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Disease-Associated α-Synuclein Aggregates as Biomarkers of Parkinson Disease Clinical Stage

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Contributions:
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Abstract

**Background and objectives:** There’s an unmet need to identify robust diagnostic biomarker that can mirror Parkinson’s disease (PD) clinical course. Here we present a novel approach to investigate disease associated αSyn aggregates as biomarkers of PD clinical stage.

**Methods:** We combined both seed amplification assay (SAA) and enzyme-linked immunosorbent assay (ELISA) to provide a quantitative test readout that reflects the clinical severity of PD patients. To attain this goal, we initially explored the potential of our test using two sets of human brain homogenates (pilot and validation sets), and then verified it with two independent human CSF cohorts; discovery (62 PD, and 34 control) and validation (49 PD and 48 control).

**Results:** We showed that oligomers-specific ELISA robustly quantified SAA end product from subjects with PD or DLB with high sensitivity and specificity scores (100%). Analysis also demonstrated that seeding activity could be detected earlier with oligomeric ELISA as the test readout rather than SAA alone. More importantly, multiplexing the assays provided robust information about the patients’ clinical disease stage. In the discovery cohort, levels of CSF seeded αSyn oligomers correlated with the severity of the clinical symptoms of PD as measured by UPDRS-motor ($r= 0.58$, $p < 0.001$) and H&Y scores ($r= 0.43$, $p < 0.01$). Similar correlations were observed in the validation cohort between the concentrations of CSF seeded αSyn oligomers and both UPDRS-motor ($r= 0.50$, $p < 0.01$) and H&Y scores ($r= 0.49$, $p < 0.01$). At 20 h, ROC analysis yielded a sensitivity of 91.9% (95% CI, 82.4%–96.5%) and a specificity of 85.3% (95% CI, 69.8%–93.5%), with an area under the curve of 0.969 for CSF seeded αSyn oligomers differentiating PD from controls in the Discovery CSF cohort, whereas, a sensitivity of 80.7% (95% CI, 69.1%–88.5%), a specificity of 76.5% (95% CI, 60.0%–87.5%), and area under the curve of 0.860 were generated with ThT Imax at the same time-point.

**Discussion:** We showed that combining SAA and ELISA assays is more promising diagnostic tool than SAA alone, providing information about the disease stage by correlating with clinical measures of disease severity.
Key words: Parkinson’s disease, biomarkers, seed amplification assay, RT-QuIC, oligomers, ELISA, alpha-synuclein

Classification of Evidence: This study provides Class III evidence that CSF seeded αSyn oligomers can accurately discriminate patients with Parkinson's Disease and normal controls and CSF seeded αSyn oligomers levels correlate with Parkinson's disease severity.

INTRODUCTION

Protein misfolding is the most conspicuous feature of all common neurodegenerative diseases with different, yet overlapping, principal proteins implicated in each disorder. Alpha-synuclein (αSyn) has been extensively studied as a pathogenic trigger, a disease marker and a therapeutic target in synucleinopathies. The primary diagnostic criteria for Parkinson’s disease (PD) rely mainly on the constellation of clinical symptoms, yet the neurodegenerative process is thought to begin many years before overt clinical symptoms are observed. Thus, there is a pressing need to identify at-risk patients prior to the onset of clinical features.

In recent years, considerable efforts have been invested in identifying specific disease markers and developing diagnostic tools for early detection of synucleinopathies, many of which have focused on the detection of misfolded αSyn aggregates in tissue and biological fluids. Among the different assays established for detection of αSyn forms, enzyme linked immunosorbent assay (ELISA) is a simple and rapid technique that permits sensitive and specific quantification of the analytes of interest and is convenient for large scale screening in a clinical set-up. In spite of the high analytical precision, robust inter- and intra-laboratory correlation, and good reproducibility of most of the ELISA assays developed to detect αSyn proteoforms, the diagnostic accuracy in distinguishing patients with PD from controls has been unsatisfactory. Encouraging results have been obtained when exploring αSyn proteoforms (oligomers and/or aggregates) as potential biomarkers for PD and dementia with Lewy bodies (DLB). A number of studies have shown that total αSyn alone in the CSF is not a reliable marker of PD diagnosis or progression, suggesting that other disease-associated forms of αSyn may be more suitable. Studies have shown that the levels of αSyn oligomers in CSF are consistently elevated in patients with PD and DLB, as well as positively correlated with PD motor functions. We previously described the development of several ELISA assays to detect αSyn oligomers in biological fluids using either the same antibody for capture and detection or conformation-specific antibodies. Despite the use of conformation-specific antibodies for αSyn oligomers, a reduction in overlap between PD and
other diagnostic groups was achieved although the sensitivity and specificity among the different studies did not exceed 80%.

