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Association of CSF α-Synuclein Seeding Amplification Assay Results With Clinical Features of Possible and Probable Dementia With Lewy Bodies


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# α-Synuclein Seed Amplification in CSF and Brain from Patients with Different Brain Distributions of Pathological α-Synuclein in the Context of Co-Pathology and Non-LBD Diagnoses

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**Objective:** The purpose of this study was to determine the sensitivity and specificity of α-synuclein seed amplification assay (αSyn-SAA) in antemortem and postmortem cerebrospinal fluid (CSF) of autopsy-confirmed patients with different distributions of pathological αSyn, co-pathologies, and clinical diagnoses.
**Methods:** The αSyn-SAA was used to test antemortem CSF samples from 119 subjects with a variety of clinical syndromes and standardized neuropathological examinations from Oregon Health and Science University (OHSU) and University of California San Diego (UCSD; 56 additional postmortem CSF samples available). The αSyn-SAA was also applied to frontal cortex and amygdala homogenates. Sensitivity and specificity were compared across distributions of αSyn pathology. Clinical data and co-pathologies were compared across αSyn-SAA positive and negative groups.
**Results:** Fifty-three individuals without and 66 with αSyn-pathology (neocortical [n = 38], limbic [n = 7], and amygdala-predominant [n = 21]) were included. There was a sensitivity of 97.8% and specificity of 98.1% of the αSyn-SAA to identify patients with limbic/neocortical pathology from antemortem CSF. Sensitivity to detect amygdala-predominant pathology was only 14.3%. Postmortem CSF and brain tissue αSyn-SAA analyses also showed higher assay positivity in samples from limbic/neocortical cases.
**Interpretation:** CSF αSyn-SAA reliably identifies αSyn seeds in patients with diffuse αSyn pathology in the context of co-pathology and non-Lewy body disease (LBD) diagnoses. The analysis of brain homogenates suggests that pathological αSyn in the amygdala might differ from pathological αSyn in the frontal cortex. The αSyn-SAA might facilitate the differential diagnosis of dementias with mixed pathologies.

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Aggregated α-synuclein (αSyn) is the main component of cytoplasmic inclusions called Lewy bodies (LBs) and Lewy neurites, which are the defining pathological features of Lewy body diseases (LBD), including Parkinson's disease (PD) and dementia with Lewy bodies (DLBs).[1,2] In addition, αSyn-laden LBs are found in the brains of as many as 50% to 60% of sporadic Alzheimer's disease (AD) cases,[3–7] 96% in familial *PSEN1* cases,[8] and in 10% to 20% of normal elders.[9,10] AD cases with αSyn pathology (sometimes called AD Lewy Body variant [AD-LBV]) present relevant clinical differences compared to AD without αSyn pathology, such as lower age of onset, lower age of death, more severe delusions, hallucinations, aberrant motor function, and sleep disorders.[7,11] Similarly, co-incidental AD pathology in DLB may lower the likelihood of patients manifesting certain core features, like visual hallucinations.[12] The αSyn pathology in AD cases affects the amygdala, limbic, and can affect neocortical areas with sparing of the brainstem and recent neuropathological studies in AD cases with amygdala-predominant αSyn pathology found different αSyn truncations and modifications compared to limbic and neocortical αSyn pathology found in PD or DLB.[13–15]

To date, neuropathological assessment at autopsy remains the gold standard to diagnose LBDs and in vivo αSyn biomarkers have been an unmet need. Recently, αSyn seed amplification assays (αSyn-SAAs; also known as protein misfolding cyclic amplification [PMCA] and real time quaking induced conversion [RT-QuIC]), have been adapted to detect misfolded αSyn aggregates (αSyn seeds) in cerebrospinal fluid (CSF) and peripheral tissues with remarkable diagnostic accuracy.[16–21] The αSyn-SAA in CSF of clinically and, in some cases, pathologically confirmed PD and DLB cases has shown impressive results, with several independent groups reporting sensitivities and specificities near or above 90%.[16–18,22–29] However, αSyn-SAA performance in neuropathologically validated cohorts with varying distribution of αSyn pathologies, co-pathologies, and non-LBD diagnoses has not been evaluated. Thus, it remains unknown if different types of αSyn pathology distributions produce differences in seeding activity. A few studies have reported detection of αSyn seeds in CSF from patients clinically diagnosed with AD (5/14 or 36% in one report[17] and 0/16 in another[18]) and from patients clinically diagnosed with AD who were pathologically confirmed to have DLB (11/17 or 65%) or incidental LBs (2/13 or 15%).[16] Despite the low number of cases, these results suggest that current assays may have different sensitivities, which may depend on αSyn pathology distribution, co-pathologies, and/or pathological αSyn species.

In this multicenter study, we evaluated the capability of αSyn-SAA to detect αSyn seeds in antemortem and postmortem CSF samples as well as brain tissue of patients who underwent autopsy and neuropathological analyses. We compared the αSyn-SAA results to clinical and neuropathological data to determine sensitivity, specificity, clinical, and pathological correlations of this assay across different distributions of αSyn-pathology in the context of co-pathology and non-LBD diagnoses.

## Methods

### *Patient Selection*

The electronic institional review board (eIRB) 725 of Oregon Health and Science University ADRC gave ethical approval for this work. IRB 170957 of University of California San Diego ADRC gave ethical approval for this work. Informed consent was obtained from each subject for the retrieval of biological samples.

Participants in brain aging studies from the Oregon Alzheimer's Disease Center (OADC) (n = 57) and University of California San Diego Shiley-Marcos Alzheimer's Disease Research Center (UCSD-ADRC; n = 62) who had (1) CSF collection during life, and (2) subsequent brain autopsy (n = 119) were included in the study. All subjects had an annual battery of clinical, neuropsychologic, and other cognitive assessments, as described by the National Alzheimer's Coordinating Center (NACC),[30] including Mini-Mental State exam (MMSE), and Unified Parkinson's Disease Rating Scale Part III (UPDRS). Blood was drawn for the determination of APOE genotype. Clinical diagnoses, assigned at the time of CSF collection, included AD (n = 75), DLB (n = 9), PD (n = 4), mild cognitive impairment (MCI; n = 11), other dementia (n = 13), including frontotemporal dementia (n = 10), mixed dementia (n = 1), and "other dementia" (n = 2), and cognitively normal controls (n = 7). Clinical diagnoses were assigned by a multidisciplinary consensus conference at each site. Pathologically, the cohort included patients with AD pathology (n = 43), AD with αSyn pathology (n = 59), αSyn pathology in isolation (n = 3), progressive supranuclear palsy (n = 2), corticobasal degeneration (n = 2), Frontotemporal lobe degeneration with TDP43 pathology (FTLD TDP-43; n = 2), vascular disease in isolation (n = 2), healthy subjects (n = 2), and patients with a mix of AD and other tauopathies (n = 4; Table 1). Cases were grouped by αSyn pathology distribution as below. Patient-level information can be found in Table S1.

