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## **$\alpha$ -synuclein seed amplification in CSF and brain from patients with different brain distributions of pathological $\alpha$ -synuclein in the context of co-pathology and non-LBD diagnoses**

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### **Abstract**

**Objective:** To determine the sensitivity and specificity of  $\alpha$ -synuclein seed amplification assay ( $\alpha$ Syn-SAA) in antemortem and postmortem CSF of autopsy-confirmed patients with different distributions of pathological  $\alpha$ Syn, co-pathologies, and clinical diagnoses.

**Methods:**  $\alpha$ Syn-SAA was used to test antemortem CSF samples from 119 subjects with a variety of clinical syndromes and standardized neuropathological examinations from OHSU and UCSD (56 additional postmortem CSF samples available). The  $\alpha$ Syn-SAA was also applied to frontal cortex and amygdala homogenates. Sensitivity and specificity were compared across distributions

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MRA, DGC, DSS, LCM, DRG, and JFQ contributed to the conception and design of the study; MRA, DGC, BHB, DSS, LCM, CMF, YM, YK, ENW, JAK, AH, and RLW contributed to the acquisition and analysis of data; MRA, DGC, BHB, LCM, DRG, and JFQ contributed to drafting the text or preparing the figures.

Potential Conflicts 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.

of  $\alpha$ Syn-pathology. Clinical data and co-pathologies were compared across  $\alpha$ Syn-SAA positive and negative groups.

**Results:** Fifty-three individuals without and 66 with  $\alpha$ Syn-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  $\alpha$ Syn-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  $\alpha$ Syn-SAA analyses also showed higher assay positivity in samples from limbic/neocortical cases.

**Interpretation:** CSF  $\alpha$ Syn-SAA reliably identifies  $\alpha$ Syn seeds in patients with diffuse  $\alpha$ Syn-pathology in the context of co-pathology and non-LBD diagnoses. The analysis of brain homogenates suggests that pathological  $\alpha$ Syn in amygdala might differ from pathological  $\alpha$ Syn in frontal cortex.  $\alpha$ Syn-SAA might facilitate the differential diagnosis of dementias with mixed pathologies.

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## Introduction

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

To date, neuropathological assessment at autopsy remains the gold standard to diagnose LBDs and *in vivo*  $\alpha$ Syn biomarkers have been an unmet need. Recently,  $\alpha$ Syn Seed Amplification Assays ( $\alpha$ Syn-SAAs) (also known as protein misfolding cyclic amplification (PMCA) and real time quaking induced conversion (RT-QuIC)), have been adapted to detect misfolded  $\alpha$ Syn aggregates ( $\alpha$ Syn seeds) in CSF and peripheral tissues with remarkable diagnostic accuracy<sup>16–21</sup>.  $\alpha$ Syn-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%<sup>16–18, 22–29</sup>. However,  $\alpha$ Syn-SAA performance in neuropathologically validated cohorts with varying distribution of  $\alpha$ Syn pathologies, co-pathologies, and non-LBD diagnoses has not been evaluated. Thus, it remains unknown if different types of  $\alpha$ Syn-pathology distributions produce differences in seeding activity. A few studies have reported detection of  $\alpha$ Syn seeds in CSF from clinically diagnosed AD patients (5/14 or 36% in one report<sup>17</sup> and 0/16 in another<sup>18</sup>) and from patients

clinically diagnosed with AD who were pathologically confirmed to have DLB (11/17 or 65%) or incidental Lewy bodies (2/13 or 15%)<sup>16</sup>. Despite the low number of cases, these results suggest that current assays may have different sensitivities, which may depend on  $\alpha$ Syn-pathology distribution, co-pathologies, and/or pathological  $\alpha$ Syn species.

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

## Methods

### Patient Selection

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)<sup>30</sup>, 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  $\alpha$ Syn-pathology (n=59),  $\alpha$ Syn-pathology in isolation (n=3), progressive supranuclear palsy (n=2), corticobasal degeneration (n=2), FTLTDP-43 (n=2), vascular disease in isolation (n=2), normal subjects (n=2), and patients with a mix of AD and other tauopathies (n=4) (Table 1). Cases were grouped by  $\alpha$ Syn-pathology distribution as below. Patient-level information can be found in Supplemental Table 1.

### CSF Analysis

CSF was collected for all 119 cases by lumbar puncture in the morning fasting condition according to a standardized protocol<sup>31</sup>. A subset of patients (n=56) had additional CSF samples obtained at the time of brain removal. CSF specimens were divided into 0.5 ml aliquots and stored at -80°C. Antemortem CSF collection occurred 1–15 years prior to autopsy (17.6% in 0–2yr, 46.3% in 2–5yr, 18.5% in 5–8yr, 6.7% in 8–10yr, 10.9% in

10–16yr). Antemortem CSF was analyzed for A $\beta$ 40, A $\beta$ 42, t-tau and p-tau (Lumipulse, Fujirebio at both sites).

