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Development of a Biochemical Diagnosis of Parkinson Disease by Detection of α-Synuclein Misfolded Aggregates in Cerebrospinal Fluid

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← Editorial

＋ Supplemental content

IMPORTANCE Parkinson disease (PD) is a highly prevalent and incurable neurodegenerative disease associated with the accumulation of misfolded α-synuclein (αSyn) aggregates. An important problem in this disease is the lack of a sensitive, specific, and noninvasive biochemical diagnosis to help in clinical evaluation, monitoring of disease progression, and early differential diagnosis from related neurodegenerative diseases.

OBJECTIVE To develop a novel assay with high sensitivity and specificity to detect small quantities of αSyn aggregates circulating in cerebrospinal fluid (CSF) of patients affected by PD and related synucleinopathies.

DESIGN, SETTING, AND PARTICIPANTS The strategy evaluated in this proof-of-concept study uses the protein misfolding cyclic amplification (PMCA) technology that detects minute amounts of misfolded oligomers by taking advantage of their ability to nucleate further aggregation, enabling a very high amplification of the signal. The technology was first adapted with synthetic αSyn oligomers prepared in vitro and used to screen in 2 blinded cohorts of CSF samples from German and Japanese patients with PD (n = 76) and individuals serving as controls affected by other neurologic disorders (n = 65), neurodegenerative diseases (n = 18), and Alzheimer disease (n = 14). The kinetics of αSyn aggregation were measured by αSyn-PMCA in the presence of CSF samples from the participants to detect αSyn oligomeric seeds present in this biological fluid. The assays were conducted from November 15, 2013, to August 28, 2015.

MAIN OUTCOMES AND MEASURES Kinetic parameters correlated with disease severity at the time of sample collection, measured by the Hoehn and Yahr scale, with the lowest grade indicating unilateral involvement with minimal or no functional impairment, and the highest grade defining patients with complete confinement to wheelchair or bed.

RESULTS Studies with synthetic αSyn aggregates showed that αSyn-PMCA enabled to detect as little as 0.1 pg/mL of αSyn oligomers. The αSyn-PMCA signal was directly proportional to the amount of αSyn oligomers added to the reaction. A blinded study of CSF samples correctly identified patients affected by PD with an overall sensitivity of 88.5% (95% CI, 79.2%-94.6%) and specificity of 96.9% (95% CI, 89.3%-99.6%). The αSyn-PMCA results for different patients correlated with the severity of the clinical symptoms of PD (Japanese cohort: r_s = −0.54, P = .006; German cohort: r_s = −0.36, P = .02).

CONCLUSIONS AND RELEVANCE The findings suggest that detection of αSyn oligomers by αSyn-PMCA in the CSF of patients affected by PD may offer a good opportunity for a sensitive and specific biochemical diagnosis of the disease. Further studies are needed to investigate the usefulness of αSyn-PMCA to monitor disease progression and for preclinical identification of patients who may develop PD.

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Parkinson disease (PD) is a devastating degenerative disorder of the brain for which there is no causative treatment or accurate early diagnosis. Initially, symptoms include motor alterations, such as tremor, rigidity, and bradykinesia with possible gait abnormalities.[1] As the disease progresses, it often manifests with cognitive problems, psychiatric alterations, and dementia. The cause of the disease is not clear, but findings from neuropathologic, biochemical, and in vivo experiments with animal models suggest that the misfolding, aggregation, and brain deposition of α-synuclein (αSyn) in the form of Lewy bodies and Lewy neurites might be the triggering event leading to the subsequent pathologic abnormalities responsible for the clinical disease.[2,3] Accumulation of αSyn aggregates also occurs in other neurologic diseases termed as a group *synuclein-aggregation disorders*, which include dementia with Lewy bodies (DLB), in which αSyn inclusions are deposited more widely in cortical areas, and multiple system atrophy (MSA), in which αSyn aggregates accumulate in glial cells.[3,4] α-Synuclein misfolding and aggregation follows a seeding-nucleation mechanism that depends on the slow formation of seeding-competent oligomers followed by the exponential growth of the polymers to form long fibrils that accumulate in damaged cells.[5,6]

Parkinson disease is diagnosed primarily by clinical examination following the UK Brain Bank Criteria[7] and its response to dopaminergic medication. The clinical diagnosis can be complemented by imaging techniques that are helpful to detect neurodegenerative damage but often cannot differentiate related diseases. Definitive diagnosis is achieved post mortem by histologic brain examination for damage in the substantia nigra and the presence of Lewy bodies and Lewy neurites.[4,8] Despite much effort to identify biochemical markers for PD, there is still not an accepted and validated surrogate biomarker.[9] Availability of a sensitive, specific, and noninvasive biochemical marker would be useful to help in clinical diagnosis, monitoring of disease progression, and early identification of affected individuals before they display substantial and irreversible neuronal loss in the substantia nigra followed by motor and nonmotor disability. Several lines of evidence point out that detection of soluble misfolded αSyn oligomers in biological fluids might represent a good strategy for biochemical diagnosis of PD: (1) soluble αSyn oligomers of small or intermediate molecular weight, rather than long fibrils, might be responsible for neurodegeneration in PD[10-12]; (2) the process of αSyn misfolding and aggregation appears to begin many years or even decades before the onset of clinical symptoms of the disease[3,13]; and (3) αSyn oligomers might be circulating in PD biological fluids.[14-16]

In this study we present proof-of-concept data for a novel procedure to specifically detect αSyn aggregates circulating in biological fluids of patients affected by PD and other synuclein aggregation disorders. Our approach takes advantage of the seeding-nucleation process of αSyn aggregation to detect misfolded oligomers using their capacity to seed the polymerization of the monomeric protein. To measure seeding activity in an efficient and sensitive manner, we invented the

Key Points

Question Does detection of α-synuclein oligomers by protein misfolding cyclic amplification (PMCA) in cerebrospinal fluid provide a sensitive and specific biochemical test for the diagnosis of Parkinson disease?