Recent studies have demonstrated the ability of seed amplification assays (SAAs) to detect αSyn disease-associated aggregates in brain homogenates and CSF samples. SAAs showed remarkable accuracy in distinguishing PD and DLB patients from control subjects. However, SAAs in their current format are mainly binary tests (positive or negative) with only semi-quantitative readout, rendering them suboptimal for monitoring longitudinal changes in levels of αSyn aggregates over the course of a disease or in response to a treatment.

The primary research question we addressed in this study: whether combining both αSyn SAA and oligomers-specific ELISA would provide an accurate and robust measurable test readout that could reflect disease severity in patients with PD. There have been efforts to generate such information, either through calculation of SAA kinetic parameters, end-point dilution analyses, or the use of a reference standard as a guide for interpreting levels in biological samples. Nevertheless, current αSyn SAA remains unsatisfactory in reflecting the severity, clinical phenotype, or prognosis of PD. Here we present a comprehensive proof-of-concept report highlighting the clinical value of our innovative multiplex test in providing robust quantitative readout of the levels of CSF seeded αSyn oligomers that correlate with disease stage.

METHODS

Study design

The primary research question we aimed to address in this study was, whether SAA-ELISA multiplex would improve the application of αSyn aggregates as disease markers for PD. Towards that goal, we have followed a stepwise approach to strengthen the integration of our novel approach. First, we explored our research question in a “Pilot set” of human brain homogenates (BH), followed by a “Validation set” of similar tissues. Next, we explored our approach in human CSF cohort of PD patients and Ctrl subjects, termed “Discovery CSF cohort”. We then sought to validate the correlation with clinical scores noted in the discovery cohort in a second independent human CSF cohort termed the “Validation CSF cohort”. For more details about the study flow, please see Figure 1. Details about the brain tissues preparation, CSF sampling, and recombinant protein expression are in the eMethods in the supplement.

Brain tissues

Frozen post-mortem samples of the frontal cerebral cortex (Brodmann area 9) from clinically diagnosed and neuropathologically-confirmed cases obtained from Newcastle Brain Tissue
Donors or next of kin provided informed consent to donate tissue and all procedures were approved by the local UK National Health Service Research Ethics Committee. Cases of PD had no evidence of dementia, while donors with AD or DLB had long-standing dementia at death. The cases we tested were divided into two sets, Pilot BH set (Ctrl= 3, PD= 3, and DLB= 3), and Validation BH set (Ctrl= 5, AD= 5, PD= 5, and DLB= 5).

**Discovery CSF cohort**

Patient selection criteria and the method of CSF collection were as described in previous publications. In total, 62 patients with idiopathic PD and 34 age-matched controls were included in the current study. As this cohort was part of larger cohort recruited at St. Olav’s Hospital at the University Hospital of Trondheim in Norway studying (Leucine Rich Repeat Kinase 2) LRRK2 mutations, the control group was composed of first-degree relatives of LRRK2 mutation carriers who were not carrying LRRK2 mutations. PD clinical diagnoses were made by experienced senior clinicians based on guidelines described by Gelb and colleagues and disease stage was assessed according to the Hoehn and Yahr (H&Y) scale. All patients with sPD were screened and tested negative for known LRRK2 mutations. Patients with age at onset ≤50 years also tested negative for known pathogenic mutations in Parkin and PINK1. All family members were screened for clinical signs of PD. All patients provided written informed consent, and the study was approved by the Regional Committee for Medical and Health Research Ethics (Ethical committee of Central Norway number 34272).

