Disease-Associated \(\alpha\)-Synuclein Aggregates as Biomarkers of Parkinson Disease Clinical Stage

Nour Majbour, PhD, Jan Aasly, MD, Ilham Abdi, MSc, Simona Ghanem, PhD, Daniel Erskine, PhD, Wilma van de Berg, PhD, and Omar El-Agnaf, PhD

*Neurology®* 2022;99:e2417-e2427. doi:10.1212/WNL.0000000000201199

**Abstract**

**Background and Objectives**
Robust biomarkers that can mirror Parkinson disease (PD) are of great significance. In this study, we present a novel approach to investigate disease-associated \(\alpha\)-synuclein (\(\alpha\)Syn) aggregates as biomarkers of PD clinical stage.

**Methods**
We combined both seed amplification assay (SAA) and ELISA to provide a quantitative test readout that reflects the clinical severity of patients with PD. To attain this goal, we initially explored the potential of our test using 2 sets of human brain homogenates (pilot and validation sets) and then verified it with 2 independent human CSF cohorts; discovery (62 patients with PD and 34 controls) and validation (49 patients with PD and 48 controls) cohorts.

**Results**
We showed that oligomers-specific ELISA robustly quantified SAA end product from patients with PD or dementia with Lewy bodies 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. Of more importance, multiplexing the assays provided robust information about the patients’ clinical disease stage. In the discovery cohort, levels of CSF-seeded \(\alpha\)Syn oligomers correlated with the severity of the clinical symptoms of PD as measured by the Unified Parkinson Disease Rating Scale (UPDRS) motor \((r = 0.58, p < 0.001)\) and Hoehn and Yahr (H&Y) scores \((r = 0.43, p < 0.01)\). Similar correlations were observed in the validation cohort between the concentrations of CSF-seeded \(\alpha\)Syn oligomers and both UPDRS motor \((r = 0.50, p < 0.01)\) and H&Y scores \((r = 0.49, p < 0.01)\). At 20 hours, receiver operating characteristic curves 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 \(\alpha\)Syn oligomers differentiating those with 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 thioflavin T maximum intensity of fluorescence at the same time point.

**Discussion**
We showed that combining SAA and ELISA assays is a more promising diagnostic tool than SAA alone, providing information about the disease stage by correlating with clinical measures of disease severity.
Glossary

αSyn = α-synuclein; AD = Alzheimer disease; BH = brain homogenate; Ctrl = control; DLB = dementia with Lewy bodies; HC = healthy control; H&Y = Hoehn and Yahr; Imax = maximum intensity of fluorescence; LRRK2 = leucine rich repeat kinase 2; MMSE = Mini-Mental State Examination; MoCA = Montreal cognitive assessment; MWCO = molecular weight cutoff; NaCl = sodium chloride; PD = Parkinson disease; RFU = relative fluorescence unit; RIPA = radioimmunoprecipitation assay; ROC = receiver operating characteristic; SAA = seed amplification assay; T50 = 50% of the maximum aggregation; ThT = thioflavin T; ThT AUC = area under the ThT fluorescence sigmoid curve; UPDRS-III = Unified Parkinson Disease Rating Scale-Part III.

Classification of Evidence

This study provides Class III evidence that CSF-seeded αSyn oligomers can accurately discriminate patients with PD and normal controls and CSF-seeded αSyn oligomers levels correlate with PD severity.

Protein misfolding is the most conspicuous feature of all common neurodegenerative diseases with different, yet overlapping, principal proteins implicated in each disorder.1-4 α-Synuclein (αSyn) has been extensively studied as a pathogenic trigger, a disease marker, and a therapeutic target in synucleinopathies.5,6 The primary diagnostic criteria for Parkinson disease (PD) rely mainly on the constellation of clinical symptoms, yet the neurodegenerative process is believed to begin many years before overt clinical symptoms are observed.7,8 Thus, there is a pressing need to identify at-risk patients before the onset of clinical features.