CSF samples were initially analyzed by the endpoint qualitative version of the  $\alpha$ Syn-SAA that has been validated for clinical use under CLIA/CAP certifications (clinical assay, SYNTap™). Each sample was analyzed in triplicate (40 $\mu$ L CSF per well) in a 96-well plate (COSTAR, cat# 3603) with a final volume reaction of 200 $\mu$ L). The reaction mixture consisted of 0.3mg/mL rec- $\alpha$ Syn (Amprion, cat# S2020) in 100mM PIPES pH 6.50, 500mM NaCl, 10 $\mu$ M ThT, and a 2.5mm borosilicate glass bead per well. Plates were sealed using an Optical Adhesive Film (ThermoFisher, cat# 4311971) and shaken at 800rpm with orbital shaking for 1min every 29min of quiescent incubation in a TIMIX 5 shaker (Edmund Buehler) placed in an incubator set to 37°C. Bottom fluorescence readings at 490nm 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 preestablished threshold for the median maximum fluorescence of the triplicate. The research and development (R&D) kinetic  $\alpha$ Syn-SAA was utilized to analyze CSF samples and brain tissues. The methods of the kinetic  $\alpha$ Syn-SAA have been reported in detail elsewhere<sup>22, 23</sup>. Briefly, CSF samples and brain homogenates (BHs) were evaluated in triplicates (40 $\mu$ L/well) in a 96-well plate (COSTAR 96, cat# 3916), in a reaction mix consisting of 0.3mg/ml rec- $\alpha$ Syn (Amprion, cat# S2021), 100mM PIPES pH 6.50 (Sigma, cat# 80635), 500mM NaCl (Lonza, cat# 51202), 10 $\mu$ M ThT (Sigma, cat# T3516), and a 3/32-inch BSA-blocked Si<sub>3</sub>N<sub>4</sub> bead (Tsubaki Nakashima). This assay was performed in a BMG FLUOstar Omega shaker/reader with orbital shaking at 800rpm for 1min and 29min of quiescent incubation at 37°C. Fluorescence at 490nm was measured every 30min 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<sup>22</sup>. 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  $F_{max}$  ( $T_{50}$ , h) of each replicate/well. The time to threshold (TTT, h) 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), 500mg samples of frozen brain tissue from the middle frontal cortex and amygdala were provided for  $\alpha$ Syn-SAA. Cases included those without  $\alpha$ Syn-pathology (n=4), amygdala-predominant  $\alpha$ Syn-pathology (n=10), and limbic/neocortical  $\alpha$ Syn-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 cOplete Mini EDTA-free protease inhibitor cocktail (Roche, cat# 11836170001). Approximately 100 $\mu$ g of brain sample was homogenized in 1.5mL tubes preloaded with 1mm zirconium beads (cat# 11079110zx) in a MP FastPrep 24 homogenizer. Two rounds of homogenization were performed for all samples (15s at 4m/s and 30s at

6m/s). If additional homogenization was needed, samples were chilled on ice for 5min in between additional homogenization rounds at 6m/s for 30s. BHs were centrifuged at 800xg for 1 minute to remove cellular debris. Supernatants were collected, vortexed, aliquoted, and stored at -80°C until  $\alpha$ Syn-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 over-dilution.

## 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- $\beta$ ,  $\alpha$ -synuclein, and TDP-43 species as appropriate and pathological diagnoses were assigned by expert neuropathologists<sup>32-35</sup>. MSA cases were excluded from this study given the known altered kinetics on  $\alpha$ Syn-SAA assays compared to PD and DLB cases<sup>36</sup>. Alzheimer's disease neuropathological change was assigned according to NACC guidelines after Braak tau stage, CERAD stage, and Thal phase was determined<sup>33, 37</sup>. Distribution of LRP was determined via  $\alpha$ -synuclein immunohistochemistry staining (OADC:  $\alpha$ Syn MJFR1, Abcam; UCSD-ADRC: pSer129  $\alpha$ Syn 81A, Biologend Laboratories) using slices from pons and/or midbrain, hippocampus, amygdala, and neocortical areas including temporal cortex and/or 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-<sup>38</sup>.

## Statistical Analysis

Clinical and pathological differences between the OADC and UCSD-ADRC cohorts were assessed to determine the necessity for stratification by site. All DLB and PD patients 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 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, CDR at most recent visit, lumbar puncture to autopsy interval, CSF A $\beta$ 40, A $\beta$ 42, t-tau, and p-tau, disease duration, postmortem interval), we used a Kruskal-Wallis test (more than two groups) or a Wilcoxon rank-sum test (two 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, 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, 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 A $\beta$ 40, A $\beta$ 42, 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 $\alpha$ Syn analysis and comparison

The neuropathological analysis of the 119 subjects revealed  $\alpha$ Syn-pathology in the brains of 66 (55%) cases. Of the 66 patients with  $\alpha$ Syn-pathology, 38 showed neocortical stage  $\alpha$ Syn-pathology, 7 showed limbic stage  $\alpha$ Syn-pathology, and 21 showed amygdala-predominant  $\alpha$ Syn-pathology. Rates of AD pathology was high across the cohort. 40/53 (75%) of cases without  $\alpha$ Syn-pathology had intermediate or high degrees of AD neuropathological change, as did 19/21 (90%) cases with amygdala predominant  $\alpha$ Syn-pathology and 39/45 (87%) cases limbic or neocortical disease (Table 1). These rates were not statistically significant across the  $\alpha$ Syn 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 (Table 1, Supplemental Table 1). No significant difference in A $\beta$ 40, A $\beta$ 42, A $\beta$ 42/40 ratio, t-tau, and p-tau in antemortem CSF were observed between the  $\alpha$ Syn-pathology groups within institution (Supplemental Table 2).

