RESEARCH ARTICLE

Detecting Misfolded α-Synuclein in Blood Years before the Diagnosis of Parkinson's Disease

Annika Kluge, MD,[1*] Eva Schaeffer, MD,[1] Josina Bunk, MD,[1] Michael Sommerauer, MD,[2,3] Sinah Röttgen, MSc,[2,3] Claudia Schulte, MSc,[4,5] Benjamin Roeben, MD,[4,5] Anna-Katharina von Thaler, PhD,[1] Julius Welzel, MSc,[1] Ralph Lucius, MD, PhD,[6] Sebastian Heinzel, PhD,[1] Wei Xiang, PhD,[7] Gerhard W. Eschweiler, MD,[8,9] Walter Maetzler, MD,[1] Ulrike Suenkel, MD,[9,10] and Daniela Berg, MD[1]

[1]*Department of Neurology, University Hospital Kiel, Christian-Albrechts-University Kiel, Kiel, Germany*
[2]*Department of Neurology, University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany*
[3]*Cognitive Neuroscience, Institute of Neuroscience and Medicine (INM-3), Research Centre Jülich, Jülich, Germany*
[4]*Department of Neurodegeneration, Hertie Institute for Clinical Brain Research, University of Tübingen, Tübingen, Germany*
[5]*German Center for Neurodegenerative Diseases, University of Tübingen, Tübingen, Germany*
[6]*Institute of Anatomy, Kiel University, Kiel, Germany*
[7]*Department of Molecular Neurology, University Hospital Erlangen, Friedrich-Alexander University Erlangen-Nürnberg, Erlangen, Germany*
[8]*Geriatric Center, University Hospital Tübingen, Tübingen, Germany*
[9]*Department of Psychiatry and Psychotherapy, University Hospital Tübingen, Tübingen, Germany*
[10]*German Center for Mental Health (DZPG), Partner Site Tübingen, Tübingen, Germany*

ABSTRACT: Background: Identifying individuals with Parkinson's disease (PD) already in the prodromal phase of the disease has become a priority objective for opening a window for early disease-modifying therapies.
Objective: The aim was to evaluate a blood-based α-synuclein seed amplification assay (α-syn SAA) as a novel biomarker for diagnosing PD in the prodromal phase.
Methods: In the TREND study (University of Tuebingen) biennial blood samples of n = 1201 individuals with/without increased risk for PD were taken prospectively over 4 to 10 years. We retrospectively analyzed blood samples of 12 participants later diagnosed with PD during the study to detect and amplify pathological α-syn conformers derived from neuronal extracellular vesicles using (1) immunoblot analyses with an antibody against these conformers and (2) an α-syn-SAA. Additionally, blood samples of n = 13 healthy individuals from the TREND cohort and n = 20 individuals with isolated rapid eye movement sleep behavior disorder (iRBD) from the University Hospital Cologne were analyzed.
Results: All individuals with PD showed positive immunoblots and a positive α-syn SAA at the time of diagnosis. Moreover, all PD patients showed a positive α-syn SAA 1 to 10 years before clinical diagnosis. In the iRBD cohort, 30% showed a positive α-syn SAA. All healthy controls had a negative SAA.
Conclusions: We here demonstrate the possibility to detect and amplify pathological α-syn conformers in peripheral blood up to 10 years before the clinical diagnosis of PD in individuals with and without iRBD. The findings of this study indicate that this blood-based α-syn SAA assay has the potential to serve as a diagnostic biomarker for prodromal PD. © 2024 The Authors. *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society.

Key Words: Parkinson's disease; α-synuclein; seed amplification assay; neuron-derived extracellular vesicles; biomarker

*__Correspondence to:__ Dr. Annika Kluge, Department of Neurology, University Hospital Kiel, Christian-Albrechts-University Kiel, Arnold-Heller-Str. 3, 24105 Kiel, Germany; E-mail: annika.kluge@uksh.de

Annika Kluge and Eva Schaeffer have contributed equally to this study.