### *CSF Analysis*

CSF was collected for all 119 cases by lumbar puncture in the morning fasting condition according to a standardized

**TABLE 1. αS-SAA Positivity as a Function of Pathology Diagnosis**

| | | | αS-SAA positivity | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Pathology** | **n** | **αSyn Pathology** | **Antemortem** | | **Postmortem** | | **Frontal** | | **Amygdala** | |
| AD | 26 | Negative | 4% | 1/26 | 20% | 3/14 | – | – | – | – |
| AD + VD | 5 | Negative | 0% | 0/5 | 0% | 0/3 | – | – | – | – |
| AD + VD + HS | 2 | Negative | 0% | 0/2 | 0% | 0/2 | – | – | – | – |
| AD + VD + AA | 1 | Negative | 0% | 0/1 | – | – | – | – | – | – |
| AD + AA | 3 | Negative | 0% | 0/3 | 0% | 0/1 | 0% | 0/1 | 0% | 0/1 |
| AD + HS | 1 | Negative | 0% | 0/1 | 0% | 0/1 | – | – | – | – |
| AD + HS + LMN encephalitis | 1 | Negative | 0% | 0/1 | – | – | – | – | – | – |
| AD + Pick's disease | 1 | Negative | 0% | 0/1 | – | – | – | – | – | – |
| AD + PART | 1 | Negative | 0% | 0/1 | – | – | – | – | – | – |
| AD + PART + METS | 1 | Negative | 0% | 0/1 | 0% | 0/1 | – | – | – | – |
| AD + VD + AA + ARTAG | 1 | Negative | 0% | 0/1 | 0% | 0/1 | 0% | 0/1 | 0% | 0/1 |
| CBD + VD + AA | 1 | Negative | 0% | 0/1 | – | – | 100%* | 1/1 | 0% | 0/1 |
| PSP | 2 | Negative | 0% | 0/2 | – | – | – | – | – | – |
| CBD | 1 | Negative | 0% | 0/1 | 0% | 0/1 | – | – | – | – |
| FTLD-TDP43 | 1 | Negative | 0% | 0/1 | 0% | 0/1 | – | – | – | – |
| FTLD-Tau | 1 | Negative | 0% | 0/1 | – | – | – | – | – | – |
| VD | 2 | Negative | 0% | 0/2 | – | – | 0% | 0/1 | 0% | 0/1 |
| Normal | 2 | Negative | 0% | 0/2 | 0% | 0/1 | – | – | – | – |
| AD + αSyn-Path | 26 | Neocortical/limbic | 100% | 26/26 | 91% | 10/11 | 100% | 4/4 | 100% | 4/4 |
| AD + αSyn-Path | 7 | Amygdala-predominant | 14% | 1/7 | – | – | 50% | 1/2 | 100% | 1/1 |
| AD + VD + αSyn-Path | 6 | Neocortical/limbic | 83% | 5/6 | 75% | 3/4 | 100% | 1/1 | 100% | 1/1 |
| AD + VD + αSyn-Path | 4 | Amygdala-predominant | 0% | 0/4 | 100% | 4/4 | 100%* | 1/1 | 100% | 1/1 |
| AD + AA + αSyn-Path | 2 | Neocortical | 100% | 2/2 | 100% | 2/2 | 100% | 1/1 | 100% | 1/1 |
| AD + AA + αSyn-Path | 3 | Amygdala-predominant | 0% | 0/3 | 50% | 1/2 | 0% | 0/2 | 50% | 1/2 |
| AD + AA + FTLD-TDP43 + αSyn-Path | 2 | Neocortical/Limbic | 100% | 2/2 | 100% | 1/1 | 100% | 1/1 | 100% | 1/1 |
| AD + AA + FTLD-TDP43 + αSyn-Path | 3 | Amygdala-predominant | 0% | 0/3 | 33% | 1/3 | 0% | 0/3 | 33% | 1/3 |
| AD + HS + αSyn-Path | 4 | Neocortical/limbic | 100% | 4/4 | 100% | 1/1 | – | – | – | – |
| AD + Pick's disease + αSyn-Path | 1 | Amygdala-predominant | 0% | 0/1 | – | – | – | – | – | – |
| AD + ARTAG + αSyn-Path | 1 | Neocortical | 100% | 1/1 | 100% | 1/1 | 100% | 1/1 | 100% | 1/1 |
| AD + VD + PSP + αSyn-Path | 1 | Amygdala-predominant | 100% | 1/1 | – | – | 0% | 0/1 | 100%* | 1/1 |
| AD + Infarcts + αSyn-Path | 1 | Amygdala-predominant | 100% | 1/1 | – | – | – | – | – | – |
| PSP + CBD + HS + αSyn-Path | 1 | Neocortical | 100% | 1/1 | – | – | – | – | – | – |
| FTLD-TDP43 + αSyn-Path | 1 | Amygdala-predominant | 0% | 0/1 | 0% | 0/1 | 0% | 0/1 | 0% | 0/1 |
| αSyn-Path | 3 | Neocortical | 100% | 3/3 | – | – | – | – | – | – |

αSyn-Path = includes neocortical, limbic, and amygdala predominant αSyn pathology; AA = includes amyloid angyopathy, leptomeningial congophilic angiopathy, and lepto/parenchymal congophilic angiopathy; AD = Alzheimer's disease; ARTAG = aging-related tau astrogliopathy; CBD = corticobasal degeneration; FTLD-TDP43 = frontotemporal lobe degeneration with TDP43 pathology; HS = hippocampal sclerosis; LMN encephalitis = limbic microglial nodular encephalitis; METS = micrometastases; PART = primary-age related tauopathy; PSP = progressive supranuclear palsy; VD = vascular disease.

*Two of 3 wells were positive.

protocol.[31] A subset of patients (n = 56) had additional CSF samples obtained at the time of brain removal at autopsy. CSF specimens were divided into 0.5 ml aliquots and stored at −80°C. Antemortem CSF collection occurred 1 to 15 years prior to autopsy (17.6% in 0–2 years, 46.3% in 2–5 years, 18.5% in 5–8 years, 6.7% in 8–10 years, and 10.9% in 10–16 years). Antemortem CSF was analyzed for Aβ40, Aβ42, t-tau, and p-tau (Lumipulse, Fujirebio at both sites).