Using a Kruskal-Wallis test, we compared patients within  $\alpha$ Syn distribution groups (none, amygdala-predominant, 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  $\alpha$ Syn groups at lumbar puncture ( $X^2=21.59$ ,  $p<0.0001$ , Supplemental Table 2) and at last visit prior to death ( $X^2=14.93$ ,  $p=0.0006$ , Supplemental Table 2). Post-hoc analyses showed that the limbic/neocortical group had higher UPDRS part III scores at lumbar puncture than those without  $\alpha$ Syn-pathology and the amygdala-predominant  $\alpha$ Syn 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  $\alpha$ Syn while 16/21 patients with amygdala predominant  $\alpha$ Syn had a clinical diagnosis of AD ( $X^2=28$ ,  $p=0.002$ , Supplemental Table 2, Supplemental Table 1). Lastly, male sex was over-represented across the three  $\alpha$ Syn distribution groups ( $X^2=6.94$ ,  $p=0.03$ , Supplemental Table 2).

### Sensitivity and specificity of the $\alpha$ Syn-SAA using CSF samples

A total of 119 antemortem CSF samples were analyzed with the clinical  $\alpha$ Syn-SAA. All but 1 of the 53 patients without  $\alpha$ Syn-pathology were negative by the clinical  $\alpha$ Syn-SAA and thus, the specificity for the clinical assay in this cohort was 98.1% (95% CI 90.1% to 99.9%) (Table 2). Of the 66 individuals with  $\alpha$ Syn-pathology, 47 were found positive by the clinical  $\alpha$ Syn-SAA; Neuropathological analysis is the gold standard to which  $\alpha$ Syn-SAA

results were compared to. Thus, samples with positive  $\alpha$ Syn-SAA results from patients with pathological  $\alpha$ Syn found at autopsy were called true-positives, while samples with negative  $\alpha$ Syn-SAA results from patients without  $\alpha$ Syn-pathology were called true-negatives. The overall sensitivity of the assay to detect  $\alpha$ Syn-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  $\alpha$ Syn distribution.  $\alpha$ Syn-SAA had sensitivity of 97.8% (95% CI 88.4% to 99.9%) in detecting  $\alpha$ Syn seeds in limbic/neocortical pathology, but only 14.3% (95% CI 5.0% to 34.6%) in detecting amygdala-predominant  $\alpha$ Syn-pathology (Table 2).

Fifty six of the 119 patients had postmortem CSF for clinical  $\alpha$ Syn-SAA analysis, 26 had no  $\alpha$ Syn-pathology and 30 had  $\alpha$ Syn-pathology at autopsy (limbic/neocortical n=20, amygdala-predominant n=10). Of the 26 patients without  $\alpha$ Syn-pathology, 23 were found negative by the  $\alpha$ Syn-SAA, for an estimated specificity of 88.5% (95% CI 71.0% to 96.0%) (Supplemental Table 2). Of the 30 individuals with  $\alpha$ Syn-pathology, 24 were found positive by  $\alpha$ Syn-SAA; thus, the sensitivity for the combined cohort was 80% (95% CI 62.7% to 90.5%). Similarly, when stratified by  $\alpha$ Syn distribution, the clinical  $\alpha$ Syn-SAA in postmortem CSF had sensitivity of 90% (95% CI 69.9% to 98.2%) to detect individuals with limbic or neocortical  $\alpha$ Syn, but sensitivity of only 60% (95% CI 31.3% to 83.2%), to detect amygdala-predominant  $\alpha$ Syn (Table 2). Despite a decrease in sensitivity of the  $\alpha$ Syn-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  $\alpha$ Syn-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  $\alpha$ Syn-SAA results was significantly higher in amygdala-predominant cases (6/10, all negative to positive) than in limbic/neocortical cases (1/20) ( $X^2=28.49$ ,  $p<0.0001$ ).

116 antemortem (51 no  $\alpha$ Syn-pathology, 44 limbic/neocortical  $\alpha$ Syn-pathology, 21 amygdala-predominant  $\alpha$ Syn-pathology) and 33 postmortem (11 no  $\alpha$ Syn-pathology, 15 limbic/neocortical  $\alpha$ Syn-pathology, 7 amygdala-predominant  $\alpha$ Syn-pathology) CSF samples were also analyzed by a research kinetic  $\alpha$ Syn-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  $\alpha$ Syn-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  $\alpha$ Syn-pathology (p<0.0001, q=20.42, DF=113) and amygdala-predominant  $\alpha$ Syn-pathology (p<0.0001, q=14.07, DF=113) groups having significantly lower  $F_{\max}$  than individuals with neocortical or limbic  $\alpha$ Syn-pathology on antemortem CSF, most likely caused by the abundance of “negative” samples (Figure 1A). Representative raw kinetic graphs are shown in Figure 1B. There were kinetic differences

in the seed amplification of amygdala-predominant cases compared to 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  $\alpha$ Syn-SAA positive cases ( $n=3$ ) precludes reliable conclusions. There were no significant differences in kinetic parameters between  $\alpha$ Syn-pathology groups using postmortem CSF in the kinetic  $\alpha$ Syn-SAA (data not shown).