Findings α-Synuclein PMCA enabled detection of attomole quantities of α-synuclein oligomers and identification in cerebrospinal fluid samples from 76 patients affected by Parkinson disease and 97 individuals with other neurologic disorders with 88.5% sensitivity and 96.9% specificity. Moreover, good correlation was observed between the α-synuclein–PMCA results and the disease progression.

Meaning Our findings suggest that α-synuclein–PMCA may provide an efficient, objective and non-invasive biochemical test for the diagnosis of Parkinson disease.

protein misfolding cyclic amplification (PMCA), which is a platform technology that enables ultrasensitive detection of misfolded aggregates through amplification of the misfolding and aggregation process in vitro.[17,18] Protein misfolding cyclic amplification is a cyclical process; each cycle is composed of 2 phases. During the first phase, the sample containing minute amounts of misfolded oligomers and an excess of monomeric protein are incubated to induce growing of the polymers. In the second phase, the sample is subjected to a mechanical force to break down the polymers, multiplying the number of nuclei.[18] After each cycle, the number of seeds increases in an exponential fashion. Protein misfolding cyclic amplification is conceptually analogous to DNA amplification by polymerase chain reaction. In both systems a template grows at the expense of a substrate in a cyclic reaction, combining growing and multiplication of the template units. The PMCA technique has been successfully applied to amplify and detect misfolded prion protein implicated in prion diseases.[17,19] Protein misfolding cyclic amplification enabled detection of the equivalent of a single particle of misfolded prion protein.[19] This very high sensitivity led to detection of misfolded prion protein in the blood and urine of infected animals at symptomatic and presymptomatic stages of the disease[20-22] as well as in the blood and urine of humans affected by variant Creutzfeldt-Jakob disease.[23,24] Over the past years, PMCA has been extensively used by many other groups to understand prion biology, the mechanisms and factors involved in prion transmission, and ultrasensitive detection of prions in biological fluids.[25,26] A recent study reported the expansion and adaptation of PMCA to amplify seeding-competent β-amyloid (Aβ) oligomers circulating in the cerebrospinal fluid (CSF) of patients with Alzheimer disease (AD) and application of PMCA to distinguish, with high sensitivity and specificity, samples obtained from patients with AD from those with other forms of dementia or neurodegenerative diseases.[27] In the present study, we adapted PMCA for highly sensitive detection of αSyn aggregates (αSyn-PMCA) and used it to distinguish CSF samples obtained from patients affected by PD from those of individuals affected by other neurologic diseases who served as controls.

Methods

Patients and Study Design

For this study we used aliquots of CSF from 76 patients clinically diagnosed with PD, 10 with DLB, and 10 with MSA, with the samples blinded. As controls we used CSF samples from 3 groups: (1) 65 patients with other neurologic diseases (ie, epilepsy, cervical spondylosis, muscular dystrophy, viral myositis, and myelopathy) as well as 2 people without neurologic diseases; (2) 14 patients with clinical diagnoses of AD; and (3) 18 people affected by other chronic neurodegenerative disorders (eg, frontotemporal dementia, ataxia, Huntington disease, amyotrophic lateral sclerosis, and progressive supranuclear palsy). Samples were collected at the Paracelsus-Elena-Klinik, Kassel, Germany (as part of the Kassel cohort previously described[28]); the Kyoto Prefectural University of Medicine, Kyoto, Japan; and the Higashi Matsudo Municipal Hospital, Matsudo, Japan. The methods of CSF collection and the overall study were approved by the local ethics committee of Kyoto Prefectural University and Landesaerztekammer Hesse, Frankfurt, Germany. Participants provided written informed consent; they did not receive financial compensation. Testing of the samples by αSyn-PMCA was conducted from November 15, 2013, to August 28, 2015.

The clinical diagnoses of probable PD, DLB, or MSA were made according to internationally standardized criteria, including the UK Brain Bank guidelines.[1,7,29] The Hoehn and Yahr scale has been widely used to classify the patients with Parkinson disease. Modified 7-grade Hoehn and Yahr scale (1, 1.5, 2, 2.5, 3, 4, and 5) defines progressive motor impairment that correlates with disease severity with progressive motor decline and deterioration in quality of life. Grade 1 in the scale corresponds to patients with unilateral involvement with minimal or no functional impairment, whereas grade 5 defines patients with complete confinement to wheelchair or bed. At this stage, the patient exhibits inability to stand, walk, rise from a chair, and get out of bed without assistance.[30,31] The grading was performed during off periods. Assigned diagnoses were independently reviewed by a board-certified neurologist (T.T., C.T., and B.M.) with subspecialty training in movement disorders and dementias. Samples of CSF were collected in the morning using polypropylene tubes following lumbar puncture at the L4/L5 or L3/L4 interspace with atraumatic needles after 1 night of fasting. The samples were centrifuged at 3000g for 10 minutes at room temperature, aliquoted, and stored at −80°C until analysis. Blood cell (red and white) counts, glucose, protein, and hemoglobin concentrations were determined, as previously described.[32]

Expression and Preparation of Seed-Free αSyn

A bacterial plasmid carrying human αSyn gene was used to over-express the protein in BL21(DE3) pLysS cells at 25°C using 0.1mM isopropyl β-D-thiogalactoside for 10 hours.[33] The bacterial pellets were lysed in 300mM sodium chloride (NaCl), 50mM sodium dihydrogenphosphate (pH 8.0), 1mM phenylmethylsulfonyl fluoride, 0.1mM tris-(2-carboxyethyl) phosphine (TCEP), and 1 mg/mL lysozyme followed by sonication on ice. After soni-

cation, the solution was centrifuged at 20 000g for 30 minutes at 4°C. Supernatant was recovered and loaded onto a nickel-affinity column (Qiagen Co). Proteins were eluted in a buffer consisting of 125mM NaCl, 300mM imidazole, 0.1mM TCEP, and 25mM NaH_2PO_4 (pH 7.4). After that, purified protein was dialyzed overnight at 4°C against phosphate-buffered saline (PBS), pH 7.4, containing protease inhibitors. Next, to remove any preformed seed or aggregate, protein solution containing αSyn was filtered through a 100-kDa cutoff filter (Amicon Ultra; Millipore). Purity was evaluated by silver staining.