CSF samples were initially analyzed by the end point qualitative version of the αSyn-SAA that has been validated for clinical use under Clinical Laboratory Improvement Amendment/College of American Pathologists (CLIA/CAP) certifications (clinical assay, SYNTap). Each sample was analyzed in triplicate (40 µl CSF per well) in a 96-well plate (COSTAR, cat #3603) with a final volume reaction of 200 µl. The reaction mixture consisted of 0.3 mg/ml rec-αSyn (Amprion, cat #S2020) in 100 mM PIPES pH 6.50, 500 mM NaCl, 10 µM ThT, and a 2.5 mm borosilicate glass bead per well. Plates were sealed using an Optical Adhesive Film (ThermoFisher, cat #4311971) and shaken at 800 rpm with orbital shaking for 1 minute every 29 minutes of quiescent incubation in a TIMIX 5 shaker (Edmund Buehler) placed in an incubator set to 37°C. Bottom fluorescence readings at 490 nm were performed using a BMG FLUOstar Omega. This clinical version of the assay was performed according to standard operational procedures in agreement with CLIA regulation. CSF samples were deemed "detected" or "not detected" based on a pre-established threshold for the median maximum fluorescence of the triplicate. The research and development (R&D) kinetic αSyn-SAA was utilized to analyze CSF samples and brain tissues. The methods of the kinetic αSyn-SAA have been reported in detail elsewhere.[22,23] Briefly, CSF samples and brain homogenates (BHs) were evaluated in triplicates (40 µl/well) in a 96-well plate (COSTAR 96, cat #3916), in a reaction mix consisting of 0.3 mg/ml rec-αSyn (Amprion, cat #S2021), 100 mM PIPES pH 6.50 (Sigma, cat #80635), 500 mM NaCl (Lonza, cat #51202), 10 µM ThT (Sigma, cat #T3516), and a 3/32-inch BSA-blocked $Si_3N_4$ bead (Tsubaki Nakashima). This assay was performed in a BMG FLUOstar Omega shaker/reader with orbital shaking at 800 rpm for 1 minute and 29 minutes of quiescent incubation at 37°C. Fluorescence at 490 nm was measured every 30 minutes for accurate estimation of kinetic parameters. The assay outcomes of the R&D kinetic assay are positive, inconclusive, or negative, based on a probabilistic algorithm that uses maximum fluorescence and kinetic parameters.[22] Maximum fluorescence ($F_{max}$, RFU) was the highest fluorescence reading within the length of the assay. A 4-parameter fit (Mars, BMG) was fit to estimate the slope (RFU/h) and the time to reach 50% of the $F_{max}$ ($T_{50}$, hours) of each replicate/well. The time to threshold (TTT, hours) was determined with a user-defined formula (Mars, BMG); threshold was set to 5,000 RFU. Scientists performing the assay were blinded to the clinical or pathological diagnoses associated with the samples.

### Brain Tissue Analysis

In a subset of patients (n = 22), 500 mg samples of frozen brain tissue from the middle frontal cortex and amygdala were provided for αSyn-SAA. Cases included those without αSyn-pathology (n = 4), amygdala-predominant αSyn-pathology (n = 10), and limbic/neocortical αSyn-pathology (n = 8). All frozen samples were provided from the UCSD-ADRC.

Frontal cortex and amygdala samples were homogenized to 10% w/v in 1XPBS (Cytiva, cat #SH30256.02) with cOmplete Mini EDTA-free protease inhibitor cocktail (Roche, cat #11836170001). Approximately 100 µg of brain sample was homogenized in 1.5 ml tubes preloaded with 1 mm zirconium beads (cat #11079110zx) in an MP FastPrep 24 homogenizer. Two rounds of homogenization were performed for all samples (15 seconds at 4 m/s and 30 seconds at 6 m/s). If additional homogenization was needed, samples were chilled on ice for 5 minutes in between additional homogenization rounds at 6 m/s for 30 seconds. BHs were centrifuged at 800×*g* for 1 minute to remove cellular debris. Supernatants were collected, vortexed, aliquoted, and stored at −80°C until αSyn-SAA analysis. BH aliquots were 10-fold serially diluted in synthetic CSF (Amprion, cat #S2022) up to $10^{-9}$ and analyzed in triplicates. Results for $10^{-8}$ dilution are shown to avoid negativity by overdilution.

### Neuropathological Assessments

Neuropathological assessments were performed in a standardized manner with various pathologies assessed using hematoxylin and eosin staining and immunohistochemistry directed against tau, amyloid-β, α-synuclein, and TDP-43 species, as appropriate, and pathological diagnoses were assigned by expert neuropathologists.[32–35] The mobility shift assay (MSA) cases were excluded from this study given the known altered kinetics on αSyn-SAA assays compared with PD and DLB cases.[36] AD neuropathological change was assigned according to NACC guidelines after Braak tau stage, CERAD stage, and Thal phase was determined.[33,37] Distribution of Lewy-related pathology (LRP) was determined via α-synuclein immunohistochemistry staining (OADC: αSyn MJFR1, Abcam; UCSD-ADRC: pSer129 αSyn 81A, Biolegend Laboratories) using slices from

pons and/or midbrain, hippocampus, amygdala, and neocortical areas, including the temporal cortex and/or the middle frontal cortex and the following staging definitions were applied: neocortical: midbrain+ pons+ hippocampus+ amygdala+ neocortex+; limbic: midbrain+ pons+ hippocampus+ amygdala+ neocortex-; amygdala-predominant: midbrain- pons- hippocampus+/− amygdala+ neocortex-.[38]

### Statistical Analysis

Clinical and pathological differences between the OADC and UCSD-ADRC cohorts were assessed to determine the necessity for stratification by site. All patients with DLB and PD were from UCSD. Sensitivity, specificity, and predictive values were calculated via chi-squared test with 95% confidence intervals calculated using the hybrid Wilson-Brown method. Differences in kinetic parameters were analyzed by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test or unpaired *t* test. Prior to testing group differences, all outcome variables were assessed for normality. For normally distributed continuous variables, we used the general linear model (GLM) to test whether there were group differences in the outcome variables (age at death, onset of cognitive symptoms, and MMSE decline rate). For non-normally distributed continuous variables (UPDRS at lumbar puncture, MMSE at lumbar puncture, UPDRS at most recent visit, MMSE at most recent visit, clinical dementia rating [CDR] at the most recent visit, lumbar puncture to autopsy interval, CSF Aβ40, Aβ42, t-tau, and p-tau, disease duration, and postmortem interval), we used a Kruskal-Wallis test (more than 2 groups) or a Wilcoxon rank-sum test (2 groups) to test for group differences. Post hoc pairwise comparisons were tested using the Dwass, Steel, Critchlow-Fligner Method. We used chi-square tests or Fisher's exact tests to test for group differences when outcome variables were categorical (biological sex, early-onset status, neuropathology diagnosis, clinical diagnosis, and NACC variables): Thal phase for amyloid plaques, Braak stage for neurofibrillary degeneration, density of neocortical neuritic plaques, NIA-AA Alzheimer's disease neuropathologic change (ADNC), density of diffuse plaques, cerebral amyloid angiopathy, arteriosclerosis, and APOE status. For the following variables, we had data from both the OADC and UCSD-ADRC cohorts: onset of cognitive symptoms, disease duration, age at death, rate of MMSE decline, MMSE at lumbar puncture, most recent MMSE score, interval between lumbar puncture and autopsy, postmortem interval, biological sex, clinical diagnosis, Thal phase, Braak tau stage, Cerad stage, ADNC, APOE genotype, CSF Aβ40, Aβ42, t-tau, and p-tau. UPDRS score at lumbar puncture was only collected at UCSD. Statistical significance was set at $p < 0.05$.