To investigate the potential effects of AD co-pathology on likelihood of  $\alpha$ Syn-SAA seeding activity, we compared antemortem CSF  $\alpha$ Syn-SAA results to CERAD scores (C0/C1 v C2/C3), Braak tau stage (B0/B1 v B2/B3) and Amyloid- $\beta$  Thal Phase (A0/A1 v A2/A3) for cases with pathological  $\alpha$ Syn (Figure 2). There were no significant associations between the likelihood of  $\alpha$ Syn-SAA positivity and CERAD score ( $p=0.7$ ), Thal phase ( $p>0.9$ ), and by Braak tau stage ( $p>0.9$ ) (Figure 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 found in levels of A $\beta$ 40, A $\beta$ 42, A $\beta$ 42/40 ratio, t-tau, and p-tau, between limbic/neocortical cases and amygdala-predominant cases as a function of  $\alpha$ Syn-SAA result within institution (data not shown). Overall,  $\alpha$ Syn-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 vs negative CSF $\alpha$ Syn-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 gold standard. The interval between lumbar puncture and death was significantly different between true positive and false negative groups, with the false negative group having on average a longer interval than the true positive group ( $Z=2.09$ ,  $p=0.04$ , Figure 3A). The two groups also differed in the distribution of  $\alpha$ Syn-pathology ( $X^2=48.69$ ,  $p<0.0001$ ); 94.7% of the false negatives fell into the amygdala-predominant group, while 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  $\alpha$ Syn group ( $X^2=3.75$ ,  $p=0.05$ ) (Figure 3B).

### Clinical significance of incidental synuclein pathology

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



### Detection of $\alpha$ Syn 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  $\alpha$ Syn-pathology, 10 amygdala-predominant, and 8 limbic/neocortical cases. In both brain regions, the 4 patients without  $\alpha$ Syn-pathology were negative by the  $\alpha$ Syn-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 frontal cortex and 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 2/3 wells positive in frontal cortex.

Within no  $\alpha$ Syn-pathology and limbic/neocortical groups, there was 100% concordance between brain homogenate results and CSF results. Of the 5 patients with amygdala-predominant  $\alpha$ Syn-pathology that also tested positive on the  $\alpha$ Syn-SAA using amygdala brain tissue, 3 also had some seeding activity on the  $\alpha$ Syn-SAA using either antemortem or postmortem CSF (Table 3). Overall, the assay detected higher seeding activity in amygdala tissue in amygdala-predominant cases, while 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  $\alpha$ Syn are still limited. Misfolded  $\alpha$ Syn 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<sup>39</sup>. Thus,  $\alpha$ Syn-related biomarkers remain a crucial need to the field. Several publications have shown promising results for  $\alpha$ Syn-SAAs performed in academic laboratories<sup>17, 24</sup>, 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  $\alpha$ Syn-SAAs can detect pathological  $\alpha$ Syn in patients with other pathologies and with clinical diagnoses other than PD or DLB, is crucial to understanding the range of their diagnostic utility.  $\alpha$ Syn-SAA offers the ability to identify  $\alpha$ Syn 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  $\alpha$ Syn-pathology who may not exhibit a 'synucleinopathy phenotype'. One factor that can complicate diagnosis is the presence of AD co-pathology which affect clinical expression particularly in PD and DLB<sup>12, 32, 40–44</sup>. Furthermore, in AD,  $\alpha$ Syn-pathology in AD-LBV is common and also associated with worse prognosis and specific clinical features<sup>7, 11</sup>. The use of  $\alpha$ Syn-SAA assays to help characterize patients in terms of their  $\alpha$ Syn-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  $\alpha$ Syn-SAA assay can be applied. We used pathologically driven categories of  $\alpha$ Syn pathology, independent of clinical diagnosis, in a cohort of patients with high degrees of co-pathology to assess the performance of the  $\alpha$ Syn-SAA assay. In these cases, the use of such a biomarker could prove useful in identifying  $\alpha$ Syn pathology that was not necessarily suspected.

Our results add to the previous reports that  $\alpha$ Syn-SAAs can robustly detect  $\alpha$ Syn seeds in limbic/neocortical stage  $\alpha$ Syn-pathology, but also show decreased sensitivity in detecting  $\alpha$ Syn 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  $\alpha$ Syn-pathology. Lastly, we also observed decreased seeding activity from amygdala-predominant cases when assaying frozen brain tissue from frontal cortex and amygdala.

The lower sensitivity of CSF  $\alpha$ Syn-SAA to detect  $\alpha$ Syn seeds in amygdala-predominant pathology may represent assay dependence on degree of brain  $\alpha$ Syn “burden”. Alternatively, negative  $\alpha$ Syn-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 amygdala homogenate from amygdala-predominant cases showed low detection, suggesting less seeding activity by these particular  $\alpha$ Syn species. Recent studies have found that  $\alpha$ Syn species in amygdala-predominant pathology found in AD may have different immunohistochemical properties than PD or DLB patients with limbic and neocortical  $\alpha$ Syn-pathology<sup>13–15</sup>. It is plausible that these amygdala-predominant  $\alpha$ Syn seeds have lower rates of amplification due to unique conformation or post-translational modifications of these  $\alpha$ Syn species. Currently, there is no method to quantify  $\alpha$ Syn seeds in a sample, thus, it is not possible to determine if  $\alpha$ Syn 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  $\alpha$ Syn seeds. The small number of amygdala-predominant cases who had seeding activity had slower time-to-threshold 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  $\alpha$ Syn seeds take longer to amplify in  $\alpha$ Syn-SAA<sup>17, 18</sup>. However, future studies of larger cohorts will be needed to confirm these preliminary observations.