Preparation of αSyn Oligomers In Vitro

To prepare αSyn seeds, purified αSyn in PBS at a concentration of 1 mg/mL was allowed to aggregate at 37°C with vigorous shaking. Periodically, an aliquot was removed and formation of oligomeric species was characterized by transmission electron microscopy, thioflavin T (ThT) fluorescence, size exclusion chromatography, Western blot, and dot blot using conformational specific antibodies according to previously described procedures.[27]

αSyn-PMCA Assay

Samples of seed-free αSyn at a concentration of 1 mg/mL in 100mM piperazine-N,N'-bis(ethanesulfonic acid) (PIPES), pH 6.5, 500mM NaCl, were placed in black 96-well plates in the presence of 5μM concentration of ThT at a final volume of 200 μL. For each test we added 40 μL of CSF samples from patients and control participants. Positive control samples consisted of a well-documented and previously screened healthy CSF sample spiked with preformed αSyn oligomeric seeds. Samples were subjected to cyclic agitation (1 minute at 500 rpm followed by 29 minutes without shaking) at 37°C. The increase in ThT fluorescence was monitored at excitation of 435 nm and emission of 485 nm, periodically, using a microplate spectrofluorometer (Gemini EM; Molecular Devices).

Immune-depletion of αSyn Species From PD CSF

To test whether seeding activity of healthy human CSF spiked with αSyn seeds and PD CSF is dependent on the presence of αSyn, immune-depletion of αSyn species from spiked human CSF and PD CSF was done using a cocktail of antibodies against αSyn. Magnetic beads (Dynal; ThermoFisher) covalently coupled with secondary antibodies were coated (4 μg antibody per 3 × 10⁷ beads) with mouse monoclonal Syn204 antibody (Abcam Co), mouse monoclonal antibody 211 specific against regions 121 to 125 of αSyn (Santa Cruz Biotechnology), and rabbit polyclonal C-20 that recognizes the C-terminal region of αSyn (Santa Cruz Biotechnology) according to the manufacturer's protocol (Dynal; ThermoFisher). Thereafter, 200 μL of PD CSF was incubated with approximately 3 × 10⁷ antibody-coated beads (approximately 1 × 10⁷ coated beads with each antibody) for 24 hours at 4°C with mixing. Material bound to the antibody-coated beads was removed using a magnet and seeding activity of depleted CSF was assessed by αSyn-PMCA.

Determination of Sensitivity, Specificity, and Predictive Values

The differences in the kinetics of aggregation between different samples were evaluated by the estimation of various kinetic

Figure 1. Optimization of Cyclic Amplification of α-Synuclein (αSyn) by Protein Misfolding Cyclic Amplification (PMCA)
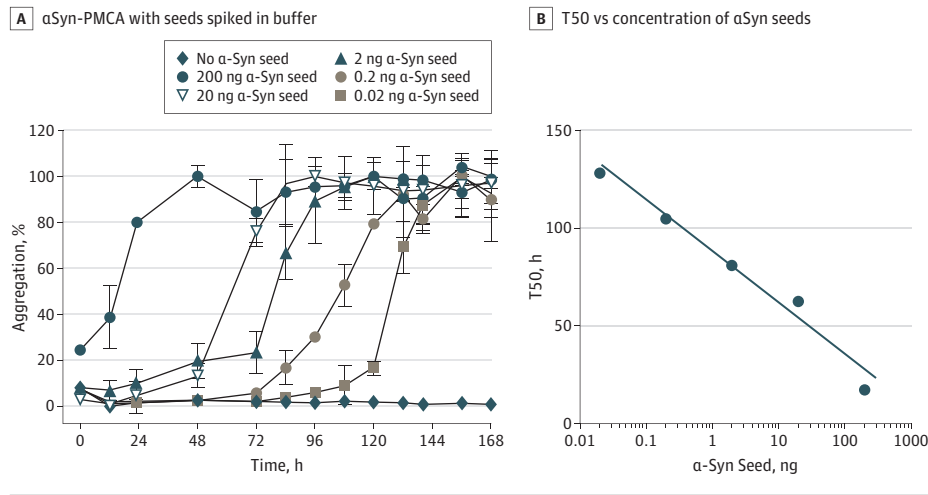
A, Purified seed-free αSyn (0.1 mg/mL) was incubated in phosphate-buffered saline, pH 7.4, at 37°C with cycles of 1-minute shaking (500 rpm) every 30 minutes. Samples were incubated in the absence or the presence of the indicated quantities of preformed seeds. The extent of aggregation was monitored by thioflavin T (ThT) fluorescence and expressed as a percentage of the maximum ThT fluorescence. Samples were run in duplicate and error bars indicate SD. B, The time to reach 50% aggregation (T50) in each condition was plotted as a function of the logarithm of the amount of αSyn added to the reaction, expressed as nanograms of monomeric αSyn.

variables, including the lag phase (time required to reach a ThT fluorescence 5 times higher than the background value of the buffer alone; approximately 20 fluorescence units), T50 (time needed to reach 50% of the maximum aggregation), and maximum fluorescence (value at which a plateau of ThT fluorescence is reached). Sensitivity, specificity, and predictive values, including the 95% CIs, were calculated by receiver operating characteristics curve analysis, using MedCalc, version 12.2.1.0 (MedCalc Software).

Preparation of Aβ1-42 and Tau Oligomeric Seeds

Highly pure synthetic Aβ1-42 was acquired from the W. Keck Facility at Yale University. The final product was characterized by amino acid analysis and mass spectrometry. To prepare seed-free Aβ1-42, lyophilized powder of Aβ1-42 was dissolved at a high pH (10mM sodium hydroxide) and filtered through a 30-kDa cutoff filter to remove remaining aggregates. To prepare Aβ1-42 seeds, a solution of seed-free Aβ1-42 (10μM) in 0.1M Tris-HCl pH 7.4 was incubated for 10 hours at 25°C with shaking. This preparation yielded a heterogeneous population of Aβ1-42 including small oligomers, protofibrils, and fibrils as analyzed by transmission electron microscopy. To make this preparation homogeneous, Aβ1-42 seeds were briefly sonicated.