## Results

### Neuropathological αSyn Analysis and Comparison

The neuropathological analysis of the 119 subjects revealed αSyn-pathology in the brains of 66 (55%) patients. Of the 66 patients with αSyn-pathology, 38 showed neocortical stage αSyn-pathology, 7 showed limbic stage αSyn-pathology, and 21 showed amygdala-predominant αSyn-pathology. Rates of AD pathology were high across the cohort. Forty of 53 (75%) of the cases without αSyn pathology had intermediate or high degrees of AD neuropathological change, as did 19 of 21 (90%) cases with amygdala predominant αSyn pathology and 39 of 45 (87%) cases limbic or neocortical disease (see Table 1). These rates were not statistically significant across the αSyn driven categories ($\chi^2 = 3.3$, $p = 0.2$). The cases that did not have significant AD neuropathological change composed a variety of tauopathies, TDP-43-opathies, and vascular disease (see Tables 1, S1). No significant difference in Aβ40, Aβ42, Aβ42/40 ratio, t-tau, and p-tau in antemortem CSF were observed between the αSyn pathology groups within institution (Table S2).

Using a Kruskal-Wallis test, we compared patients within αSyn distribution groups (none, amygdala-predominant, and limbic/neocortical) on several standardized clinical and pathological variables to determine if there were important group differences. UPDRS part III scores were significantly different between αSyn groups at lumbar puncture ($\chi^2 = 21.59$, $p < 0.0001$; see Table S2) and at last visit prior to death ($\chi^2 = 14.93$, $p = 0.0006$; see Table S2). Post hoc analyses showed that the limbic/neocortical group had higher UPDRS part III scores at lumbar puncture than those without αSyn-pathology and the amygdala-predominant αSyn group (Wilcoxon $z = -3.71$, $p = 0.0006$ and Wilcoxon $z = -3.44$, $p = 0.002$, respectively). The limbic/neocortical group also had higher UPDRS III scores at last visit prior to death compared to the amygdala-predominant group (Wilcoxon $z = -3.70$, $p = 0.0007$). The majority of patients diagnosed with DLB (8/9) and PD (4/4) showed limbic/neocortical αSyn, whereas 16 of 21 patients with amygdala predominant αSyn had a clinical diagnosis of AD ($\chi^2 = 28$, $p = 0.002$; see Tables S1, S2). Last, male sex was over-represented across the 3 αSyn distribution groups ($\chi^2 = 6.94$, $p = 0.03$; see Table S2).

### Sensitivity and Specificity of the αSyn-SAA Using CSF Samples

A total of 119 antemortem CSF samples were analyzed with the clinical αSyn-SAA. All but 1 of the 53 patients without αSyn-pathology were negative by the clinical αSyn-SAA and, thus, the specificity for the clinical assay

in this cohort was 98.1% (95% confidence interval [CI] = 90.1% to 99.9%; Table 2). Of the 66 individuals with αSyn-pathology, 47 were found positive by the clinical αSyn-SAA; neuropathological analysis is the gold standard to which αSyn-SAA results were compared. Thus, samples with positive αSyn-SAA results from patients with pathological αSyn found at autopsy were called true-positives, whereas samples with negative αSyn-SAA results from patients without αSyn-pathology were called true-negatives. The overall sensitivity of the assay to detect αSyn-pathology in any form was 71.2% (95% CI = 59.4% to 80.7%). However, significant differences were observed when stratifying sensitivity analysis by pathological αSyn distribution. The αSyn-SAA had sensitivity of 97.8% (95% CI = 88.4% to 99.9%) in detecting αSyn seeds in limbic/neocortical pathology, but only 14.3% (95% CI = 5.0% to 34.6%) in detecting amygdala-predominant αSyn-pathology (see Table 2).

Fifty-six of the 119 patients had postmortem CSF for clinical αSyn-SAA analysis, 26 had no αSyn-pathology, and 30 had αSyn-pathology at autopsy (limbic/neocortical n = 20 and amygdala-predominant n = 10). Of the 26 patients without αSyn pathology, 23 were found negative by the αSyn-SAA, for an estimated specificity of 88.5% (95% CI = 71.0% to 96.0%; see Table S2). Of the 30 individuals with αSyn pathology, 24 were found positive by αSyn-SAA; thus, the sensitivity for the combined cohort was 80% (95% CI = 62.7% to 90.5%). Similarly, when stratified by αSyn distribution, the clinical αSyn-SAA in postmortem CSF had sensitivity of 90% (95% CI = 69.9% to 98.2%) to detect individuals with limbic or neocortical αSyn, but sensitivity of only 60% (95% CI = 31.3% to 83.2%), to detect amygdala-predominant αSyn (see Table 2). Despite a decrease in sensitivity of the αSyn-SAA between antemortem and postmortem CSF samples, there was no significant difference in postmortem interval between patients that tested positive or negative using postmortem CSF in both limbic/neocortical ($p = 0.45$) and amygdala-predominant groups ($p = 0.12$).

Of the 56 individuals with both antemortem and postmortem CSF, 46 (82.1%) showed concordant αSyn-SAA results, 9 (16.1%) changed from negative results antemortem to positive results on the postmortem assay, and 1 (1.8%) changed from positive to negative. Interestingly, changes between antemortem and postmortem CSF αSyn-SAA results were significantly higher in amygdala-predominant cases (6/10, all negative to positive) than in limbic/neocortical cases (1/20; $\chi^2 = 28.49$, $p < 0.0001$).

One hundred sixteen antemortem (51 no αSyn pathology, 44 limbic/neocortical αSyn pathology, and 21 amygdala-predominant αSyn pathology) and 33 postmortem (11 no αSyn pathology, 15 limbic/neocortical αSyn pathology, and 7 amygdala-predominant αSyn pathology) CSF samples were also analyzed by a research kinetic αSyn-SAA to accurately estimate kinetic parameters and further characterize seeding activity in these samples. Fewer samples were run using this assay because some samples had been exhausted in the previous analysis. The kinetic assay provides a diagnostic output based on a probabilistic algorithm, which deems samples as "negative," "positive," or "inconclusive. The kinetic αSyn-SAA "negative" and "positive" determinations were consistent with the CLIA-regulated version of the assay for the antemortem and postmortem analyzed in parallel (data not shown). $F_{max}$ was analyzed between groups, with no αSyn-pathology ($p < 0.0001$, q = 20.42, DF = 113) and amygdala-predominant αSyn-pathology ($p < 0.0001$, q = 14.07, DF = 113) groups having significantly lower $F_{max}$ than individuals with neocortical or limbic αSyn pathology on antemortem CSF, most likely caused by the abundance of "negative" samples (Fig 1A). Representative raw kinetic graphs are shown in Figure 1B. There were kinetic differences in the seed amplification of amygdala-

**TABLE 2. Sensitivity, Specificity, and Predictive Values for Antemortem and Postmortem CSF αSyn-SAA Against αSyn-Pathology**

| Variable | Value, % (95% CI) |
|---|---|
| Antemortem (n = 119) | |
| Sensitivity | 71.2 (59.4–80.7) |
| *Limbic/neocortical* | 97.8 (88.4–99.9) |
| *Amygdala* | 14.3 (5.0–34.6) |
| Specificity | 98.1 (90.1–99.9) |
| Positive predictive value | 97.9 (89.1–99.9) |
| Negative predictive value | 73.2 (62.0–82.2) |
| Postmortem (n = 56) | |
| Sensitivity | 80.0 (62.7–90.5) |
| *Limbic/neocortical* | 90.0 (69.9–98.2) |
| *Amygdala* | 60.0 (31.3–83.2) |
| Specificity | 88.5 (71.0–96.0) |
| Positive predictive value | 88.9 (71.9–96.2) |
| Negative predictive value | 79.3 (61.6–90.2) |

αSyn-Path = includes neocortical, limbic, and amygdala predominant αSyn pathology; CI = confidence interval; CSF = cerebrospinal fluid.