Since  $\alpha$ Syn-pathology commonly coexists in AD and may be associated with faster clinical progression<sup>45</sup>, identifying this pathology with a biomarker would improve clinical monitoring and create options for clinical trials targeting  $\alpha$ Syn in these patients. If amygdala-predominant type  $\alpha$ Syn-pathology is an early stage or precursor of more widespread concomitant LB pathology in AD, then detecting its presence through

biomarkers such as  $\alpha$ Syn-SAA would be useful. However, the effect of amygdala-predominant  $\alpha$ Syn-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  $\alpha$ Syn-pathology is lower in some cases, or whether different types of  $\alpha$ Syn-SAAs could provide detection of this pathology. We also report for the first time that  $\alpha$ Syn 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  $\alpha$ Syn upon neuropathological analysis. In these cases, positive postmortem CSF results could indicate that the  $\alpha$ Syn 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. Since we observed an increase in sensitivity when analyzing amygdala-predominant postmortem CSF,  $\alpha$ Syn 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  $\alpha$ Syn seeds and other CSF components in postmortem CSF and their potential effects on  $\alpha$ Syn-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 27/75 (36%) of the clinically diagnosed AD patients had  $\alpha$ Syn 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 DLB patients, meaning that it is more challenging to diagnose these patients with mixed pathologies accurately<sup>32, 44, 46</sup>.

Clinically, our cohort included only 4 PD and 9 DLB cases, and pathologically, there were no cases with brainstem-only  $\alpha$ Syn-pathology which are limitations of the study. Our study adds valuable new information about the accuracy of  $\alpha$ Syn-SAA in the context of co-pathology and non-LBD diagnoses. In another study, CSF from 4 cases with incidental  $\alpha$ Syn-pathology in brainstem-only have been analyzed by  $\alpha$ Syn-SAA<sup>25</sup>. Three of these cases were positive, suggesting that brainstem pathological  $\alpha$ Syn shares propagation features with limbic and neocortical rather than amygdala-predominant pathological  $\alpha$ Syn. Since brainstem-only pathological  $\alpha$ Syn is an early event, these results are consistent detection of  $\alpha$ Syn seeds in CSF of prodromal PD cases, like isolated REM sleep behavior disorder (iRBD)<sup>25, 29, 47</sup>. Finally, other minor weaknesses include potential differences in interpretation of the NACC guidelines between the two institutions and the impossibility to determine if patients with  $\alpha$ Syn-SAA negative antemortem CSF and pathological  $\alpha$ Syn upon autopsy represent true false negatives or if the pathology developed after antemortem CSF collection. Additionally, the limbic/neocortical group was skewed towards male participants. This is congruent with numerous studies identifying a sex-link for risk of synucleinopathy<sup>48–50</sup>. Larger numbers of cases with additional distributions of pathological