For tau, bacterial plasmid carrying full-length human tau (*htau40*; GenBank NP_005901.2) gene was overexpressed in BL21(DE3) *Escherichia coli* bacteria and purified as described previously.[34] To prepare *htau40* seeds, purified seed-free *htau40* (50μM) was incubated with 25μM heparin (average molecular weight approximately 18 kDa) in aggregation buffer (10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 100mM NaCl) with shaking for 3 days at 37°C. To make *htau40* seeds homogeneous, the aggregated *htau40* preparation was sonicated briefly.

Statistical Analysis

Differences on the kinetics of aggregation with or without immune-depleted samples were analyzed by 2-way analysis of variance, using time and conditions as the variables. Differences in maximum fluorescence between the PD and control samples were evaluated by unpaired, 2-tailed *t* test. Spearman rank correlation was used to evaluate the correlation between T50 and the Hoehn and Yahr Scales. All statistical analyses were performed by using GraphPad Prism, version 5.0 (GraphPad Software). The level of significance was set at *P* < .05.

Results

Implementation of αSyn-PMCA

To optimize and implement the conditions for cyclic amplification of αSyn misfolding (αSyn-PMCA), we used oligomeric seeds prepared in vitro from purified recombinant human αSyn. Currently, it is not known which of the different intermediates in the process of αSyn aggregation is most relevant for PD pathology. Therefore, we decided to work with a heterogeneous mixture of oligomers of different sizes generated during αSyn aggregation. The kinetics and structural features of the species produced during αSyn aggregation over time were studied by the ThT fluorescence assay, which detects the formation of amyloid fibrils,[35] as well as transmission electron microscope, and gel electrophoresis followed by silver staining (eFigure 1 in the Supplement). Based on these results, we identified that incubation of purified αSyn (1 mg/mL) for 96 hours resulted in the formation of a heterogeneous population of αSyn aggregates comprising small to large oligomeric species and small fibrils. This preparation was sonicated to increase the homogeneity of the aggregates. The material produced after 96 hours of incubation under these conditions migrated as sodium dodecyl sulfate-resistant aggregates in the range of 80 to 135 kDa, with the major species having 135-kDa molecular weight (eFigure 1C in the Supplement).

One of the hurdles for developing a reproducible αSyn-PMCA reaction was to avoid or substantially minimize the spontaneous aggregation of the protein to provide a good window

Figure 2. Performance of α-Synuclein (αSyn) Protein Misfolding Cyclic Amplification (PMCA) in Cerebrospinal Fluid (CSF) Samples
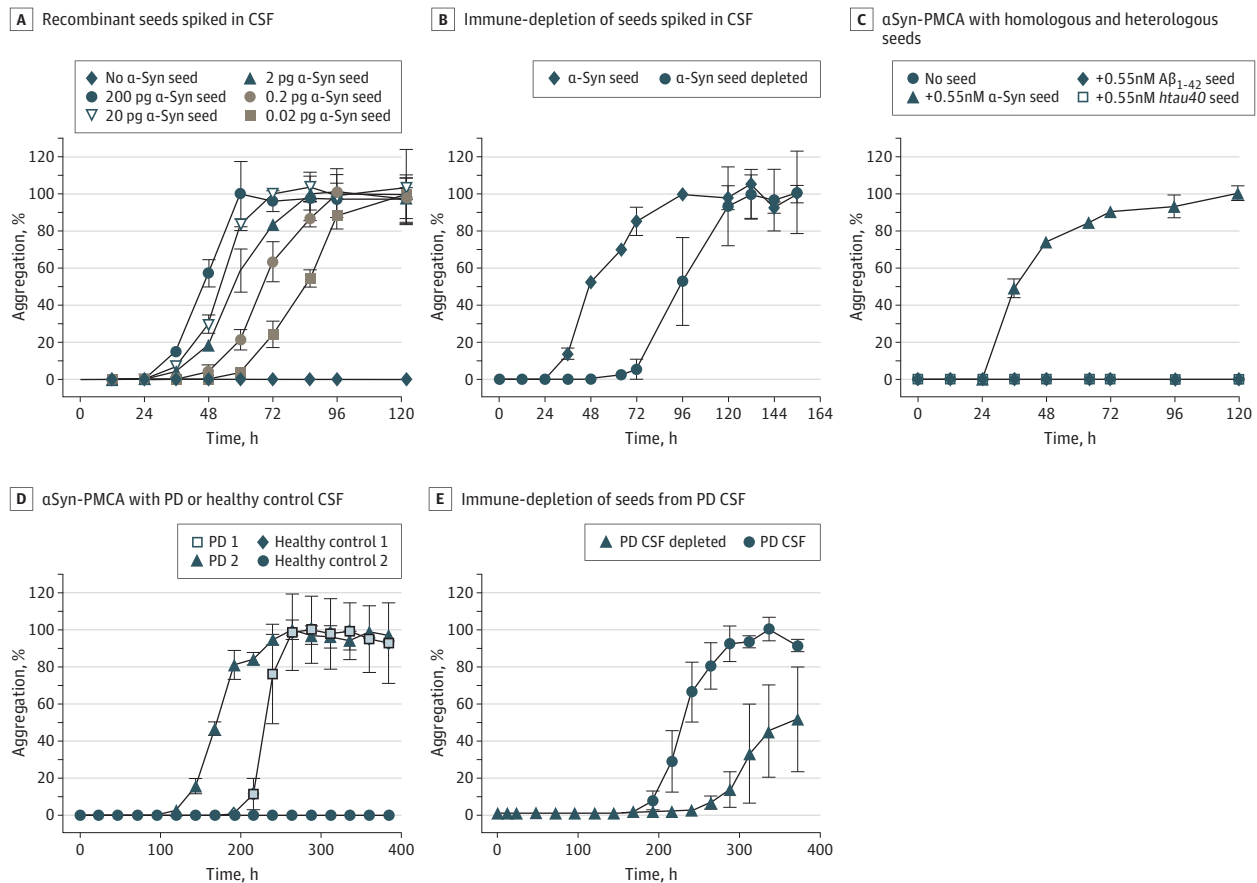
A, Purified seed-free αSyn (1 mg/mL in 100mM piperazine-N,N'-*bis* (ethanesulfonic acid, pH 6.5, and 500mM NaCl) was incubated in the presence of 40 µL of human CSF from a healthy individual serving as a control spiked with the indicated quantities of preformed seeds. Samples were subjected to αSyn-PMCA, consisting of cycles of 1 minute of shaking (500 rpm) every 30 minutes of samples incubated at 37°C. The extent of aggregation was monitored by thioflavin T fluorescence and expressed as a percentage of the maximum fluorescence. B, Human CSF spiked with 20 pg of preformed αSyn seeds was immune-depleted using magnetic beads coated with 3 different antibodies recognizing different sequence epitopes in the αSyn sequence. Samples obtained before and after immune depletion were used for αSyn-PMCA as described in A. C, To study the specificity of αSyn-PMCA for homologous or heterologous seeds,