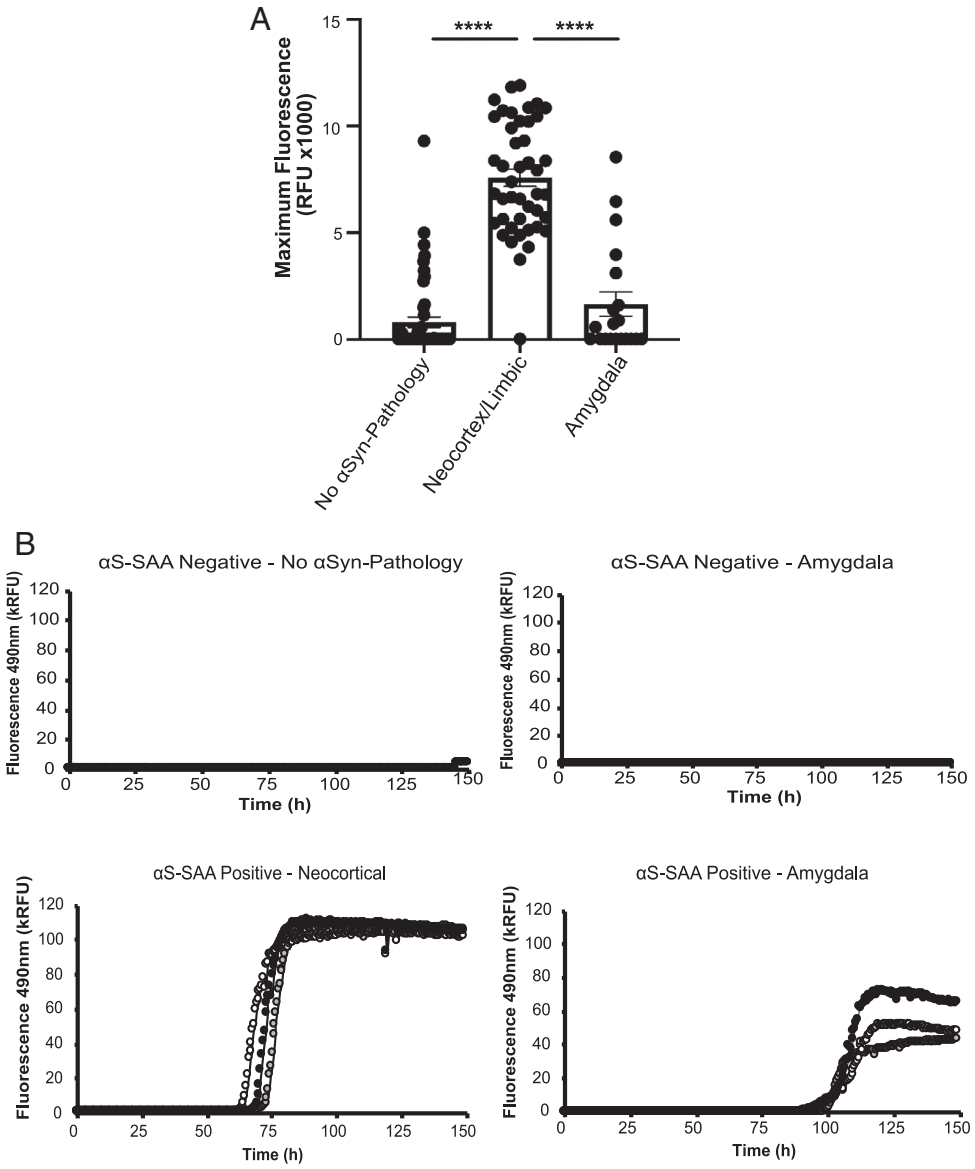


**FIGURE 1: Kinetic parameters of research SAA stratified by alpha-synuclein distribution. (A) Maximum fluorescence signal from R&D αSyn-SAA using antemortem CSF among no αSyn-pathology (n = 51), neocortex/limbic (n = 44), and amygdala-predominant (n = 21) groups. (B) Representative figures of raw kinetic data from the research SAA using antemortem CSF. Included are "negative" samples that are from no αSyn pathology and amygdala-predominant individuals, and "positive" samples that are from neocortical and amygdala-predominant individuals. Statistical analysis using one-way anlysis of variance (ANOVA) with Tukey's multiple comparisons post hoc (A). Error bars represent standard error of the mean (SEM). αSyn-SAA = α-synuclein seed amplification assay; CSF = cerebrospinal fluid; R&D = research and development; SAA = seed amplification assay.**

predominant cases compared with neocortical/limbic cases (TTT [$p = 0.0007$] and $T_{50}$ [$p = 0.0002$]) where amygdala-predominant cases had slower seeding activity. However, the small number of amygdala-predominant αSyn-SAA positive cases (n = 3) precludes reliable conclusions. There were no significant differences in kinetic parameters between αSyn pathology groups using post-mortem CSF in the kinetic αSyn-SAA (data not shown).

To investigate the potential effects of AD co-pathology on likelihood of αSyn-SAA seeding activity, we compared antemortem CSF αSyn-SAA results to CERAD scores (C0/C1 vs C2/C3), Braak tau stage (B0/B1 vs B2/B3) and Amyloid-β Thal Phase (A0/A1 vs A2/A3) for cases with pathological αSyn (Fig 2). There were no significant associations between the likelihood of αSyn-SAA positivity and CERAD score ($p = 0.7$), Thal phase ($p > 0.9$), and by Braak tau stage ($p > 0.9$; see Fig 2). We also evaluated the effect of proteins associated to AD biomarkers in CSF as they could interfere with the amplification process in the assay. No significant differences were

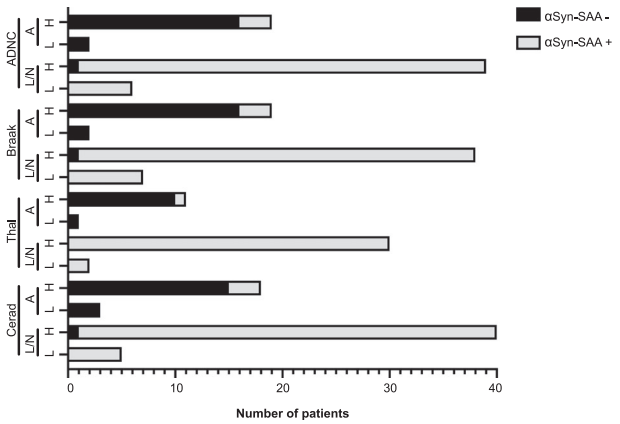


**FIGURE 2: Differences in neuropathology scores between synuclein-pathology groups as a function of SAA results. Bars represent the distribution of SAA positive or SAA negative within high ("H") or low ("L") categorization of ADNC, Braak, Thal, and Cerad neuropathological staging. Patients are further classified by limbic/neocortical ("L/N") or amygdala-predominant ("A") groups. Statistical analysis using Fisher's exact test within synuclein-pathology group. αSyn-SAA = α-synuclein seed amplification assay; ADNC = Alzheimer's disease neuropathologic change; SAA = seed amplification assay.**

found in levels of Aβ40, Aβ42, Aβ42/40 ratio, t-tau, and p-tau, between limbic/neocortical cases and amygdala-predominant cases as a function of αSyn-SAA result within institution (data not shown). Overall, αSyn-SAA positivity or lack thereof is not associated with the presence of AD co-pathology or commonly used AD CSF biomarkers.