$\alpha$ Syn, particularly brainstem-only and olfactory-only, should be further investigated to get a full picture of the relationship between brain pathology and CSF  $\alpha$ Syn-SAA positivity. Lastly, further work is needed to fully interrogate differences in the seeding activity between pathological  $\alpha$ Syn 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  $\alpha$ Syn-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  $\alpha$ Syn-pathology, our results indicate that the  $\alpha$ Syn-SAA is highly predictive of neocortical or limbic  $\alpha$ Syn-pathology in aging patients for whom  $\alpha$ Syn-pathology is not clinically suspected. This feature makes  $\alpha$ Syn-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  $\alpha$ Syn-pathology. However, there was substantially lower sensitivity to detect amygdala-predominant  $\alpha$ Syn pathology in brain tissue and CSF, which may have distinct biochemical properties and seeding potential that reduces detection in current generation of  $\alpha$ Syn-SAAs.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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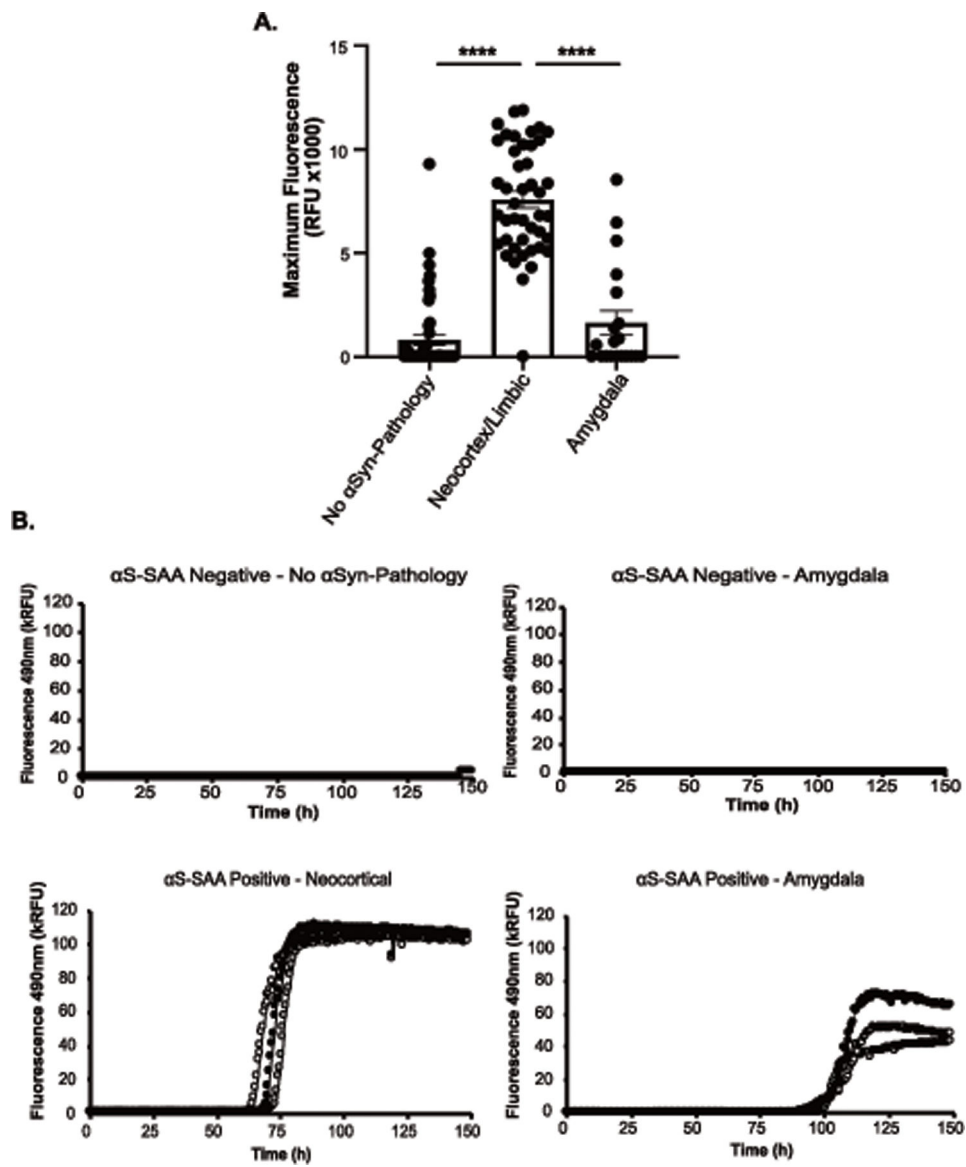
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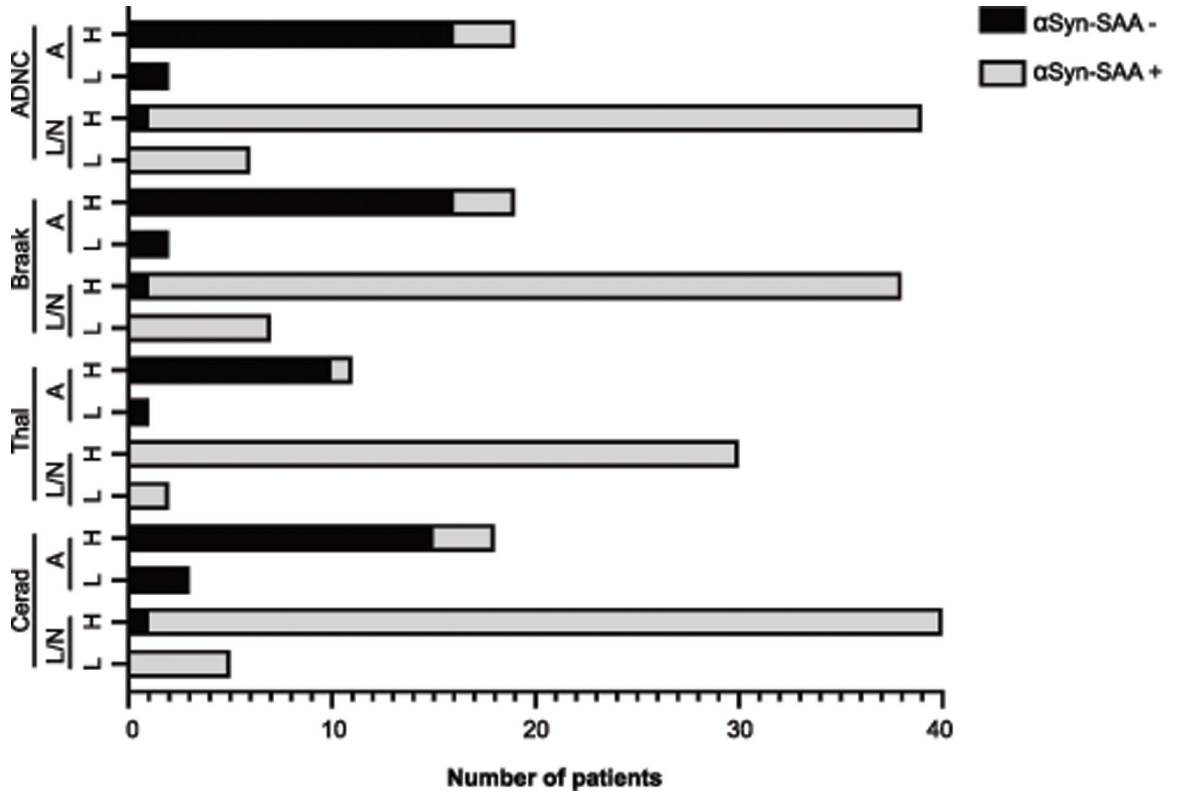
### Summary for Social Media If Published