human CSF (40 µL) as such (no seed) or spiked with 0.55-nM seeds of αSyn, β-amyloid 1-42, or *htau40* was subjected to αSyn-PMCA using the procedure described in A. The molar concentration of seeds was expressed considering the molecular weight of the respective monomer. D, Human CSF samples (40 µL) from 2 patients from PD and 2 from healthy individuals were subjected to αSyn-PMCA as described in A. E, Samples of CSF from 2 patients affected by PD were immune depleted as described in B. Samples obtained before or after immune depletion were used for αSyn-PMCA using the conditions described in A. Values correspond to the mean (SE) of the 2 samples used, each done in duplicate. Curves before and after immune depletion were significantly different as evaluated by 2-way analysis of variance (*P* < .001). Samples in all other panels were run in duplicate; error bars indicate SD.

of time to observe the aggregation seeded by exogenous oligomers. A critical step was to completely remove minute amounts of preformed seeds in the αSyn monomer preparation, which was achieved through filtration with a defined cutoff filter. Several factors were modified to improve the efficiency and reproducibility of αSyn-PMCA, such as the concentration of αSyn, temperature, pH of the reaction, and shaking speed. We found that using a 100-µg/mL concentration of seed-free αSyn at 37°C and cycles of 29-minute incubation followed by 1 minute of shaking (500 rpm), the protein aggregates only after the addition of exogenous seeds during the time in which the experiments are done (**Figure 1**A). Spontaneous aggregation occurs at a slower rate. This condition allowed us to be able to detect as little as 0.02 ng of seeds, which, considering an average molecular weight of αSyn oligomer of approximately 135

kDa (based on the results shown in eFigure 1C in the Supplement), would translate into 1.5×10^{-16}M of αSyn oligomers or 150 attomoles of oligomers in 200 µL of the reaction (Figure 1B). However, this estimation was done with in vitro–generated αSyn oligomers, which may or may not represent the biologically produced aggregates circulating in human biological fluids. Therefore, extrapolation of these quantities to patients' samples should not be done without further experiments. The time to reach 50% aggregation (T50) is directly proportional to the logarithmic concentration of added seeds (Figure 1B). When small aliquots of CSF (control or disease) were added to the reaction, we found that CSF has an inhibitory effect on αSyn-PMCA and no aggregation was observed in any of the samples. To overcome this inhibition, we slightly modified the conditions (αSyn concentration and pH of the reaction) for the

Figure 3. Performance of α-Synuclein Protein Misfolding Cyclic Amplification (αSyn-PMCA) Assay on Human Cerebrospinal Fluid (CSF) Samples
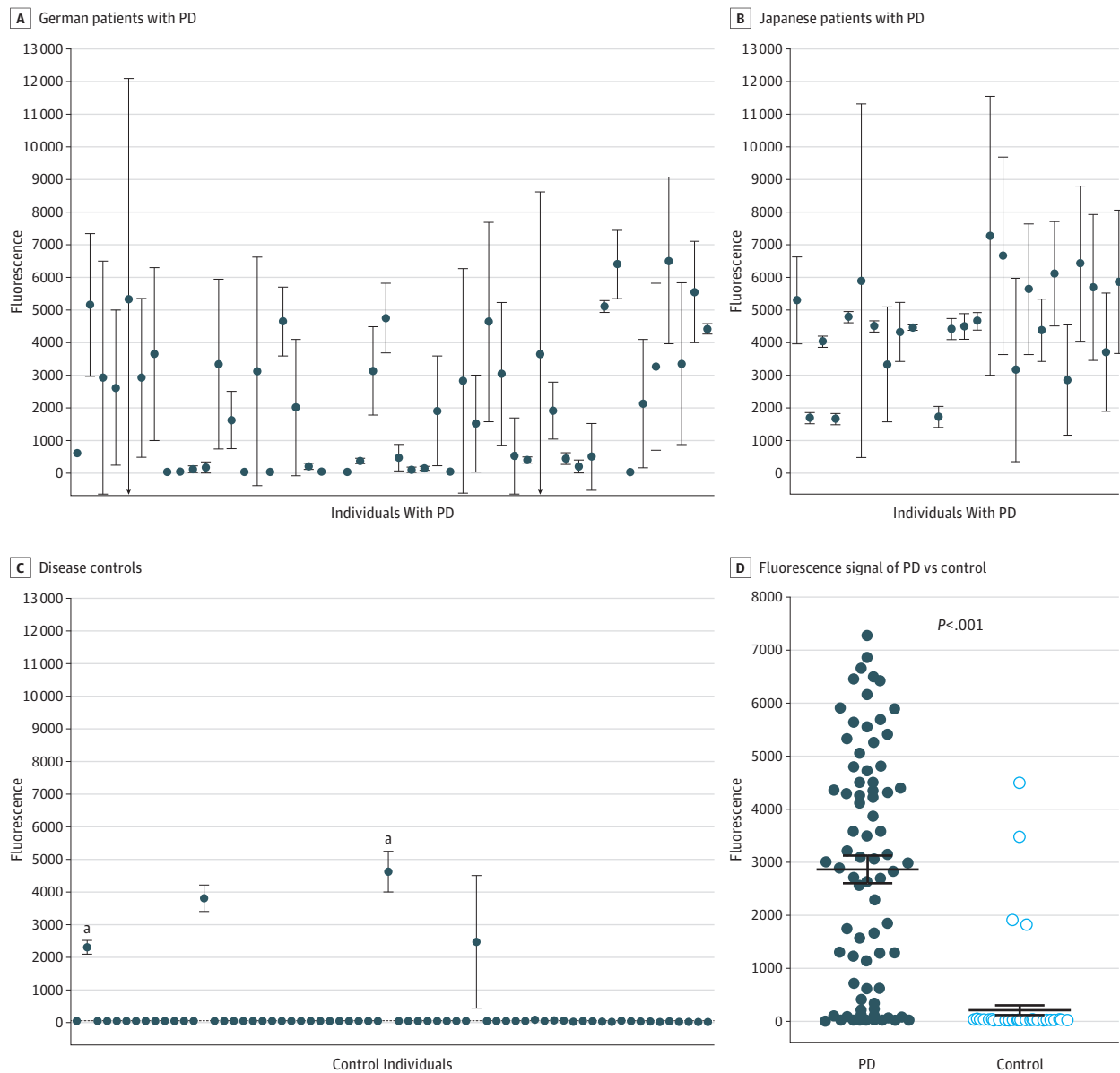
Human CSF samples obtained from 2 cohorts of patients with Parkinson disease (PD) (n = 76) collected in Germany (A) and Japan (B) and samples obtained from individuals serving as controls affected by other neurologic diseases (C) (n = 65) were analyzed by αSyn-PMCA. The graphs show the ThT fluorescence at 314 hours of incubation. Each point indicates a different patient. Experiments were done in duplicate and error bars represent SD. D, Even though the results