**Validation CSF cohort**

A detailed description of the cohort has been published elsewhere. The cohort included 49 patients with PD and 48 age-matched healthy controls (HC). Patients presenting with parkinsonian symptoms, i.e. bradykinesia/hypokinesia, rigidity, tremor and/or postural instability (de novo patients) and patients already under treatment which fulfilled acknowledged diagnostic criteria for PD (United Kingdom Parkinson’s Disease Society Brain Bank clinical diagnostic criteria) were recruited from the outpatient clinic for movement disorders of the VU University Medical Center by health care professionals and movement disorders specialists. All consecutive patients attending the outpatient clinic that fulfilled the inclusion criteria and did not meet any of the exclusion criteria were asked to participate in this project in the period 2008-2010. The healthy controls were recruited through an advertisement on the website http://www.parkinson-vereniging.nl and in the magazine ‘Papaver’ of the Dutch Parkinson Foundation (Parkinson Vereniging) and from spouses and acquaintances of the patients that visit the outpatient clinic for movement disorders. The controls were matched with the
parkinsonian patients for age and gender. Mini-Mental State Examination (MMSE) and/ or neuropsychological assessment in the patients was used to assess global cognitive function. Severity of motor symptoms and disease stage in the ‘on’ state were rated using the Unified Parkinson’s Disease Rating Scale-Part-III (UPDRS-III) and the modified Hoehn & Yahr classification, respectively. The study was approved by the local medical ethical committee of VU University Medical Center, Amsterdam. All patients gave written informed consent at study entry for the use of clinical information and CSF material for scientific research purposes.

αSyn seed amplification assay

eTable 1 in the supplement summarizes the differences between the different seed amplification protocols employed in the current study. For BH samples, we followed a modified version of Shahnawaz et al., 2017, a well-established protocol for αSyn seeding amplification assay. Briefly, 160 µL of reaction mix composed of 0.1 M piperazine-N, N’ bis (ethanesulfonic acid) (PIPES), pH 6.5, 0.5 M sodium chloride (NaCl), 10 µM Thioflavin T (ThT), and 0.1 mg/mL wild-type untagged monomeric αSyn (filtered through a 100 kD MWCO filter immediately prior to use), were distributed in 96-well black plate with clear bottom (Nunc, Thermo Fisher) at a final volume of 200 µL per well. For each test, we loaded 40 µl of BH of 0.1 mg/mL total protein concentration. The plate was then sealed with a sealing tape and incubated in Omega FLUOstar plate reader (BMG Labtech, Aylesbury, Buckinghamshire, UK) at 37°C for 120 hours with intermittent shaking cycles: double orbital with 1 min shake (500 rpm,) and 15 min rest throughout the indicated incubation time.

For CSF seeding assay, we adapted the protocol from Groveman et al. 2018 to use wild-type αSyn rather than the K23Q mutated form. Wells were pre-loaded with 6 silica beads (Sigma-Aldrich), and 85 µL of a reaction mix prepared to give final reaction concentrations of 40 mM phosphate buffer (pH 8.0), 170 mM NaCl, 0.1 mg/mL recombinant monomeric αSyn (filtered through a 100 kD MWCO filter immediately prior to use), 10 µM ThT and 0.0015% sodium dodecyl sulfate (SDS) were distributed according to the plate layout. Then 15 µL CSF per sample were spiked in triplicates into corresponding wells. The plate was then sealed with a sealing tape and incubated in Omega FLUOstar plate reader (BMG Labtech, Aylesbury, Buckinghamshire, UK) at 42°C with intermittent shaking cycles: double orbital with 1 min shake (500 rpm,) and 1 min rest throughout the indicated incubation time. For both protocols, ThT fluorescence readings were taken every 45 min with a bottom read using 450 ± 10 nm (excitation) and 480 ± 10 nm (emission) wave-lengths. The sample was considered positive if 2 or more of the replicates were above the calculated threshold. The threshold was calculated as

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the average fluorescence for all samples within the first 10 h of incubation, plus 3 times the 
Standard Deviations (SD) \(^{36}\).