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The pathological hallmark of Parkinson's disease (PD) are Lewy bodies, which contain misfolded α-synuclein (α-syn) as a major component. It is now well accepted that misfolded α-syn has the capacity to spread throughout the nervous system in a prion-like manner.[1] This has important implications as PD (1) encompasses the whole nervous system, manifesting besides the typical motor symptoms with a plethora of non-motor symptoms, and (2) is characterized by a prodromal phase, lasting years to decades. This prodromal phase of PD is defined by an already-ongoing neurodegeneration, including propagation of misfolded α-syn which, however, has not reached a degree at which the clinical disease defining motor symptoms occurs.[2] Diagnosing PD at this early stage would allow the implementation of disease-modulating therapies with more efficiency than currently possible in the clinical phase of the disease, in which a large proportion of dopaminergic neurons have already degenerated.

α-Syn seed amplification assays (SAAs) have revolutionized the diagnosis of prion diseases and have become increasingly interesting for the understanding of neurodegenerative disorders in which misfolding of proteins plays a major part in pathophysiology.[3,4] Applying the prion hypothesis for α-syn in PD, several studies examined the role of SAAs to detect pathological α-syn conformers as biomarkers in different tissues and body fluids like skin, olfactory mucosa, and cerebrospinal fluid (CSF).[5-30] In research settings, especially studies using skin and CSF have shown a high sensitivity and specificity to detect individuals with (clinical) PD. Moreover, studies in clinical PD are complemented by the finding of positive α-syn SAAs in individuals with isolated rapid eye movement sleep behavior disorder (iRBD) as suspected prodromal PD patients, including a variety of studies using CSF and skin samples and, most recently, using a blood-based SAA.[21,31]

However, a blood-based and thus less-invasive, reliable diagnostic, and prognostic biomarker has not been established for the clinical routine yet. Moreover, the aforementioned studies in prodromal PD included primarily individuals with iRBD, meaning that there is little data on the substantial number of PD patients who develop PD without iRBD as a prodromal symptom. The only study that included individuals with hyposmia as a prodromal marker and measured SAA in the CSF provides only cross-sectional data so far.[27]

Recently, we were able to show that an α-syn SAA amplifying pathological α-syn derived from neuronal extracellular vesicles (NE) in blood can detect clinical PD in 100% correspondence with clinical diagnosis.[31] We here evaluate in a proof-of-concept study the ability of this blood-based α-syn SAA to detect α-syn seeding in the prodromal phase utilizing consecutively collected samples of the longitudinal TREND study. As individuals with fairly unspecific prodromal signs like hyposmia and depression were included in the TREND study, this proof-of-concept study also provides α-syn seeding data of the large subgroup of non-RBD individuals in the prodromal phase. Additionally, we analyzed cross-sectional data of individuals with iRBD from another center (Cologne) for validation.

Patients and Methods

Participants

TREND Cohort

The TREND study enrolled 1201 participants, aged 50 to 80 years, without a diagnosis of clinical PD at baseline, who have been examined by a comprehensive assessment battery, including biennial blood sampling since 2009 (https://www.trend-studie.de/english/). At the time of analysis (spring 2022), 20 individuals had been diagnosed with clinical PD during the course of the study based on clinical examination (Movement Disorder Society diagnostic criteria for PD). For the present analysis, serum samples of 12 of these PD converters with a sufficient amount of blood to perform the SAA before and after clinical conversion were analyzed. Thirteen age- and gender-matched individuals of the TREND study without a diagnosis of clinical PD after at least 9 years of follow-up, a low probability of being in the prodromal phase of PD[32] and sufficient serum samples, were taken as control group (Table 1). Exclusion criteria for all study participants comprised (1) inability to provide written informed consent (ie, Montreal Cognitive Assessment [MoCA] <18 points) and (2) other diseases affecting the central nervous system. The study protocol was approved by the Ethical Committee of the Medical Faculty of the University of Tübingen (no.: 90/2009BO2).