of αSyn-PMCA were positive or negative, we graphed the values of fluorescence at 314 hours for PD and control samples to analyze the significance of differences, which was evaluated by an unpaired, 2-tailed *t* test (D).

ª Persons who were diagnosed with PD after sample collection.

technology to work with CSF and to further decrease the limit of detection (**Figure** 2A). For this purpose, we increased the concentration of αSyn monomer to 1 mg/mL and changed the buffer of the reaction to 100mM PIPES, pH 6.5, 500mM NaCl. Under these conditions, we were able to detect as little as 0.02 pg of αSyn seeds, which translates to 0.15 attomoles of oligomeric αSyn (assuming an average molecular weight of 135 kDa).

To prove that αSyn-PMCA detects αSyn seeds, we attempted to immune deplete αSyn aggregates from the seed preparation containing 20 pg of oligomers by incubating the

sample with a cocktail of anti-αSyn antibodies, as indicated in the Methods section. When immune-depleted samples were used for αSyn-PMCA, we observed slower kinetics of αSyn aggregation, comparable to (or even slower than) when the reaction was done in the presence of 0.02 pg of seeds (Figure 2B). This result suggests that immune-depletion was able to remove more than 99% of the αSyn-aggregated material from the mixture.

To investigate whether αSyn-PMCA can detect aggregates composed of other amyloidogenic proteins, we prepared

Table. Sensitivity, Specificity, and Predictive Value for αSyn-PMCA in CSF Samples[a]

Variable	Value, % (95% CI)
Sensitivity for PD	88.5 (79.2-94.6)
Sensitivity for DLB	100 (94.9-100)
Sensitivity for MSA	80.0 (79.5-94.6)
Specificity against disease controls	96.9 (89.3-99.6)
Specificity against controls and neurodegenerative diseases	94.0 (86.5-98.0)
Positive predictive value[b]	94.7 (88.0-98.3)
Negative predictive value[b]	87.6 (78.7-93.7)

Abbreviations: αSyn-PMCA, α-synuclein protein misfolding cyclic amplification; CSF, cerebrospinal fluid; DLB, dementia with Lewy bodies; MSA, multiple system atrophy; PD, Parkinson disease.

[a] Data were analyzed by receiver operating characteristic using results from 76 samples from patients with PD, 10 DLB, and 10 MSA, and 83 patients serving as controls (65 affected by unrelated diseases and 18 with other neurodegenerative diseases, but not Alzheimer disease). Two samples originally provided as controls were later confirmed to be obtained at the preclinical stage of PD or DLB. These samples were included in the disease group for the purpose of the analysis.

[b] Predictive values were determined considering all synucleinopathy samples and control patients affected by other neurologic and neurodegenerative diseases (but not Alzheimer disease).

Aβ1-42 and tau oligomeric species and used them to seed monomeric αSyn. No signal was detectable in the presence of Aβ or tau seeds (Figure 2C), even when the concentration of these particles was relatively high (equivalent to 2 ng of αSyn seeds). These Aβ or tau seeds induced aggregation efficiently in the respective Aβ1-42 assay[27] or tau PMCA assay. These results indicate that, under the conditions and concentrations used, there is no cross-seeding between other protein aggregates and αSyn and that αSyn-PMCA is specific for detecting αSyn oligomers.

Detection of αSyn Oligomers in the CSF of Patients With PD

To study the usefulness of the αSyn-PMCA assay to detect seeding-competent αSyn oligomers in biological fluids, we analyzed aliquots of CSF from 2 different cohorts of PD samples provided blinded by collaborators. In total, 76 CSF samples of clinically diagnosed PD were used. As controls we used 65 samples collected from healthy people or patients with other neurologic diseases, 18 from patients affected by other chronic neurodegenerative diseases, and 14 from AD cases. Figure 2D shows the kinetics of aggregation of 2 representative samples from the PD and control groups. The result indicates that CSF from patients with PD induced αSyn aggregation with a lag phase of approximately 120 and 220 hours, whereas αSyn did not aggregate in the presence of CSF from any of the control samples. Before evaluating a larger set of samples, we wanted to analyze the effect of freezing and thawing on the αSyn-PMCA reaction, since it is likely that samples provided from different sources may have been subjected to distinct freezing/thawing cycles. For this purpose we obtained samples from 1 of the PD cases shown in Figure 2D (PD1) and performed a different number of freezing/thawing cycles. These samples were used for αSyn-PMCA and, as shown in eFigure 2 in the Supplement, there was no significant influence of this procedure on

the outcome of the reaction. Similar results were obtained even after 6 freezing/thawing cycles.