Multiple cut-offs for SAA product quantification were selected to estimate the time-point at which 
“soluble” αSyn aggregates “oligomers” can be robustly quantified in patients with optimal 
specificity. Evidence suggests that soluble αSyn aggregates (oligomers) are more practical to 
quantify than later mature insoluble aggregates “amyloid fibrils”, thus oligomers better serve as 
disease biomarkers compared to mature insoluble fibrils, we have therefore used sandwich-
based ELISA that would quantify specifically early αSyn oligomers in liquid-phase. Another 
important element that we took into consideration in selecting the optimal run time, is the assay 
ability to produce rapid and reproducible output, that would be appealing for future integration of 
the assay into clinical practice. For BH SAA, the optimal timepoint was optimized by collecting 
 aliquots of 5 µL from each well for each sample at 0, 48, 72, 96, and 120 h of the assay total run 
time (120 h). For CSF SAA, the same volume was aliquoted at 0, 20 and 60 h of the assay total run 
time (60 h). All aliquots were stored at −80 °C until analysis in ELISA.

**αSyn oligomers-specific ELISA**

All samples were analysed using our in-house oligomer-specific ELISA \(^{26, 32}\), with minor 
modification; Syn-O2 was used for capture (at 0.5 µg/mL, overnight incubation at 4°C), and 
biotinylated Syn-O2 (at 0.5 µg/mL, 1 h incubation at 37°C) was used for detection with HRP-
conjugated streptavidin (Sigma Aldrich) (at 1:5000 dilution, 30 min incubation at 37°C) as the 
reporter. Plates were initially blocked to eliminate non-specific signal for 1 h at 37°C, and 
samples were diluted at 1:1000 in 50% RIPA prior loading, and incubated for 1 h at 37°C. 
Chemiluminescence expressed in relative light units was immediately measured using 
PerkinElmer Envision plate reader (PerkinElmer, Finland). Specified calibrators were used to 
generate an 8-point standard curve to which a 4-parameter logistic (4PL) curve of all plates was 
fitted and used to quantify unknown concentrations using GraphPad Prism software. The 
concentrations of oligomeric and total αSyn were extrapolated from corresponding standard 
curves. For each case analysed by SAA, individual replicates were tested in duplicates using 
ELISA (i.e. 3 replicates x 2 duplicates per case) and the average was calculated. Each ELISA 
run was performed using 384-well maxisorp plate and completed within ~ 4-5 h.

**Statistical analyses**

GraphPad Prism (version 8.3.0) software was used for data analysis, including 1) calculating 
SAA kinetic parameters (maximum intensity of fluorescence [I\(\text{max}\)] at final point [60 h], and at 
20 h of the assay run, time needed to reach 50% of the maximum aggregation [T\(\text{S}50\)], and area 
under the ThT fluorescence sigmoid curve [ThT AUC]) for CSF samples, 2) extrapolating αSyn
levels in the ELISA (total and seeded αSyn oligomers), 3) discriminatory power analysis by receiver operating characteristic curves (ROC) to evaluate diagnostic accuracy, and 4) correlation analysis. Briefly, all calculated parameters were tested for normality and deemed inappropriate for parametric analyses. Therefore, the Mann-Whitney U-test was used for comparisons between PD and HC diagnostic groups for the named variables. Spearman correlations were calculated to explore possible associations between seeded αSyn levels, SAA kinetic parameters and PD clinical stage assessed. \( P < 0.05 \) was set as the level of statistical significance.

**Data Availability**
Anonymized data not published within this article will be made available by request from any qualified investigator.

**Standard Protocol Approvals, Registrations, and Patient Consents**
Experimental procedures were carried out in accordance with the respective institutional ethical rules and regulations. The study was approved by the local ethics committee of Qatar Biomedical Research Institute.

**RESULTS**

**Patient population and demographics**
Demographics, clinical characteristics and CSF biomarkers levels of the study Discovery and Validation CSF cohorts are summarized in Table 1. The Discovery CSF cohort included 62 PD and 34 controls. Average age of the PD discovery cohort was 57 ± 10 years old, with average H&Y stage 2 ± 0.5, average UPDRS Part 3 score 23.5 ± 9.1, and average MoCA score of 25.8 ± 2.9 with 36 males and 26 females. Control subjects were aged 50 ± 16 years, with 16 males and 18 females. The Validation CSF cohort included 49 patients with PD with average age of 63 ± 10, average H&Y stage 2 ± 0.5, average UPDRS Part 3 score 21.7 ± 8.6, and average MMSE score of 28 ± 1.6 with 30 males and 19 females. Healthy controls were aged 50 ± 16 years, with 16 males and 32 females.