In recent years, considerable efforts have been made in identifying specific disease markers and developing diagnostic tools for the early detection of synucleinopathies, many of which have focused on the detection of misfolded αSyn aggregates in tissue and biological fluids.9,10 Among the different assays established for the detection of αSyn, 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 setup.9 In spite of high analytical precision, robust interlaboratory and intralaboratory correlations, 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.11-19 Encouraging results have been obtained when exploring αSyn proteoforms (oligomers and/or aggregates) as potential biomarkers for PD and dementia with Lewy bodies (DLB).20-30 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.11-19 Studies have shown that the levels of αSyn oligomers in CSF are consistently elevated in patients with PD and DLB and positively correlated with PD motor functions.20,30 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.26,31,32 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 (BHs) and CSF samples.33-44 SAAs showed remarkable accuracy in distinguishing patients with PD and DLB from healthy controls. However, SAAs in their current format are mainly binary tests (positive or negative) with only semiquantitative 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 was 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, endpoint dilution analyses, or the use of a reference standard as a guide for interpreting levels in biological samples.36,39,45,46 Nevertheless, current αSyn SAA remains unsatisfactory in reflecting the severity, clinical phenotype, or prognosis of PD.46 In this study, 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. Toward 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 BHs,
followed by a “validation set” of similar tissues. Next, we explored our approach in human CSF cohort of patients with PD and control (Ctrl) participants, 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 (links.lww.com/WNL/C291).

**Brain Tissues**

Frozen postmortem samples of the frontal cerebral cortex (Brodmann area 9) from clinically diagnosed and neuro-pathologically confirmed cases were obtained from Newcastle Brain Tissue Resource, Translational and Clinical Research Institute, Newcastle University, UK. Donors or next of kin provided informed consent to donate the tissue, and all procedures were approved by the local UK National Health Service Research Ethics Committee. Cases of PD had no evidence of dementia, whereas donors with Alzheimer disease (AD) or DLB had long-standing dementia at death. The cases we tested were divided into 2 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 this study. Because this cohort was part of a larger cohort recruited at St. Olav’s Hospital at the University Hospital of Trondheim in Norway studying leucine-rich repeat kinase 2 (LRRK2) variations, the control group was composed of first-degree relatives of LRRK2 mutation carriers who were not carrying LRRK2 variations. PD clinical diagnoses were made by experienced senior clinicians based on guidelines described by Gelb et al., and disease stage was assessed according to the Hoehn and Yahr (H&Y) scale. All patients with sporadic PD were screened and tested negative for known LRRK2 variations. Patients with age at onset 50 years or younger 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 (HCs). Patients presenting with parkinsonian symptoms, that is, 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 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 disorder 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 HCs were recruited through an advertisement on the website parkinson-vereniging.nl and in the magazine “Papaver” of the Dutch Parkinson Foundation (Parkinson Vereniging) and from

---

**Figure 1 Flowchart of the Study Cohorts**

Flowchart presenting the number of cases per cohort and the primary question answered by each samples’ set. αSyn = α-synuclein; DLB = dementia with Lewy bodies; HC = healthy control; PD = Parkinson disease.
spouses and acquaintances of the patients who 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 were used to assess global cognitive function. Severity of motor symptoms and disease stage in the “on” state were rated using the Unified Parkinson Disease Rating Scale-Part III (UPDRS-III) and the modified H&Y 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 (links.ww.com/WNL/C291) summarizes the differences between the different seed amplification protocols used in this study. For BH samples, we followed a modified version of Shahnawaz et al., a well-established protocol for αSyn seeding amplification assay. In brief, 160 μL of reaction mix composed of 0.1 M pipazine-N, N’ bis (ethanesulfonic acid), 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 molecular weight cutoff [MWCO] filter immediately before use) were distributed in a 96-well black plate with clear bottom (Nunc; Thermo Fisher, Waltham, MA) at a velocity in a 96-well black plate with clear bottom (Nunc; Thermo Fisher, Waltham, MA) 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, United Kingdom) at 37°C for 120 hours with intermittent shaking cycles: double orbital with a 1-minute shake (500 rpm) and 15 minutes rest throughout the indicated incubation time.