To investigate whether the seeding activity detected by αSyn-PMCA was indeed associated with αSyn aggregates present in the CSF of patients, we performed immune-depletion experiments using the same conditions as described in Figure 2B. Cerebrospinal fluid samples from 2 patients with PD were subjected to immune-depletion as described in Methods, and the supernatant was used in the αSyn-PMCA reaction. Figure 2E shows the mean result obtained for the 2 samples analyzed, and the data indicate a significant reduction on the aggregation kinetics in immune-depleted samples. As before, although aggregation was not completely blocked, the reduction was such that it clearly proves the concept that removal of αSyn species from the CSF significantly reduces the αSyn-PMCA signal.

Testing the remaining samples from patients and control individuals was done blindly, and the results are illustrated in **Figure 3** as the fluorescence after 314 hours of incubation, when the majority of the samples that aggregate have reached the plateau. In this representation, we defined a cutoff level of 50 fluorescence units to declare samples as positive, since this value corresponds to approximately 3 SDs over the background levels. Of the 76 PD samples tested, 67 (88%) were considered positive for αSyn-PMCA (Figure 3A and B). Conversely, of the 65 unrelated disease controls tested, 61 (94.0%) were correctly scored as negative by αSyn-PMCA. The overall differences between samples from patients with PD and controls was significant ($P < .001$) (Figure 3C). Retesting of the same samples on a different day gave the same number of positive and negative samples, illustrating the reproducibility of the test. Two of the samples provided originally as controls for our Japanese cohort (Figure 3C) were obtained from people who were clinically diagnosed as having PD 1 and 4 years after sample collection. Both of these samples were positive by αSyn-PMCA, indicating that the technology may have the potential to detect disease at the presymptomatic stages. None of the samples detected as negative by αSyn-PMCA in the control group showed conversion into PD in follow-up studies.

To further analyze the specificity of the test, 2 other groups of control samples were tested, including samples collected from patients affected by other neurodegenerative diseases (n = 18) and from people with a clinical diagnosis of AD (n = 14). The results show that only a small proportion (3 of 18 [17%]) of the samples from other neurodegenerative diseases were positive in the αSyn-PMCA assay (eFigure 3A in the Supplement). This result indicates that the assay specifically detects synucleinopathies and not neurodegeneration in general. The number of false-positive results in the AD samples was higher (5 of 14 [36%]) (eFigure 3B in the Supplement), but this is likely owing to the fact that many patients with AD (>50%) also have αSyn abnormalities.[36-38]

To investigate whether αSyn-PMCA may also be useful to detect αSyn aggregates in the CSF of patients affected by other synuclein-aggregation disorders, we tested 10 samples obtained from patients with DLB and 10 from those with MSA. All 10 of the DLB samples and 8 of the MSA samples were positive in αSyn-PMCA testing (eFigure 4 in the Supplement). Over-

Figure 4. Correlation Between α-Synuclein Protein Misfolding Cyclic Amplification (αSyn-PMCA) Values and Disease Severity
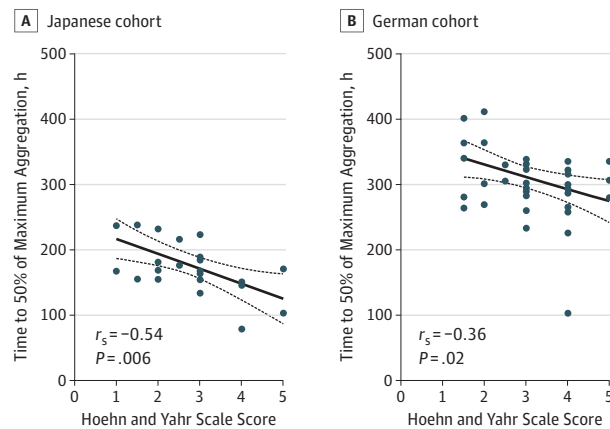
The time to reach 50% aggregation in the αSyn-PMCA assay was plotted as a function of the severity of the disease at the time of sample collection, measured by the Hoehn and Yahr scale in the Japanese (A) and German (B) cohorts. Scores on the scale indicate clinical severity of the patients. Only samples that tested positive in the assay and had clinical information were included in the analysis. Spearman correlation in both studies was significant. Solid line represents the best fit to the data; dotted lines, 95% CIs.

all sensitivity and specificity were calculated by receiver operating characteristic analysis, using the MedCalc software (**Table**). Sensitivity for diagnosis was 88.5% for PD, 100% for DLB, and 80% for MSA. Specificity (counting the 2 presymptomatic samples as positives) was 96.9% and 94.0% for the unrelated disease controls and all controls (ie, unrelated diseases and neurodegenerative diseases), respectively.

Correlation Between Disease Severity and αSyn-PMCA

To study the possibility that αSyn-PMCA may help to measure disease progression, we attempted to correlate the kinetic parameters of the reaction with disease severity in the PD group, measured by the modified Hoehn and Yahr scale.[30,31] As shown in **Figure 4**, both cohorts of patients with PD showed a significant negative correlation between T50 in αSyn-PMCA and the score on the Hoehn and Yahr scale (Japanese cohort: $r_s = -0.54$, $P = .006$ and German cohort: $r_s = -0.36$, $P = .02$). If confirmed in a larger number of patients and individuals serving as controls, the ability of αSyn-PMCA to distinguish PD from controls and monitor progression of PD might be useful in the clinic.