### *Comparisons of Subjects with Positive Versus Negative CSF αSyn-SAA Results*

UPDRS part III scores at the time of lumbar puncture were significantly lower in the antemortem false-negative group compared to the true-positive group ($Z = -3.12$, $p = 0.002$), considering pathological analysis as the gold standard. The interval between lumbar puncture and death was significantly different between the true-positive and false-negative groups, with the false-negative group having, on average, a longer interval than the true-positive group ($Z = 2.09$, $p = 0.04$; Fig 3A). The 2 groups also differed in the distribution of αSyn pathology ($\chi^2 = 48.69$, $p < 0.0001$); 94.7% of the false negatives fell into the amygdala-predominant group, whereas 93.6% of the true positives fell into the limbic/neocortical group. Similarly, in postmortem CSF, 66.7% of false negatives were in the amygdala-predominant group and 75% of true positives were in the limbic/neocortical αSyn group ($\chi^2 = 3.75$, $p = 0.05$; Fig 3B).

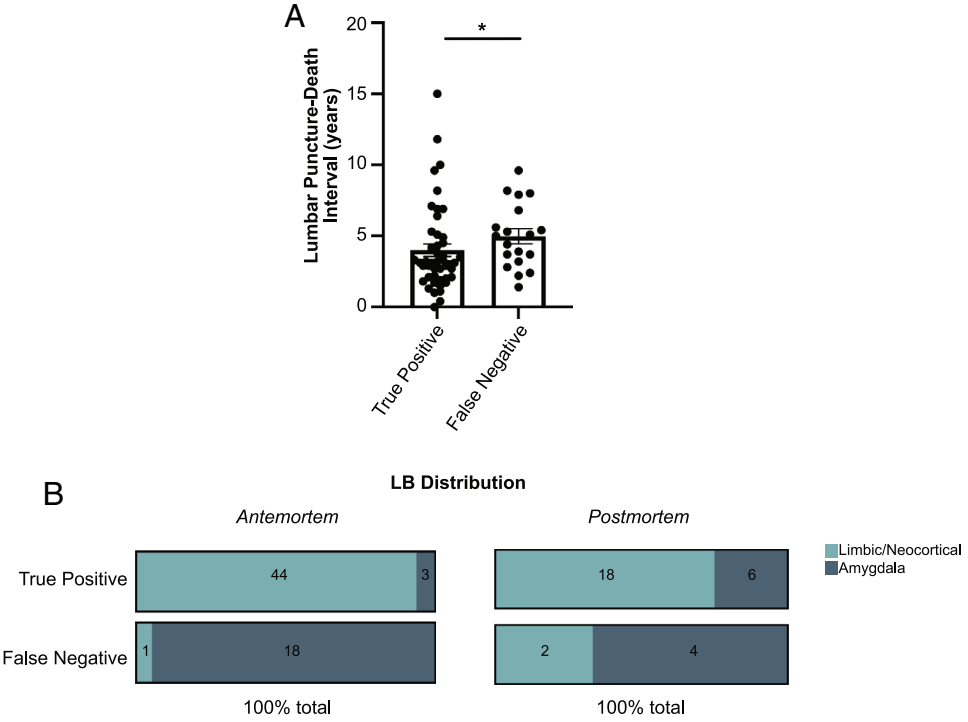


**FIGURE 3: Clinical and pathological differences between true positive and false negative. (A) Interval in years from lumbar puncture to death between true positives (n = 47) and false negative (n = 19) groups. (B) Distribution of neocortex/limbic and amygdala-predominant LRP in true positive and false negative groups for antemortem and postmortem CSF analysis. Number of patients in each category is indicated on the bar. Statistical analysis using Wilcoxon rank-sum test with post hoc pairwise comparisons from the Dwass, Steel, Critchlow-Fligner method (A) or chi-square (B). Error bars represent standard error of the mean (SEM). CSF = cerebrospinal fluid; LB = Lewy bodie; LRP = Lewy-related pathology.**

**TABLE 3. Patient Categorization from Brain Homogenate Samples**

| Case information | | | | | | | | | | Brain tissue | | AM CSF | | PM CSF |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Case | Sex | Age at onset | Age at death | Primary Pathology | Thal Phase | CERAD Stage | Braak Stage | ADNC | LRP Classification | Frontal Cortex | Amygdala | LP to death (y) | Result | Result |
| 1 | Male | NA | 84 | Normal | A0 | C0 | I | Not | None | − | − | 1.8 | − | |
| 2 | Male | 65 | 71 | CBD | A3 | C1 | I | Low | None | 2/3 | − | 2.9 | − | |
| 3 | Female | 65 | 76 | AD | A3 | C3 | VI | High | None | − | − | 4.7 | − | − |
| 4 | Female | 58 | 66 | AD | A3 | C3 | VI | High | None | − | − | 3.0 | − | − |
| 5 | Female | 72 | 84 | AD | A3 | C2 | VI | High | Amygdala | − | | 2.7 | − | |
| 6 | Female | 83 | 90 | AD + PSP | A3 | C2 | VI | High | Amygdala | − | 2/3 | 0.9 | + | |
| 7 | Female | 84 | 91 | AD | A3 | C3 | VI | High | Amygdala | 2/3 | + | 4.6 | − | |
| 8 | Male | 75 | 84 | AD | A3 | C2 | VI | High | Amygdala | 2/3 | + | 4.9 | − | + |
| 9 | Female | 56 | 66 | FTLD TDP-43 | A0 | C0 | I | Not | Amygdala | − | − | 3.8 | − | − |
| 10 | Male | 69 | 76 | AD | A3 | C3 | VI | High | Amygdala | − | − | 5.5 | − | − |
| 11 | Male | 55 | 73 | AD | A3 | C3 | V | High | Amygdala | − | − | 1.9 | − | − |
| 12 | Male | 77 | 86 | AD | A3 | C3 | V | High | Amygdala | − | + | 5.3 | − | + |
| 13 | Female | 79 | 87 | AD | A3 | C3 | IV | Intermediate | Amygdala | − | + | 5.3 | − | + |
| 14 | Female | 90 | 100 | AD | A3 | C3 | V | High | Amygdala | − | − | 8.0 | − | − |
| 15 | Male | 54 | 67 | LBD | A1 | C2 | I | Low | Limbic/neocortical | + | + | 8.1 | + | |
| 16 | Male | 72 | 81 | LBD | A3 | C2 | V | High | Limbic/neocortical | + | + | 3.7 | + | |
| 17 | Male | 59 | 71 | LBD | A3 | C2 | IV | Intermediate | Limbic/neocortical | + | + | 3.5 | + | |
| 18 | Male | 63 | 71 | LBD | A3 | C2 | III | Intermediate | Limbic/neocortical | + | + | 1.2 | + | |
| 19 | Male | 66 | 71 | LBD | A3 | C3 | VI | High | Limbic/neocortical | + | + | 1.7 | + | + |
| 20 | Male | 62 | 73 | LBD | A3 | C1 | II | Low | Limbic/neocortical | + | + | 6.8 | + | + |
| 21 | Male | 52 | 72 | LBD | A2 | C2 | V | Intermediate | Limbic/neocortical | + | + | 9.3 | + | + |
| 22 | Female | 51 | 59 | LBD | A3 | C3 | VI | High | Limbic/neocortical | + | + | 2.7 | + | + |