1. Twitter handles of the authors: @edwardwilsoniii
2. Alpha-synuclein seed amplification assays ( $\alpha$ Syn-SAAs) are highly accurate detecting  $\alpha$ Syn seeds in CSF from LBD cohorts. Accuracy in patients with co-pathologies and non-LBD clinical diagnoses is not well known.  $\alpha$ Syn-SAA results have not been correlated with the distribution of  $\alpha$ Syn-pathology in the brain.
3. Here we examine the ability of the  $\alpha$ Syn-SAA to detect  $\alpha$ Syn seeds in a multicenter cohort of autopsy-validated cases with different  $\alpha$ Syn-pathology distributions. Antemortem CSF, and when available postmortem CSF, amygdala and frontal cortex tissues were tested by  $\alpha$ Syn-SAA.
4. High accuracy of the  $\alpha$ Syn-SAA is confirmed in limbic and neocortical  $\alpha$ Syn-pathology cases. Lower detection is observed in cases with amygdala-predominant  $\alpha$ Syn-pathology. Similar results were found in brain tissues. These results indicate differences between frontal cortex and amygdala pathological  $\alpha$ Syn.
5.  $\alpha$ Syn-SAAs present high sensitivity and specificity for the identification of cases with limbic and neocortical  $\alpha$ Syn-pathology, even in the presence of co-pathology and independent of clinical diagnosis.  $\alpha$ Syn-SAA could assist in the differential diagnosis in dementias with mixed pathology, especially AD, DLB, and AD-LBV.



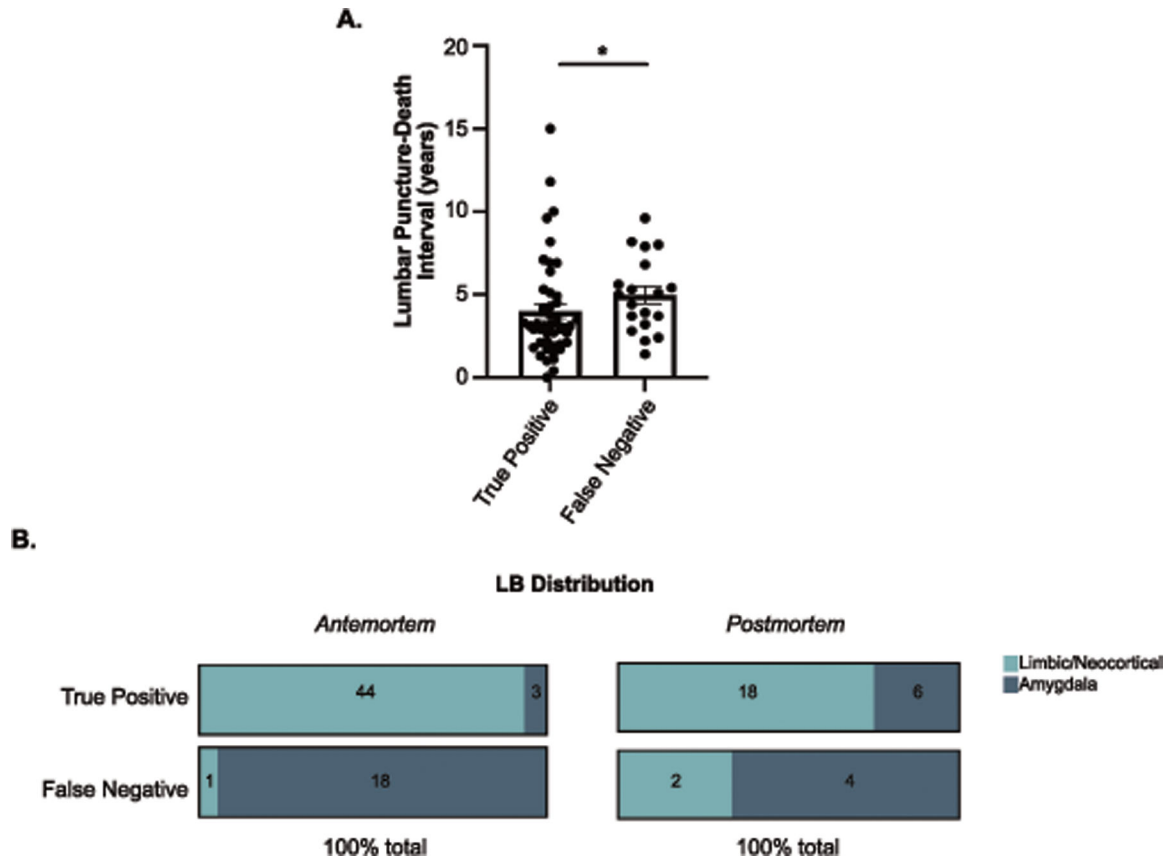


**Figure 1. Kinetic parameters of Research SAA stratified by alpha-synuclein distribution.**  
 A) Maximum Fluorescence Signal from R/D  $\alpha$ Syn-SAA using antemortem CSF between no  $\alpha$ Syn-pathology (n=51), Neocortex/Limbic (n=44), and Amygdala-predominant (n=21) groups. B) Representative figures of raw kinetic data from the Research SAA using antemortem CSF. Included are “negative” samples that are from no  $\alpha$ Syn-pathology and amygdala-predominant individuals, and “positive” samples that are from neocortical and amygdala-predominant individuals. Statistical analysis using one-way ANOVA with Tukey’s multiple comparisons post hoc (A). Error bars represent Standard Error of the Mean (SEM).