For CSF SAA, we adapted the protocol from Groveman et al. to use wild-type αSyn rather than the K23Q mutated form. Wells were preloaded with 6 silica beads (Sigma-Aldrich, St. Louis, MO), 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 before use), 10 μM ThT, and 0.0015% sodium dodecyl sulfate was distributed according to the plate layout. Then, 15 μL of CSF per sample was spiked in triplicates into corresponding wells. The plate was then sealed with a sealing tape and incubated in Omega FLUOstar plate reader (BMG Labtech) at 42°C with intermittent shaking cycles: double orbital with a 1-minute shake (500 rpm) and 1-minute rest throughout the indicated incubation time. For both protocols, ThT fluorescence readings were taken every 45 minutes with a bottom read using 450 ± 10 nm (excitation) and 480 ± 10 nm (emission) wavelengths. The sample was considered positive if 2 or more of the replicates were above the calculated threshold. The threshold was calculated as the average fluorescence for all samples within the first 10 hours of incubation and 3 times the SDs.

Multiple cutoffs for SAA product quantification were selected to estimate the time point at which “soluble” αSyn aggregate “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 with 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 time point was optimized by collecting aliquots of 5 μL from each well for each sample at 0, 48, 72, 96, and 120 hours of the assay total run time (120 hours). For CSF SAA, the same volume was aliquoted at 0, 20, and 60 hours of the assay total run time (60 hours). All aliquots were stored at −80°C until analysis in ELISA.

**αSyn Oligomers-Specific ELISA**

All samples were analyzed using our in-house oligomers-specific ELISA, with a 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 hour incubation at 37°C) was used for detection with HRP-conjugated streptavidin (Sigma Aldrich) (at 1:5,000 dilution, 30 minutes incubation at 37°C) as the reporter. Plates were initially blocked to eliminate nonspecific signal for 1 hour at 37°C, and samples were diluted at 1:1,000 in 50% radioimmunoprecipitation assay (RIPA) buffer before loading and incubated for 1 hour 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 curve of all plates was fitted and used to quantify unknown concentrations using GraphPad Prism software. The concentrations of oligomeric total αSyn were extrapolated from corresponding standard curves. For each case analyzed by SAA, individual replicates were tested in duplicates using ELISA (i.e., 3 replicates × 2 duplicates per case), the average was calculated. Each ELISA run was performed using a 384-well maxisorp plate and completed within ~4–5 hours.

**Statistical Analyses**

GraphPad Prism (version 8.3.0) software was used for data analysis, including (1) calculating SAA kinetic parameters (maximum intensity of fluorescence [Ikmax] at final point [60 hours], and at 20 hours of the assay run, the time needed to reach 50% of the maximum aggregation [T50], 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) receiver operating characteristic (ROC) curves to evaluate diagnostic accuracy, and (4) correlation analysis. In brief, 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 aSyn levels, SAA kinetic parameters, and PD clinical stage assessed. \( p < 0.05 \) was set as the level of statistical significance.

**Standard Protocol Approvals, Registrations, and Patient Consents**

Experimental procedures were performed in accordance with the respective institutional ethical rules and regulations. The study was approved by the local ethics committee of Qatar Biomedical Research Institute.

**Data Availability**

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

## Results

### Patient Population and Demographics

Demographics, clinical characteristics, and CSF biomarker levels of the study discovery and validation CSF cohorts are summarized in Table 1. The discovery CSF cohort included 62 patients with PD and 34 controls. The average age of the PD discovery cohort was 67 ± 10 years, with average H&Y stage 2 ± 0.5, average UPDRS-III score 23.5 ± 9.1, and average Montreal cognitive assessment (MoCA) score of 25.8 ± 2.9 with 36 male and 26 female participants. Controls were aged 50 ± 16 years, with 16 male and 18 female participants. The validation CSF cohort included 49 patients with PD and 34 controls. The average age of the PD validation cohort was 66 ± 7.5 years, average H&Y stage 2 ± 0.5, average UPDRS-III score 23 ± 9, average MoCA score 27 ± 2.4 with 22 male and 27 female participants. HCs were aged 50 ± 16 years, with 16 male and 32 female participants.