**ELISA demonstrates high sensitivity and specificity for SAA end product seeded by brain homogenates from patients with PD and DLB**
We initially seeded the *in vitro* assembly of recombinant monomeric wildtype human αSyn with BH from frontal cortex in a Pilot BH set of samples consisting of three cases per group of PD, DLB and Ctrl subjects. A lag phase of 40–60 h was observed (Figure 2a), followed by a rapid increase in fluorescence and a plateau at 90–120 hours. DLB samples seeded faster and resulted in higher fluorescence intensities than PD, whereas no increase in fluorescence was
observed in the Ctrl group. We calculated both Imax and ThT AUC of the SAA aggregation curve and compared the values against the three groups. The DLB group showed a trend towards a higher Imax and AUC compared to the PD group, and as expected, both were higher compared to the Ctrl group with one PD sample overlapping with Ctrl (Figure 2b, c). SAA quantified end product using ELISA, showed that the levels of seeded αSyn oligomers in both PD and DLB groups were also higher than the Ctrl group with no overlap (Figure 2d).

**Brain homogenates of PD and DLB seeding activity detected earlier using oligomeric-ELISA**

For SAA-ELISA multiplex protocol, we optimized the dilution factor (1:1000), dilution buffer (50% RIPA) and time point cut-off (data not shown). The cut-off was defined as the most appropriate time point time where positive samples can be robustly measured above the ELISA limit of quantification (LLoQ). On average, PD and DLB BH samples gave a positive response within ~55–65 h, and ~15–30 h for PD CSF samples, thus 60 h and 20 h were selected as the assay optimal time point for BH and CSF, respectively. We performed time-dependent analyses to validate our results from the pilot set and to detect seeded αSyn oligomers at the earliest possible timepoint. The Validation BH set cohort included human brain tissues from Ctrl (n= 5), AD (n= 5), PD (n= 5), and DLB (n= 5) cases. αSyn SAA assay was carried out as described above, and the seeding activity of BH samples was monitored by assessing the formation of early soluble αSyn aggregates “oligomers” using our oligomers-specific ELISA \(^{25, 26}\). Samples were collected from individual replicates of each BH case at different time points over 120 h (0, 48, 72, 96, and 120 h), then frozen prior to ELISA analysis. Levels of αSyn oligomers increased in a time-dependent manner up to a plateau at approximately 90 h. Interestingly, BH seeding activity was detected earlier with ELISA than SAA alone (72 versus 120 h) (Figure 3a-c), while maintaining optimal specificity for discriminating synucleinopathies from controls (eFigure 1 in the supplement).

**CSF seeded αSyn oligomers robustly discriminate PD patients from controls in the Discovery CSF cohort**

We then analysed CSF samples from PD patients (n=62) and age-matched control subjects (Ctrl) (n=34) to better understand the added value of our SAA-ELISA multiplex approach. The demographics of all subjects are shown in Table 1. The time needed to reach 50% of the maximum aggregation (T50) varied greatly among PD positive CSF samples (Figure 4a), whereas all Ctrl CSF samples with negative results based on the maximum fluorescence failed to reach the T50 value (Figure 4a). In order to quantify the levels of αSyn oligomers in SAA seeded by CSF, samples from each replicate of each case at
20 h of the assay run were analysed using ELISA assays for measuring total or αSyn oligomers. In comparing relative fluorescence units (RFU) values for SAA data, a notable overlap was noted between diagnostic groups at 20 h, which was significantly reduced at the end point (Figure 4b). Whereas, the levels of CSF seeded αSyn oligomers were significantly higher in PD (mean± SD = 321 ± 180, n= 62) compared to Ctrls (mean± SD = 47 ± 40, n= 34) at 20 h (p < 0.001, Mann-Whitney U test) (Figure 4c), with minimal overlap compared to RFU at the same timepoint. The levels of total αSyn did not differ significantly between the two groups (mean± SD = 799 ± 219, n= 62, and 554 ± 201, n= 34 for PD and Ctrl respectively) (Figure 4c).