RBD Cohort

Additionally, 20 iRBD patients from the University Hospital Cologne were recruited as validation cohort. These patients were recruited from the general population by newspaper advertisements and a structured screening process with eventually an overnight video polysomnography.[33] Biobanking of various tissues and fluids, including plasma and serum sampling, is being realized in this cohort.[17,34,35] All patients provided written informed consent, and the study was approved by the Ethical Committee of the Medical Faculty of the University of Cologne (19-1644).

Isolation of Extracellular Vesicles and Purification of NEs

Details of materials and techniques are given in the Supporting Information Data S1. NEs were isolated as described earlier except the use of blood serum.[31]

TABLE 1 *Clinical characteristics*

PD converters versus healthy controls			
	PD converters n = 12	**Healthy controls, n = 13**	***P*-value**
Age, mean (SD) (y)	68 (4) at baseline	72 (4) at baseline	0.16
Male gender, n (%)	9 (75)	9 (70)	0.99
Prodromal PD probability, mean (SD) (%)	23.3 (31.8) at baseline	3.5 (6.3) at time point of collection of samples or one visit before	0.004
Prodromal clinical symptoms of PD converters at baseline			
Nonmotor symptoms			
Polysomnographic-proven iRBD	n = 1		
Hyposmia	n = 10		
Mild cognitive impairment	n = 2		
Current depression	n = 0		
Motor symptoms			
MDS-UPDRS-III, mean (SD), points	2 (3)		

Abbreviations: PD, Parkinson's disease; SD, standard deviation; iRBD, isolated rapid eye movement sleep behavior disorder; MDS-UPDRS-III, Movement Disorder Society Unified Parkinson's Disease Rating Scale, Part III.

As described in our previous study, immunoblots and SAA were performed using NEs based on the increased presence of pathological α-syn species in these specific vesicles. The successful precipitation of NEs was confirmed by immunoblotting and transmission electron microscopy (TEM) imaging (Fig. S1).

Dot Blot Analysis

For dot blot analyses, 10 µg of total NE protein was applied in 2.5-µL dots onto nitrocellulose membranes (Amersham Biosciences, 10600001), air dried for 5 h, and blocked in Tris-buffered saline (TBS) with 5% (w/v) nonfat dry milk for 1 h. Primary antibodies (Table S1) were incubated overnight in 1% TBS-Tween containing 5% nonfat dry milk; secondary fluorescent-conjugated antibodies (Table S1) were incubated for 1 h after washing the membranes with 1% TBS-Tween and also in 1% TBS-Tween containing 5% nonfat dry milk. Using the Amersham Typhoon Biomolecular Imager (GE Lifesciences), signals were detected and digitalized. Total protein staining (Direct Blue 71, Sigma-Aldrich, St. Louis, MO, 212407) was used as loading control. Antibody signal intensities were normalized to loading control (total protein).

α-Syn Seed Amplification Assay

The α-syn SAA protocol was performed as described earlier with three adaptions (see Table S2)[1]: this analysis used serum instead of plasma samples. Pre-analyses revealed no significant differences regarding the use of serum versus plasma samples (see Fig. S2C,D).[2] Due to the availability of plates that were used in the previous work, a completely comparable plate was used in this study (Thermo Fisher Scientific, 437111, 96-well black plate, nontreated surfaces, nonsterile, Waltham, MA, USA).[3] For the α-syn SAA, 10 µg total protein of control extracellular vesicles (EVs) and PD-EVs or control-NEs and PD-NEs was incubated with 500 ng (previously: 100 ng) of recombinant monomeric α-syn in a total volume of 100 µL of PBS in a nonsticky dark 96-well plate. Pre-analysis revealed that this increased concentration of recombinant monomeric α-syn results in only one seed amplification round needed to observe typical seeding courses (Fig. S2C,D). The same batch of recombinant α-syn was used for all analyses; moreover, one 96-well plate containing all patient and control samples as well as the positive and negative controls was used.