### ELISA Demonstrates High Sensitivity and Specificity for SAA End Product Seeded by BHs From Patients With PD and DLB

We initially seeded the in vitro assembly of recombinant monomeric wild-type human aSyn with BH from frontal cortex in a pilot BH set of samples consisting of 3 cases per group of patients with PD, those with DLB, and Controls. A lag phase of 40–60 hours 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 3 groups. The DLB group showed a trend toward a higher Imax and AUC compared with the PD group, and as expected, both were higher compared with the Ctrl group with 1 PD sample overlapping with Ctrl (Figure 2, B and C). SAA quantified end product using ELISA showed that the levels of seeded aSyn oligomers in both PD and DLB groups were also higher than the Ctrl group with no overlap (Figure 2D).

### BHs of PD and DLB Seeding Activity Detected Earlier Using Oligomeric ELISA

For SAA-ELISA multiplex protocol, we optimized the dilution factor (1:1,000), dilution buffer (50% RIPA), and time point cutoff (data not shown). The cutoff was defined as the most appropriate time point where positive samples can be robustly measured above the ELISA limit of quantification. On average, PD and DLB BH samples gave a positive response within ~55–65 hours and ~15–30 hours for PD CSF samples; thus, 60 and 20 hours 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 aSyn oligomers at the earliest possible time point. The

### Table 1 Demographics and CSF Biomarkers by Diagnostic Group

<table>
<thead>
<tr>
<th></th>
<th>Discovery cohort</th>
<th>Validation cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ctrl (n = 34)</td>
<td>PD (n = 62)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>50 (43–62.5)</td>
<td>57.5 (49.2–66)</td>
</tr>
<tr>
<td>Gender, male</td>
<td>16 (47)</td>
<td>36 (58)</td>
</tr>
<tr>
<td>Disease duration, y</td>
<td>NA</td>
<td>5 (2–7)</td>
</tr>
<tr>
<td>MoCA score</td>
<td>NA</td>
<td>27 (24–28)</td>
</tr>
<tr>
<td>MMSE</td>
<td>NA</td>
<td>29 (29–30)</td>
</tr>
<tr>
<td>H&amp;Y score</td>
<td>2 (2–3)</td>
<td>29 (28–30)</td>
</tr>
<tr>
<td>UPDRS-III score</td>
<td>0.51 (0.42–0.63)</td>
<td>0.7 (0.59–0.94)</td>
</tr>
<tr>
<td>SAA t-aSyn, ng/mL</td>
<td>0.03 (0.01–0.06)</td>
<td>0.30 (0.20–0.39)</td>
</tr>
<tr>
<td>SAA a-aSyn, ng/mL</td>
<td>0.01 (0.00–0.03)</td>
<td>0.41 (0.20–0.87)</td>
</tr>
</tbody>
</table>

Abbreviations: Ctrl = healthy controls; H&Y = Hoehn and Yahr stage; IQR = interquartile range; MMSE = Mini-Mental State Examination; MoCA = Montreal cognitive assessment; NA = not applicable; o-aSyn = oligomeric a-synuclein; PD = Parkinson disease; SAA = seed amplification assay; t-aSyn = total a-synuclein; UPDRS-III = Unified Parkinson Disease Rating Scale-Part III. Data are expressed as median (IQR) or n (%).
validation BH set included human brain tissues from Ctrl (n = 5), AD (n = 5), PD (n = 5), and DLB (n = 5) cases. αSyn SAA assay was performed as described earlier, 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 hours (0, 48, 72, 96, and 120 hours) and then frozen before ELISA analysis. Levels of αSyn oligomers increased in a time-dependent manner up to a plateau at approximately 90 hours. Of interest, BH seeding activity was detected earlier with ELISA than SAA alone (72 vs 120 hours) (Figure 3, A–C), while maintaining optimal specificity for discriminating synucleinopathies from controls (eFigure 1, links.lww.com/WNL/C291).