ROC analysis was performed to evaluate the diagnostic accuracy of SAA RFU values measured at 20 h, and 60 h as well as CSF seeded αSyn oligomers as disease predictors (eFigure 2). The ROC curve demonstrated that cut-off values of 0.087 mg/mL for CSF seeded αSyn oligomers, 17,314 RFU for Imax at 60 h, 10,644 RFU for Imax at 20 h, and 721,500 RFU for ThT AUC were the most reliable measures to distinguish patients with PD from Ctrls (eFigure 2). The above-mentioned cut-off values yielded a sensitivity of 91.9% (95% CI, 82.4%–96.5%) and a specificity of 85.3% (95% CI, 69.8%–93.5%), with an area under the curve (ROC AUC) of 0.969 for CSF seeded αSyn oligomers at 20 h (Figure 4 f-i). However, the cut-off value for Imax at 20 h yielded a sensitivity of 80.7% (95% CI, 69.1%–88.5%) and a specificity of 76.5% (95% CI, 60.0%–87.5%), with ROC AUC of 0.860 (eTable 2) (eFigure 2).

**Correlation between disease severity and CSF seeded αSyn oligomers**

In order to investigate whether the level of αSyn oligomers in samples seeded with CSF from PD patients could reflect the severity of the disease. Initially, we explored whether SAA kinetic parameters kinetic derived from αSyn-SAA-positive PD samples correlated with CSF seeded αSyn oligomers in the Discovery CSF cohort. There were no notable correlations among kinetic parameters and CSF seeded αSyn oligomers, other than a weak correlation with Imax at 20 h (r= 0.25, p < 0.05) (eFigure 3). In the same cohort, exploring the correlation between the levels of CSF seeded αSyn oligomers at 20 h with disease severity in the PD group showed strong positive correlation with both UPDRS-motor (r= 0.58, p < 0.001) and H&Y (r= 0.48, p < 0.001) scores (Figure 5 a, b). To further test the strength of the correlations, we have re-analysed the same data set excluding 3 data points with very high concentrations of seeded αSyn oligomers (highlighted in a red square). The strength of correlations was slightly reduced, but were still significant (r= 0.52, p < 0.001, and r= 0.41, p < 0.01 with UPDRS-motor and H&Y scores, respectively). We didn’t observe any correlation between CSF seeded αSyn oligomers and cognitive scores of the PD patients, however, high scores of both MoCA and MMSE scales were registered for most PD patients rendering the patients almost cognitively intact (eFigure 4).
We also calculated correlation coefficients with clinical data from PD subjects using kinetic parameters positive αSyn SAA. No significant correlations were found. Imax at 60 h and ThT showed a weak correlation with H&Y scores, a correlation that was absent when the same high 3 data points were excluded (eFigure 5).

In the Validation CSF cohort, correlations were calculated similarly to those in the Discovery CSF cohort. Strong positive correlations were also observed between CSF seeded αSyn oligomers and UPDRS-motor (r= 0.5, p < 0.01), and H&Y (r= 0.49, p < 0.01) scores (Figure 5 c, d). When two cases with very high levels of CSF seeded oligomers were excluded, both correlations were still significant (r= 0.50, p < 0.01, and r= 0.43, p < 0.01 with UPDRS-motor and H&Y scores, respectively).

Classification of Evidence: This study provides Class III evidence that CSF seeded αSyn oligomers can accurately discriminate patients with Parkinson's Disease and normal controls and CSF seeded αSyn oligomers levels correlate with Parkinson's disease severity.

Discussion

A major priority for PD is the development of quantitative, specific, and reproducible tests that can reflect the progression of the disease and its response to therapy. Misfolded αSyn protein is implicated in a group of neurodegenerative diseases, making it an attractive biomarker candidate for both diagnostic and therapeutic applications. Among the multiple approaches employed to detect αSyn aggregates in human samples are: 1) antibody-based immunoassays, such as ELISA; and 2) assays that exploit the self-propagating property of αSyn aggregates, mostly known as seed amplification assays. Here we describe our novel approach combining both αSyn SAA and ELISA, to develop a quantitative and robust test that correlates with disease severity in patients with PD. ELISA assays are mostly quantitative, time efficient, convenient for analysing large sample sets, and more appropriate for diagnostic lab settings. αSyn SAAs, however, are remarkably specific for discriminating PD and DLB patients from other non-synucleinopathies, potentially providing a definitive diagnosis.

In the current study, we established a novel approach of multiplexed SAA and ELISA platforms to derive meaningful quantitative information about the seed concentration in a CSF sample that could relay information about PD severity.