The plate was covered with silicon lids (Thermo Fisher Scientific, AB0566) and a PARAFILM M sealing film (Bemis, Neenah, WI, USA), incubated at 37°C, and agitated at 1000 rpm using a plate shaker (MTS 4, IKA); 1 µL of thioflavin T (ThT) (Sigma-Aldrich, T3516, 1 mM stock solution, freshly prepared before each measurement) was added before each measurement point. The plate was shaken continuously. ThT fluorescence was monitored over time at an excitation of 410 nm and an emission of 475 nm using a microplate reader (Infinite 200 PRO, Tecan, Männedorf, Schweiz), and measurements were stopped when ThT fluorescence signals plateaued. Recombined prepared α-syn fibrils (10 µL of 0.68 ng/µL) and 500 ng monomeric α-syn were used as reference.

Analyses show total ThT signals. For this purpose, the raw numbers of the plate reader were used and are shown in arbitrary units (AU). Single analyses were performed in the presence of negative and positive controls. No replicate assays were performed due to limited material and based on our observation in previous analyses, in which no significant test–retest variability in absolute ThT values was observed (examples are shown in Fig. S3). ThT signal increased could not be induced by the exosome precipitation reagent and/or used beads (Fig. S4). One single well was evaluated per individual and per time point. α-Syn SAAs were performed blinded to the clinical status.

We calculated the threshold to determine a positive seeding as the average of ThT raw values of control samples during the first 10 h of recording plus 5 standard deviations (SD). The peak of the ThT fluorescence response (F_{max}), the ThT value measured at the end of the SAA (F60), the area under the curve, the time to reach 50% of F_{max} (T50), and the lag phase (time required to reach the threshold) were determined on linear interpolated data using the ThT values per patient as inputs (Table S3). Analyses were performed with custom scripts using a Python 3.10.11 environment. The key Python packages used include numpy version 1.24.3 for numerical computations.

Clinical Assessments

TREND Study

Motor impairment before and after conversion to clinical PD was assessed using the Movement Disorder Society Unified Parkinson's Disease Rating Scale, Part III (MDS-UPDRS-III). Olfactory performance was evaluated using the 12-item Sniffin' Sticks test, mood using the Beck Depression's Inventory (BDI) and Geriatric Depression Scale, and cognitive function using the CERAD plus neuropsychological battery (Consortium to Establish a Registry for Alzheimer's Disease).[36-39] *z*-Scores of the CERAD plus subtests were used to define five cognitive domains (memory, language, executive function, visuospatial, and attention/working memory), which were then applied using thresholds of −1 SD in at least two different domains to define mild cognitive impairment (MCI). MCI forms were subdivided into amnestic MCI (including memory impairment) and non-amnestic MCI (other impairment than memory function). The occurrence of iRBD was analyzed using the Rapid Eye Movement Sleep Behavior Disorder Screening Questionnaire (RBDSQ). One participant was diagnosed with iRBD using polysomnography. The likelihood of an individual to be in the prodromal phase of PD was calculated according to Heinzel et al.[32] For PD converters the prodromal probability score was calculated for the first time point at which serum analysis yielded a positive α-syn SAA. Probability scores for healthy controls were calculated for the one time point blood samples were analyzed, except for 2 healthy controls for whom clinical data were, due to restrictions imposed by COVID-19, available only at the last visit before blood sampling.

iRBD Cohort

All iRBD patients underwent a comprehensive clinical assessment with a battery comparable to the one used in the TREND study, including MDS-UPDRS-III, 12-item Sniffin' Sticks test, BDI, and RBDSQ as well as MoCA for the assessment of cognition. Longitudinal follow-up with yearly visits is currently realized at the Department of Neurology of the University of Cologne.

Statistical Analyses

For statistical analyses of clinical parameters, SPSS 27.0 (SPSS Inc., IBM, USA) was used. Group comparisons were performed using the Fisher's exact test for dichotomous variables and the Mann-Whitney-U-Test for nonparametric/parametric variables. Correlations of clinical data (MDS-UPDRS-III) and signal intensities were performed using 2-tailed Spearman's correlations and considered statistical thresholds Bonferroni corrected for multiple testing.