**CSF-Seeded αSyn Oligomers Robustly Discriminate Patients With PD From Controls in the Discovery CSF Cohort**

We then analyzed CSF samples from patients with PD (n = 62) and age-matched Ctrls (n = 34) to better understand the added value of our SAA-ELISA multiplex approach. The demographics of all participants are listed in Table 1.

The time needed to reach 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). To quantify the levels of αSyn oligomers in SAa seeded by CSF, samples from each replicate of each case at 20 hours of the assay run were analyzed using ELISA assays for measuring total or αSyn oligomers. In comparing relative fluorescence unit (RFU) values for SAA data, a notable overlap was noted between diagnostic groups at 20 hours, which was significantly reduced at the end point (Figure 4B). Whereas the levels of CSF-seeded αSyn oligomers were significantly higher in patients with PD (mean ± SD = 321 ± 180, n = 62) compared with Ctrls (mean ± SD = 47 ± 40, n = 34) at 20 hours (p < 0.001, Mann-Whitney U test) (Figure 4C), with minimal overlap compared with RFU at the same time point. The levels of total αSyn did not differ significantly between the 2 groups (mean ± SD = 799 ± 219, n = 62, and 554 ± 201, n = 34 for patients with PD and Ctrls, respectively) (Figure 4C).

ROC analysis was performed to evaluate the diagnostic accuracy of SAA RFU values measured at 20 hours and 60 hours and CSF-seeded αSyn oligomers as disease predictors (eFigure 2, links.lww.com/WNL/C291). The ROC curve demonstrated that cutoff values of 0.087 mg/mL for CSF-seeded αSyn oligomers, 17,314 RFU for Imax at 60 hours, 10,644 RFU for Imax at 20 hours, and 721,500 RFU for ThT AUC were the most reliable measures to distinguish patients with PD from Ctrls (eFigure 2). The abovementioned cutoff 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 hours (Figure 4, F–I). However, the cutoff value for Imax at 20 hours 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 an ROC AUC of 0.860 (eTable 2 and eFigure 2).

**Correlation Between Disease Severity and CSF-Seeded αSyn Oligomers**

To investigate whether the level of αSyn oligomers in samples seeded with CSF from patients with PD 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 between the levels of CSF-seeded αSyn oligomers at 20 hours and a severity of 87.5%, with an ROC AUC of 0.860 (eTable 2 and eFigure 2).
UPDRS motor and H&Y scores, respectively). We did not observe any correlation between CSF-seeded αSyn oligomers and cognitive scores of patients with PD; however, high scores of both MoCA and MMSE scales were registered for most patients with PD, rendering the patients almost cognitively intact (eFigure 4).

We also calculated correlation coefficients with clinical data from patients with PD using kinetic parameters positive αSyn SAA. No significant correlations were found. Imax at 60 hours 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 and D). When 2 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 PD and normal controls, and CSF-seeded αSyn oligomers levels correlate with PD 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 used 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 SAAs.

In this study, 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 analyzing large sample sets, and more appropriate for diagnostic laboratory settings. αSyn SAAs, however, are remarkably specific for discriminating patients with PD and DLB from other non-synucleinopathies, potentially providing a definitive diagnosis.

In this 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 2 assays because
both PD and DLB groups scored positive in SAA and showed high levels of seeded aSyn oligomers in the ELISA. Likewise, controls scored negative on SAA and no seeded aSyn oligomers were detected above the ELISA limit of quantification.

Based on the abovementioned findings, we expanded our analyses to a larger set of human brain tissues, where our data showed high degrees of consistency in identifying patients with PD and DLB from Ctrlrs or those with AD. Moreover, the amplification of misfolded aSyn in PD and DLB was robustly detected by ELISA at an earlier time point compared with SAA. The detection of misfolded aSyn by ELISA was ideally specific between 70 and 90 hours of seed amplification, whereas a similar segregation was only achieved at 120 hours using SAA alone. Those findings emphasized that the implementation of aSyn oligomer-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 analyzed 2 independent CSF cohorts of patients with PD and Ctrlrs, named the discovery and validation CSF cohorts, respectively. In the discovery CSF cohort, we analyzed CSF samples from 62 patients with PD and 34 Ctrlrs. At 20 hours, 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 aSyn oligomers were assessed between the same groups at the same time point. As expected, at 60 hours, 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 aSyn in human CSF but also an earlier reading of the test output.

