemination and integration into routine expert laboratories for the benefit of patients.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest. Author disclosures are available in the Supporting Information.

CONSENT STATEMENT

All human subject participants gave their written informed consent prior to the examinations to save and use their biological fluid samples for research purposes.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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