Inconclusive cases have 2 of 3 replicate wells that were positive. Brain tissue samples were analyzed at $10^{-8}$ dilution. Positive results indicate 3 of 3 replicates were positive and negative results indicate 0 of 3 replicates were positive. Amygdala tissue could not be obtained for case 5.

AD = Alzheimer's disease; ADNC = Alzheimer's disease neuropathological change; AM = antemortem; CBD = corticobasal degeneration; CSF = cerebrospinal fluid; FTLD TDP-43 = frontotemporal lobar degeneration TAR DNA-binding protein 43; LBD = Lewy-body disease; LRP = Lewy related pathology; NA = not applicable; PSP = progressive supranuclear palsy; PM = postmortem.

### *Clinical Significance of Incidental Synuclein Pathology*

Last, we explored how clinical diagnosis related to clinical αSyn-SAA performance, in order to better understand whether subtle clinical predictors were present among patients without a diagnosis of a synucleinopathy whose antemortem CSF tested positive by αSyn-SAA. In this analysis, we examined all patients who were clinically diagnosed with AD, without concomitant PD or DLB, and whose antemortem CSF αSyn-SAA results were positive versus negative. There was a significant difference in patient biological sex, where αSyn-SAA-positive patients had a significantly greater proportion of men (23/29, 79.3%) compared to αSyn-SAA-negative patients (25/46, 54.3%, $\chi^2 = 7.84$, $p = 0.005$). Clinically diagnosed patients with AD with positive αSyn-SAA CSF had higher UPDRS part III scores (6.71 +/− 8.6) than those with negative αSyn-SAA CSF (1.82 +/− 4.92) at most recent visit prior to death ($Z = 2.53$, $p = 0.01$).

### *Detection of αSyn Seeds from Frontal Cortex and Amygdala Brain Samples*

We next analyzed a subset of patients (n = 22) from the UCSD-ADRC cohort who had frozen brain tissue

available for analysis, including 4 no αSyn-pathology, 10 amygdala-predominant, and 8 limbic/neocortical cases. In both brain regions, the 4 patients without αSyn-pathology were negative by the αSyn-SAA, consistent with the results for antemortem CSF in both kinetic and clinical assays (Table 3). In agreement with the high sensitivity in CSF for limbic/neocortical cases, seeding activity was detected in both the frontal cortex and the amygdala of all 8 analyzed cases. However, there was a significant decrease in seeding activity in both frontal cortex and amygdala of the amygdala-predominant cases. Of the 10 amygdala-predominant cases, 4 cases showed no seeding activity in both frontal cortex and amygdala. There were 2 cases with seeding activity detected in the amygdala, with one of them showing 2 of 3 wells positive in the frontal cortex.

Within no αSyn pathology and limbic/neocortical groups, there was 100% concordance between brain homogenate results and CSF results. Of the 5 patients with amygdala-predominant αSyn pathology that also tested positive on the αSyn-SAA using amygdala brain tissue, 3 also had some seeding activity on the αSyn-SAA using either antemortem or postmortem CSF (see Table 3). Overall, the assay detected higher seeding activity in amygdala tissue in amygdala-predominant cases, whereas neocortical cases presented high levels of seeding activity in both brain regions.

## Discussion

Although there have been large strides in the understanding of the molecular basis of synucleinopathies, in vivo methods for detecting αSyn are still limited. Misfolded αSyn aggregation likely begins years to decades before the onset of symptoms, allowing for the potential ability to identify patients in the earliest stages of their diseases. The development of a sensitive and specific diagnostic tool for synucleinopathies would allow for early diagnosis of patients where often there is the highest level of clinical uncertainty and when disease modifying therapies are of the greatest potential use.[39] Thus, αSyn-related biomarkers remain a crucial need to the field. Several publications have shown promising results for αSyn-SAAs performed in academic laboratories,[17,24] but the performance of the assay within a regulated CLIA environment, and against pathology-confirmed samples, has been a gap. Moreover, the knowledge of whether current generations of αSyn-SAAs can detect pathological αSyn in patients with other pathologies and with clinical diagnoses other than PD or DLB is crucial to understanding the range of their diagnostic utility. The αSyn-SAA offers the ability to identify αSyn seeds in living patients and studies have focused largely on cases with clinical DLB, PD, and MSA and where performed, autopsy was used as a validation of the clinical diagnosis. However, these assays offer the potential ability to identify patients with αSyn pathology who may not exhibit a "synucleinopathy phenotype." One factor that can complicate diagnosis is the presence of AD co-pathology, which affects clinical expression particularly in PD and DLB.[12,32,40–44] Furthermore, in AD, αSyn pathology pathology in AD-LBV is common and also associated with worse prognosis and specific clinical features.[7,11] The use of αSyn-SAA assays to help characterize patients in terms of their αSyn pathology is immediately clinically applicable and potentially valuable in clinical trials to recruit homogenous populations; but detailed studies in well-characterized pathologically validated cohorts has been needed to understand how the current αSyn-SAA assay can be applied. We used pathologically driven categories of αSyn pathology, independent of clinical diagnosis, in a cohort of patients with high degrees of co-pathology to assess the performance of the αSyn-SAA assay. In these cases, the use of such a biomarker could prove useful in identifying αSyn pathology that was not necessarily suspected.

Our results add to the previous reports that αSyn-SAAs can robustly detect αSyn seeds in the limbic/neocortical stage αSyn pathology, but also show decreased sensitivity in detecting αSyn seeds in amygdala-predominant cases. An additional unique feature to this study is the number of subjects with postmortem CSF, providing a proximal time point to the autopsy assessment. Classification using postmortem CSF showed a sensitivity of 80% and specificity of 88.5%, however, when stratified by pathology distribution, again, the assay performed significantly better in detecting limbic/neocortical than amygdala-predominant αSyn-pathology. Last, we also observed decreased seeding activity from amygdala-predominant cases when assaying frozen brain tissue from the frontal cortex and the amygdala.