Caughey et al. 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.33-46 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 1 report showing T50 to correlate with the disease severity in patients with PD assessed by H&Y scale \( (r = -0.54, p = 0.006) \). A recent comparative study using the Parkinsons Progression Markers Initiative longitudinal cohort accentuated the shortcomings of the current aSyn SAA at quantifying disease severity or clinical features.46 Similarly, in our study, SAA kinetic parameters failed to correlate with PD-specific clinical features in 2 independent CSF cohorts. The only notable correlations between SAA kinetic parameters and H&Y scores were sensitive to the effect of potential outliers, which underlined the existence of a true correlation. However, it was remarkable that CSF-seeded aSyn 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 aSyn SAA could provide clinical information about PD severity beyond diagnostic performance.

Although our SAA-ELISA multiplex was initially tested in postmortem 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. In addition, analyzing 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 oligomer-specific ELISA is useful in patients with other synucleinopathies and in assessing therapies targeting aSyn aggregation. In summary, our findings further support the growing evidence of aSyn 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 aSyn aggregates in PD.

**Acknowledgment**
The authors thank Dr. Houari Abdesselem, the manager of OE laboratory, for his valuable support. This paper is dedicated to the memory of our dear friend and colleague Jan Aasly who passed away on June 19, 2022.

**Study Funding**
This study was supported by Qatar Biomedical Research Institute (IGP4-ID-2020-001). The Newcastle Brain Tissue Resource is funded in part by a grant from the UK Medical Research Council (G0400074), by NIHR Newcastle Biomedical Research Center awarded to the Newcastle upon Tyne NHS Foundation Trust and Newcastle University, and by a grant from the Alzheimer’s Society and Alzheimer’s Research UK as part of the Brains for Dementia Research Project.

**Disclosure**
The authors report no relevant disclosures. Go to Neurology.org/N for full disclosures.

**Publication History**
Received by *Neurology* November 20, 2021. Accepted in final form July 19, 2022. Submitted and externally peer reviewed. The handling editor was Peter Hedera, MD, PhD.

**Appendix Authors**

<table>
<thead>
<tr>
<th>Name</th>
<th>Location</th>
<th>Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nour Majbour, PhD</td>
<td>Neurological Disorders Research Centre, Qatar Biomedical Research Institute (QBRI), Hamad Bin Khalifa University (HBKU), Qatar Foundation, Doha; MRC Prion Unit at University College London (UCL), UCL Institute of Prion Diseases, UCL, United Kingdom</td>
<td>Additional contributions: designed and performed the experiments; drafting/revision of the article for content, including medical writing for content; major role in the acquisition of data; study concept or design; and analysis or interpretation of data</td>
</tr>
</tbody>
</table>

Continued
Appendix (continued)