PD diagnosis is not always accurate and potentially overlaps with other neurodegenerative disorders, we therefore first evaluated our new approach to probe for misfolded αSyn in pathologically well-characterized human brain tissues. The initial analyses of SAA end-products
using oligomeric ELISA revealed a strong correlation between the two assays, since both PD and DLB groups scored positive in SAA, and also showed high levels of seeded αSyn oligomers in the ELISA. Likewise, controls scored negative on SAA, no seeded αSyn oligomers were detected above the ELISA limit of quantification.

Based on the above findings, we expanded our analyses to a larger set of human brain tissues, where our data showed high degrees of consistency in identifying PD and DLB patients from Ctrl or AD subjects. Moreover, the amplification of misfolded αSyn in PD and DLB was robustly detected by ELISA at an earlier timepoint compared to SAA. The detection of misfolded αSyn by ELISA was ideally specific between 70 to 90 h of seed amplification, whereas a similar segregation was only achieved at 120 h using SAA alone. Those findings emphasized that the implementation of αSyn oligomers-specific ELISA significantly provided an early and quantitative discrimination between the groups.

To thoroughly assess the added value of our proposed approach at providing information about the disease clinical stage, we analysed two independent CSF cohorts of PD patients and Ctrls, named the Discovery and the Validation CSF cohorts. In the Discovery CSF cohort, we analysed CSF samples from 62 PD patients and 34 Ctrl subjects. At 20 h, a significant overlap was observed between PD and Ctrl groups when maximum ThT counts were compared, however, the overlap was considerably less when CSF seeded αSyn oligomers were assessed between the same groups at the same timepoint. As expected, at 60 h both Imax and ThT AUC significantly discriminated between PD and Ctrl groups. Those results illustrated that our novel approach provided not only a specific quantitation of misfolded αSyn in human CSF, but also an earlier reading of the test output.

Caughey and others have previously proposed the use of SAA end-point dilution analysis, lag phase and/or time-to-threshold values as potential parameters to estimate seeding dose in the initial test sample with the broad assumption they would reflect disease stage or clinical profile. Considering that PD is a progressive disorder with neurodegeneration taking place years before the appearance of the symptoms, robust quantitative monitoring of disease related biomarkers is crucial for any drug development efforts. To date, there has been only one report showing T50 to correlate with the disease severity in PD patients assessed by H&Y scale (r = −0.54, P = .006). A recent comparative study utilizing the Parkinson’s Progression Markers Initiative (PPMI) longitudinal cohort, accentuated the shortcomings of the current αSyn SAA at quantifying disease severity or clinical features. Similarly, in our study, SAA kinetic parameters failed to correlate with PD-specific clinical features in two independent CSF cohorts. The only notable correlations between SAA kinetic parameters and H&Y scores were sensitive...
to the effect of potential outliers, which undermined the existence of a true correlation. However, it was remarkable that CSF seeded αSyn oligomers correlated with disease severity assessed by both H&Y and UPDRS-motor scores in both CSF cohorts. To our knowledge, our approach demonstrated for the first time that αSyn SAA could provide clinical information about PD severity beyond diagnostic performance.

Although our SAA-ELISA multiplex was initially tested in post-mortem human tissues, the lack of autopsy data to provide a definitive diagnosis of PD that would help us better appreciate the correlation with disease severity in the CSF cohorts remains a limitation of this study. Also, analysing longitudinal cohorts would also be essential as a next step to extract further information about the potential use of our approach.

Further studies are warranted to explore whether our approach combining SAA with oligomers-specific ELISA is useful in patients with other synucleinopathies and in assessing therapies targeting αSyn aggregation.

In summary, our findings further support the growing evidence of αSyn SAA as a robust clinical diagnostic tool for patients with PD. Furthermore, we have established and validated a novel approach to provide clinical information about underlying disease severity in patients with PD, and thus perhaps a promising tool to support clinical trials targeting αSyn aggregates in PD.