The lower sensitivity of CSF αSyn-SAA to detect αSyn seeds in amygdala-predominant pathology may represent assay dependence on degree of brain αSyn "burden." Alternatively, negative αSyn-SAA CSF samples in the amygdala-predominant group could be explained by localized brain pathology that does not enter the CSF. However, direct analysis of the amygdala homogenate from amygdala-predominant cases showed low detection, suggesting less seeding activity by these particular αSyn species. Recent studies have found that αSyn species in amygdala-predominant pathology found in AD may have different immunohistochemical properties than patients with PD or DLB with limbic and neocortical αSyn-pathology.[13–15] It is plausible that these amygdala-predominant αSyn seeds have lower rates of amplification

due to unique conformation or post-translational modifications of these αSyn species. Currently, there is no method to quantify αSyn seeds in a sample, thus, it is not possible to determine if αSyn seeds were extracted with similar efficiencies from amygdala and frontal lobe tissues. Lower concentrations in the amygdala homogenates could explain negative results. However, we found positivity in dilutions up to $10^{-9}$ in some cases which is higher than previously shown in the literature (not shown), suggesting the homogenization protocol did not artificially decrease the amount of αSyn seeds. The small number of amygdala-predominant cases who had seeding activity had slower TTT and $T_{50}$ values than limbic/neocortical cases (TTT = $p = 0.0007$ and $T_{50} = p = 0.0002$). This is potentially of interest given that in vitro models have shown that lower levels of synthetic αSyn seeds take longer to amplify in αSyn-SAA.[17,18] However, future studies of larger cohorts will be needed to confirm these preliminary observations.

Because αSyn pathology commonly co-exists in AD and may be associated with faster clinical progression,[45] identifying this pathology with a biomarker would improve clinical monitoring and create options for clinical trials targeting αSyn in these patients. If amygdala-predominant type αSyn pathology is an early stage or precursor of more widespread concomitant LB pathology in AD, then detecting its presence through biomarkers such as αSyn-SAA would be useful. However, the effect of amygdala-predominant αSyn-pathology in AD appears to have less clinical impact in some cases or may take years to convert to a more widespread seeding. Further work is needed to determine why the seeding potential of amygdala-predominant αSyn pathology is lower in some cases, or whether different types of αSyn-SAAs could provide detection of this pathology. We also report for the first time that αSyn seeds can be amplified from postmortem CSF samples. This is relevant because it could offer some insights when antemortem CSF samples are negative but there is detectable brain pathological αSyn upon neuropathological analysis. In these cases, positive postmortem CSF results could indicate that the αSyn pathological process started after antemortem CSF collection or that the disease process was too early at the time of antemortem CSF collection. However, we observed a reduction in sensitivity when testing postmortem CSF from neocortical/limbic cases, driven by 2 samples that were negative. Because we observed an increase in sensitivity when analyzing amygdala-predominant postmortem CSF, αSyn seed degradation or overall CSF instability is unlikely to explain the difference. Preliminary observations suggest that brain debris or cellular breakdown products could contaminate the sample during postmortem CSF collection, which effects could be minimized at least partially by centrifugation. Nevertheless, the instability of αSyn seeds and other CSF components in postmortem CSF and their potential effects on αSyn-SAA have not been systematically studied and require further exploration.

The assay's ability to identify clinically unexpected synuclein pathology is an area of great potential. Our results indicate that 27 of 75 (36%) of the clinically diagnosed patients with AD had αSyn aggregates in their antemortem CSF and were later autopsy-confirmed to have limbic/neocortical LB disease. DLB can be misdiagnosed as AD during life, and the presence of moderate to severe AD-related tau pathology is associated with a lower likelihood of visual hallucinations and cognitive fluctuations, and worse performance on tests of episodic memory and naming in patients with DLB, meaning that it is more challenging to diagnose these patients with mixed pathologies accurately.[32,44,46]

Clinically, our cohort included only 4 PD and 9 DLB cases, and, pathologically, there were no cases with brainstem-only αSyn pathology, which are limitations of the study. Our study adds valuable new information about the accuracy of αSyn-SAA in the context of co-pathology and non-LBD diagnoses. In another study, CSF from 4 cases with incidental αSyn pathology in the brainstem only have been analyzed by αSyn-SAA.[25] Three of these cases were positive, suggesting that brainstem pathological αSyn shares propagation features with limbic and neocortical rather than amygdala-predominant pathological αSyn. Because brainstem-only pathological αSyn is an early event, these results are consistent detection of αSyn seeds in CSF of prodromal PD cases, like isolated rapid eye movement (REM) sleep behavior disorder (iRBD).[25,29,47] Finally, other minor weaknesses include potential differences in interpretation of the NACC guidelines between the 2 institutions and the impossibility to determine if patients with αSyn-SAA negative antemortem CSF and pathological αSyn upon autopsy represent true false negatives or the pathology developed after antemortem CSF collection. Additionally, the limbic/neocortical group was skewed toward male participants. This is congruent with numerous studies identifying a sex-link for risk of synucleinopathy.[48–50] Larger numbers of cases with additional distributions of pathological αSyn, particularly brainstem-only and olfactory-only, should be further investigated to get a full picture of the relationship between brain pathology and CSF αSyn-SAA positivity. Last, further work is needed to fully interrogate differences in the seeding activity between pathological αSyn from different brain regions. It is unknown if the differences reflect the conformation of the seeds (strains), interactions with co-localized co-pathology, or perhaps brain region specific components (proteins, lipids, polysaccharides,

nucleic acids, etc.) that may have an effect of the αSyn-SAA. Our data suggest that AD co-pathology is unlikely to explain the differences based on CSF measures, CERAD scores, Braak-tau stages, and Thal phases.

In this large, multicentered autopsy-validated cohort of patients with a variety of stages of αSyn pathology, our results indicate that the αSyn-SAA is highly predictive of neocortical or limbic αSyn pathology in aging patients for whom αSyn pathology is not clinically suspected. This feature makes αSyn-SAA a diagnostic tool with great potential for clinical trials aiming to initiate interventions early in the disease process or to select-out patients with co-incidental αSyn pathology. However, there was substantially lower sensitivity to detect amygdala-predominant αSyn pathology in brain tissue and CSF, which may have distinct biochemical properties and seeding potential that reduces detection in current generation of αSyn-SAAs.

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## Author Contributions

M.R.A., D.G.C., D.S.S., L.C.M., D.R.G., and J.F.Q. contributed to the conception and design of the study. M.R.A., D.G.C., B.H.B., D.S.S., L.C.M., C.M.F., Y.M., Y.K., E.N.W., J.A.K., A.H., and R.L.W. contributed to the acquisition and analysis of data. M.R.A., D.G.C., B.H.B., L.C.M., D.R.G., and J.F.Q. contributed to drafting the text or preparing the figures.

## Potential Conflict of Interest

Dr. Concha, Ms. Farris, and Mr. Ma are inventors on several patents related to PMCA technology (SAA) and are associated to Amprion Inc., a biotech company focused on the commercial utilization of SAA for diagnosis. All other authors have no conflicts of interest to disclose.

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