<table>
<thead>
<tr>
<th>Name</th>
<th>Location</th>
<th>Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jan Aasly, MD</td>
<td>Department of Neuroscience, Norwegian University of Science and Technology (NTNU), Trondheim; Department of Neurology, St. Olav's Hospital, Trondheim, Norway</td>
<td>Additional contributions: contributed the clinical CSF cohort; drafting/revision of the article for content, including medical writing for content; and major role in the acquisition of data</td>
</tr>
<tr>
<td>Ilham AbdI, MSc</td>
<td>Neurological Disorders Research Centre, Qatar Biomedical Research Institute (QBRI), Hamad Bin Khalifa University (HBKU), Qatar Foundation, Doha</td>
<td>Additional contributions: designed and performed the experiments; drafting/revision of the article for content, including medical writing for content; and analysis or interpretation of data</td>
</tr>
<tr>
<td>Simona Ghanem, PhD</td>
<td>Neurological Disorders Research Centre, Qatar Biomedical Research Institute (QBRI), Hamad Bin Khalifa University (HBKU), Qatar Foundation, Doha</td>
<td>Additional contributions: defined the clinical CSF cohort; drafting/revision of the article for content, including medical writing for content; and major role in the acquisition of data</td>
</tr>
<tr>
<td>Daniel Erskine, PhD</td>
<td>Amsterdam UMC, location VUmc, Vrije Universiteit Amsterdam, Department of Anatomy and Neurosciences, Amsterdam Neuroscience, the Netherlands</td>
<td>Additional contributions: characterized the human brain tissues; drafting/revision of the article for content, including medical writing for content; and major role in the acquisition of data</td>
</tr>
<tr>
<td>Wilma van de Berg, PhD</td>
<td>Translational and Clinical Research Institute, Newcastle University, United Kingdom</td>
<td>Additional contributions: contributed the clinical CSF cohort; drafting/revision of the article for content, including medical writing for content; and major role in the acquisition of data</td>
</tr>
<tr>
<td>Omar El-Agnaf, PhD</td>
<td>Neurological Disorders Research Centre, Qatar Biomedical Research Institute (QBRI), Hamad Bin Khalifa University (HBKU), Qatar Foundation, Doha</td>
<td>Drafting/revision of the article for content, including medical writing for content; study concept or design; and analysis or interpretation of data</td>
</tr>
</tbody>
</table>

References

1. Trojanowski JQ, Lee VM. “Fatal attractions” of proteins. A comprehensive hypo-
   thetical mechanism underlying Alzheimer’s disease and other neurodegenerative
2. Bossy-Wetzel E, Schwarzenbacher R, Lipton SA. Molecular pathways to neuro-
3. Forman MS, Trojanowski JQ, Lee VME. Neurodegenerative diseases: a decade of
4. Kwon S, Iba M, Kim C, Masliah E. Immunotherapies for aging-related neurodegen-
5. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-
7. Postuma RB, Berg D, Stern M, et al. MDS clinical diagnostic criteria for Parkinson’s


Disease-Associated α-Synuclein Aggregates as Biomarkers of Parkinson Disease Clinical Stage
Nour Majbour, Jan Aasly, Ilham Abdi, et al.
Neurology 2022;99:e2417-e2427 Published Online before print September 12, 2022
DOI 10.1212/WNL.0000000000201199

This information is current as of September 12, 2022

Updated Information & Services
including high resolution figures, can be found at:
http://n.neurology.org/content/99/21/e2417.full

References
This article cites 50 articles, 3 of which you can access for free at:
http://n.neurology.org/content/99/21/e2417.full#ref-list-1

Subspecialty Collections
This article, along with others on similar topics, appears in the following collection(s):
Alzheimer disease
http://n.neurology.org/cgi/collection/alzheimers_disease
Assessment of cognitive disorders/dementia
http://n.neurology.org/cgi/collection/assessment_of_cognitive_disorders_dementia
Cognitive neuropsychology in dementia
http://n.neurology.org/cgi/collection/cognitive_neuropsychology_in_dementia
Dementia with Lewy bodies
http://n.neurology.org/cgi/collection/dementia_with_lewy_bodies
Neuropsychological assessment
http://n.neurology.org/cgi/collection/neuropsychological_assessment
Parkinson disease/Parkinsonism
http://n.neurology.org/cgi/collection/parkinsons_disease_parkinsonism

Permissions & Licensing
Information about reproducing this article in parts (figures, tables) or in its entirety can be found online at:
http://www.neurology.org/about/about_the_journal#permissions

Reprints
Information about ordering reprints can be found online:
http://n.neurology.org/subscribers/advertise

Neurology ® is the official journal of the American Academy of Neurology. Published continuously since 1951, it is now a weekly with 48 issues per year. Copyright © 2022 The Author(s). Published by Wolters Kluwer Health, Inc. on behalf of the American Academy of Neurology. All rights reserved. Print ISSN: 0028-3878. Online ISSN: 1526-632X.