References

# Table 1. Demographics and CSF biomarkers by diagnostic group

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<th>Discovery cohort</th>
<th>Validation cohort</th>
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<td></td>
<td>Ctrl (n= 34)</td>
<td>PD (n= 62)</td>
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<td><strong>Age (y), mean ± SD</strong></td>
<td>50 (43 - 62.5)</td>
<td>57.5 (49.2 - 66)</td>
</tr>
<tr>
<td><strong>Gender (male), n (%)</strong></td>
<td>16 (47%)</td>
<td>36 (58 %)</td>
</tr>
<tr>
<td><strong>Disease duration (y)</strong></td>
<td>NA</td>
<td>5 (2 - 7)</td>
</tr>
<tr>
<td><strong>MoCA score</strong></td>
<td>NA</td>
<td>27 (24 - 28)</td>
</tr>
<tr>
<td><strong>MMSE</strong></td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>H&amp;Y score</strong></td>
<td>NA</td>
<td>2 (2 - 3)</td>
</tr>
<tr>
<td><strong>UPDRS-III score</strong></td>
<td>NA</td>
<td>26 (21 - 35)</td>
</tr>
<tr>
<td><strong>SAA t-αSyn (ng/mL)</strong></td>
<td>0.51 (0.42 - 0.63)</td>
<td>0.7 (0.59 - 0.94)</td>
</tr>
<tr>
<td><strong>SAA o-αSyn (ng/mL)</strong></td>
<td>0.03 (0.01 - 0.06)</td>
<td>0.30 (0.20 - 0.39)</td>
</tr>
</tbody>
</table>

Data are expressed as median (IQR), or n (%). Ctrl, healthy controls; H&Y, Hoehn and Yahr stage; MMSE, Mini-Mental State Exam; MoCA, Montreal Cognitive Assessment; NA, not applicable; o-αSyn, oligomeric α-synuclein; PD, Parkinson’s disease; SAA, seed amplification assay; t-αSyn, total α-synuclein; UPDRS-III, Unified Parkinson’s Disease Rating Scale.
Figure legends

Figure 1. Flowchart of the study cohorts.
Flowchart presenting the number of cases per cohort, and the primary question answered by each samples’ set.
Figure 2. SAA and ELISA analysis of the pilot BH samples set
RT-QuIC reactions seeded in triplicate with brain homogenate (BH) from control (Ctrl, blue), Parkinson's disease (PD, red) or dementia with Lewy bodies (DLB, green) cases. The solid line of each sample trace represents the average ThT signal of triplicate wells. The coloured ribbon represents the standard error (±SD) (a). Comparison of Imax, area under the ThT curve (ThT AUC), and seeded αSyn oligomers for each group. The dots represent the single cases, and the lines reflect the group's average. Statistical analysis was not conducted at this stage due to the small sample size, however, the differences between the groups are clearly pronounced (b-c).
Figure 3. SAA and ELISA analysis of the validation BH samples set
RT-QuIC reactions seeded in triplicate with brain homogenate (BH) from subjects with αSyn pathology (PD, n=1 and DLB, n=4), or without αSyn pathology (AD, n=3 and Ctrl, n=2). The solid line represents the average ThT signal per group (a). Monitoring the changes of αSyn oligomers' levels over multiple timepoints (0, 48, 72, 96, and 120 hr) for each group for ELISA (b), and SAA (c).
Figure 4. RT-QuIC reactions seeded in triplicate with CSF from control and PD subjects.
RT-QuIC reactions seeded in triplicate with CSF samples from Ctrl subjects (blue) and patients with PD (red). The solid line represents the average ThT signal of triplicate wells. The coloured ribbon represents the standard error (a). Comparison of the mean maximum fluorescence at SAA endpoint, and at 20 h of the assay run for each diagnostic group, respectively (b). Comparison of seeded CSF αSyn oligomers and total, respectively at 20 h of the assay run for each diagnostic group (c). Floating bars show the min to max with the line at the mean.
Figure 5. Correlation analysis in discovery and validation cohorts
Scatter plots showing the correlation analysis in the discovery cohort between CSF seeded αSyn oligomers and UPDRS-motor scores and H&Y scores, respectively (a, b). Scatter plots showing the correlation analysis in the validation cohort between UPDRS-motor and H&Y scores with seeded CSF αSyn oligomers (c, d). The subplots present the same dataset excluding extreme datapoints highlighted with the red square. The solid line highlights the calculated regression line. P values and Spearman $r$ are displayed for each correlation.
Disease-Associated α-Synuclein Aggregates as Biomarkers of Parkinson Disease Clinical Stage

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