**Figure 2. Differences in neuropathology scores between synuclein-pathology groups as a function of SAA result.**

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.



**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 Dwass, Steel, Critchlow-Fligner method (A) or chi-square (B). Error bars represent Standard Error of the Mean (SEM).

**Table 1.**

$\alpha$ S-SAA positivity as a function of pathology diagnosis.

Pathology	n	$\alpha$ Syn Pathology	$\alpha$ S-SAA positivity							
			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 + $\alpha$ Syn-Path	26	Neocortical/Limbic	100%	26/26	91%	10/11	100%	4/4	100%	4/4
AD + $\alpha$ Syn-Path	7	Amygdala-predominant	14%	1/7	-	-	50%	1/2	100%	1/1
AD + VD + $\alpha$ Syn-Path	6	Neocortical/Limbic	83%	5/6	75%	3/4	100%	1/1	100%	1/1
AD + VD + $\alpha$ Syn-Path	4	Amygdala-predominant	0%	0/4	100%	4/4	100%*	1/1	100%	1/1
AD + AA + $\alpha$ Syn-Path	2	Neocortical	100%	2/2	100%	2/2	100%	1/1	100%	1/1
AD + AA + $\alpha$ Syn-Path	3	Amygdala-predominant	0%	0/3	50%	1/2	0%	0/2	50%	1/2
AD + AA + FTLD-TDP43 + $\alpha$ Syn-Path	2	Neocortical/Limbic	100%	2/2	100%	1/1	100%	1/1	100%	1/1
AD + AA + FTLD-TDP43 + $\alpha$ Syn-Path	3	Amygdala-predominant	0%	0/3	33%	1/3	0%	0/3	33%	1/3
AD + HS + $\alpha$ Syn-Path	4	Neocortical/Limbic	100%	4/4	100%	1/1	-	-	-	-
AD + Pick's disease + $\alpha$ Syn-Path	1	Amygdala-predominant	0%	0/1	-	-	-	-	-	-
AD + ARTAG + $\alpha$ Syn-Path	1	Neocortical	100%	1/1	100%	1/1	100%	1/1	100%	1/1
AD + VD + PSP + $\alpha$ Syn-Path	1	Amygdala-predominant	100%	1/1	-	-	0%	0/1	100%*	1/1
AD + Infarcts + $\alpha$ Syn-Path	1	Amygdala-predominant	100%	1/1	-	-	-	-	-	-
PSP + CBD + HS + $\alpha$ Syn-Path	1	Neocortical	100%	1/1	-	-	-	-	-	-

Pathology	n	αSyn Pathology	αS-SAA positivity							
			Antemortem		Postmortem		Frontal		Amygdala	
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	-	-	-	-	-	-

AD:Alzheimer’s disease, VD: Vascular disease, HS: hippocampal sclerosis, AA: Includes amyloid angyopathy, leptomenigial congophilic angiopathy, and lepto/parenchymal congophilic angiopathy, LMN Encephalitis: Limbic Microglial Nodular Encephalitis , PART: Primary-Age Related Tauopathy, METS: Micrometastases, ARTAG: Aging-related tau astrogliopathy, CBD: corticobasal degeneration, PSP: progressive supranuclear palsy, FTLD-TDP43: Frontotemporal lobe degeneration with TDP43 pathology, and αSyn-Path: Includes neocortical, limbic, and amygdala predominant αSyn pathology.

\* 2/3 wells were positive.

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**Table 2.**

Sensitivity, specificity, and predictive values for antemortem and postmortem CSF  $\alpha$ Syn-SAA against  $\alpha$ Syn-pathology.

<b>ANTEMORTEM (n=119)</b>	
<b>Variable</b>	<b>Value, % (95% CI)</b>
Sensitivity	71.2 (59.4–80.7)
<i>Limbic/Neocortical</i>	97.8 (88.4–99.9)
<i>Amygdala</i>	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)
<b>POSTMORTEM (n=56)</b>	
<b>Variable</b>	<b>Value, % (95% CI)</b>
Sensitivity	80.0 (62.7–90.5)
<i>Limbic/Neocortical</i>	90.0 (69.9–98.2)
<i>Amygdala</i>	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)

**Table 3.**

Patient categorization from brain homogenate samples.

Case	Sex	Age at onset	Age at death	Case Information				ADNC	LRP Classification	Brain Tissue		AM CSF	PM CSF	
				Primary Pathology	Thal Phase	CERAD Stage	Braak Stage			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	+	+

Abbreviations: AM: Antemortem, PM: Postmortem, ADNC: Alzheimer's disease neuropathological change NA: not applicable. LRP: Lewy Related Pathology, CSF: cerebrospinal fluid. CBD: corticobasal degeneration, AD: Alzheimer's disease, PSP: progressive supranuclear Palsy, FTLTDP-43: frontotemporal lobar degeneration TAR DNA-binding protein 43, LBD: Lewy-body disease. Inconclusive cases have 2/3 replicate wells that were positive. Brain tissue samples were analyzed at  $10^{-8}$  dilution. Positive results indicate 3/3 replicates were positive and negative results indicate 0/3 replicates were positive. Amygdala tissue could not be obtained for case 5.