Discussion

Despite much progress to understand the molecular basis of PD, we still lack disease-modifying drugs that can effectively prevent, cure, or even delay the progression of the disease. The lack of more efficient therapeutic interventions for this disease is due in part to the absence of an early and sensitive diagnosis to permit the identification of people on the way to develop the disease before substantial and irreversible brain damage has occurred. A biochemical diagnostic procedure will

also be useful to monitor the progression of the disease and the efficacy as well as target engagement of novel treatments and their potential mechanism of action.[39]

Extensive evidence supports the concept that the hallmark event in PD and other neurodegenerative disorders is the formation and cerebral accumulation of misfolded protein aggregates.[40] This process likely begins years or decades before the onset of clinical disease, indicating that detection of soluble misfolded oligomeric forms of abnormal proteins in biological fluids may provide an early and sensitive strategy to identify people who will develop the disease. Our strategy for efficient and specific detection of soluble misfolded oligomers is to use a platform technology for the cyclic amplification of the process of protein misfolding and aggregation implicated in various diseases.[18] The PMCA technique takes advantage of the biological activity of misfolded aggregates to nucleate further aggregation, enabling very high amplification of the signal. Protein misfolding cyclic amplification was initially developed to detect infectious prions responsible for prion diseases.[17,25] The PMCA technique and some modified versions using shaking to fragment aggregates (real-time quaking-induced conversion),[41] addition of prion-binding compounds,[42,43] or more sophisticated detection techniques (eg, surround optical fiber immunoassay)[44] have been successfully applied to detect prions in biological fluids, such as CSF, blood, and urine of animals and humans, even at the preclinical stage of the disease.[20-24,45-50] The principles behind PMCA have been extended to reach high-efficiency detection of misfolded Aβ oligomers in the CSF of patients affected by AD.[27]

In this study we implemented PMCA for specific and sensitive detection of αSyn aggregates in the CSF of people affected by PD. Blinded studies resulted in 88% sensitivity and 97% specificity for correctly identifying PD from other neurologic disorders. A major advantage of αSyn-PMCA is that the current format of the technology is all-or-none (ie, signal is observed only in the presence of patient samples), offering a much easier interpretation of the results and no overlapping with control samples. Considering that clinical diagnosis of PD is not very accurate and substantially overlaps with the diagnosis of other neurodegenerative disorders,[51] it is possible that the sensitivity and specificity of the test could be even higher if neuropathologically confirmed samples were used. Furthermore, our findings show a positive correlation between disease severity and the kinetic factors of αSyn aggregation, suggesting that αSyn-PMCA might be useful to monitor disease progression and possibly to study the efficacy of therapeutic interventions. Additional studies are needed to fully investigate these applications. Further experiments should also focus on analyzing the possibility of using αSyn-PMCA to detect αSyn aggregates in samples collected at the preclinical stage of the disease. In this sense, an interesting anecdotal finding of this study is the positive detection of αSyn aggregates in 2 samples initially collected as controls from people who subsequently developed clinical symptoms of PD. None of the control samples that were negative by αSyn-PMCA showed conversion into PD or other synuclein-aggregation disorders. If this observation is supported in a larger set of samples, αSyn-PMCA may offer a good opportunity for a much-needed pre-

clinical diagnosis of PD. In future studies we will also attempt to implement the conditions to perform αSyn-PMCA using blood samples to investigate the possible existence of αSyn oligomers in patients' blood. Availability of a blood-based biochemical diagnosis of PD and related synuclein-aggregation disorders will be a major advance in the field.

Our results indicate that αSyn-PMCA specifically detects αSyn aggregates, since a signal was not observed when the reaction was seeded by Aβ or tau aggregates. These data do not necessarily exclude the possibility of cross-seeding; rather, they only show that, at the low concentrations of seeds detectable in CSF, the presence of aggregates other than αSyn does not affect the test. Cross-seeding interaction between αSyn, Aβ, and tau have been reported in various in vitro and in vivo experiments[52-55] and are a plausible explanation for the rather common presence of multiple aggregates in diseased brains.[56,57]

Limitations

This study has some limitations. Samples used for our study are defined by clinical diagnosis and are not pathologically confirmed. Because clinical diagnosis of PD is not 100% accurate, it is possible that some of the samples labeled as PD are not so and also that some of the negative controls might have synuclein aggregates. A future study using pathologically confirmed samples will be able to estimate the true sensitivity and specificity of the test. In its current format, αSyn-PMCA cannot differentiate PD from other synucleinopathies (eg, MSA and DLB) that also accumulate αSyn aggregates. It is likely that αSyn aggregates implicated in distinct synucleinopathies may adopt

different conformations that could be differentiated after amplification. This process is precisely what happens in the prion field, and PMCA has been shown to distinguish the different "prion strains." Another limitation of the assay is that it uses human CSF to detect misfolded αSyn aggregates. Collection of human CSF is considered a moderately invasive procedure and cannot be used as a routine diagnostic test for PD. We are currently attempting to optimize a blood-based αSyn-PMCA assay. Considering that αSyn oligomers could be present in blood plasma in very low quantities compared with CSF, substantial optimization of the technique will be needed.

Conclusions

The findings obtained in the present study further support the idea that PMCA is a platform technology that could be used to detect with high efficiency the presence of misfolded aggregates circulating in the biological fluids of patients affected by neurodegenerative diseases associated with the misfolding and aggregation of proteins. Combining the use of prion protein-PMCA, Aβ-PMCA, and αSyn-PMCA and further implementation of the technology to detect tau and TDP-43 aggregates may provide a complete panel of tests to screen patients affected by neurodegenerative diseases, offer specific diagnosis, and distinguish patients with mixed disease states. The availability of such tests could be a major advance for disease diagnosis and help in the development of patient-specific therapeutic interventions.

Author Contributions: Drs Shahnawaz and Soto had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.
Study concept and design: Shahnawaz, Soto.
Acquisition, analysis, or interpretation of data: All authors.
Drafting of the manuscript: Shahnawaz, Soto.
Critical revision of the manuscript for important intellectual content: All authors.
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