Results

Demographics

TREND Cohort

In total, serum samples of 12 incident PD patients (mean age at baseline: 68 years, SD: 4 years; men n = 9) were collected at several time points before and at one or two time points after clinical diagnosis. Moreover, samples of 13 control subjects (mean age: 72 years, SD: 4 years; men n = 9) without conversion to PD during a 6- to -10- year follow-up were analyzed at one time point. Age and gender distribution among groups were comparable (age: $P = 0.16$; gender: $P = 0.99$).

RBD Cohort

Mean age of n = 20 iRBD patients was 66 years (SD: 8 years), including n = 18 men. Median disease duration of iRBD was 7 years (2–30 years).

Detection and Amplification of Neuron-Derived α-Syn

Isolation of Serum NEs

NE immunoprecipitation revealed significantly increased signal of NCAM-L1 when compared to serum-derived EVs using Western blot analysis. The commonly used EV markers (CD63, CD9) exhibited strong signals in immunoblot analyses for EVs as well as for NEs (Fig. S1A–C,E, G). Besides the positive EV markers, calnexin as a potential contamination marker was positive for the EV and

NE pool (Fig. S1B, S1G). Immunoblot analyses of NEs further revealed a significant increase in neuronal marker proteins such as neuron-specific enolase and protein gene product 9.5, confirming the neuronal origin of the precipitated NEs (Fig. S1A,B,F). Additionally, α-syn was detected in Western blot analyses within the NE fraction (Fig. S1A,C,D). Further confirmation of the presence of vesicles was reached using TEM analyses (Fig. S1H).

Immunoblot

After the NE enrichment, pathological α-syn forms were visualized by immunoblotting (Fig. 1A,B; Fig. S2B), using the structure-specific α-syn antibody MJFR-14-6-4-2 that was raised against fibrillary α-syn conformers. This antibody exhibited concentration-dependent binding to recombinant, preformed α-syn fibrils (0.625–5 ng protein) and no interaction with monomeric α-syn (Fig. S2A). Immunoblots of NEs of all PD converters (at least once before clinical PD diagnosis) and 1 of the 13 controls revealed an increased antibody signal against pathological α-syn conformers. For the control individual with a positive dot blot, the calculated prodromal PD probability according to the MDS research criteria was 6.6%. The time period from first-time increased antibody signal to clinical PD diagnosis varied between 1 and 10 years (Fig. 1A,B).

α-Syn Seed Amplification Assay

Examples of the capacity of pathological neuron-derived α-syn to seed amyloid protein aggregation using the α-syn SAA can be observed in Figure S2C,D. The formation of amyloid proteins was monitored by an increase in ThT fluorescence. Recombinant α-syn fibrils and monomers were used as references (Fig. S2E). A concentration-dependent increase in ThT signals was observed for in vitro–produced α-syn fibrils (Fig. S2F) and for different concentrations of monomers (Fig. S2G).

All control samples of the TREND cohort exhibited a negative α-syn SAA (Fig. S2H). All individuals with PD exhibited a positive α-syn SAA after clinical conversion, with significantly increased F_{max} values compared to healthy control NEs (PD: $F_{max} = 184.6$ AU, 94.9–217.8, controls: $F_{max} = 2.8$ AU, 1.8–3.7, $P < 0.001$). Regarding the prodromal phase, all PD patients exhibited a positive α-syn SAA before clinical diagnosis (Fig. 1C,D,E; Fig. S5A,B). Time of first positive α-syn SAA to clinical conversion varied between 1 and 10 years. In all individuals F_{max} ThT signal intensities increased over time after the first increase was observed. All detailed SAA kinetic parameters of the PD patients at all measured time points are presented in Table S3; all SAAs can be observed in Figure S5B.

In the analysis of the iRBD cohort, a positive α-syn SAA was observed in 6 of 20 individuals with iRBD (30%; Fig. S6). Median F_{max} values of iRBD individuals with a positive α-syn SAA were 68.1 AU (48.0–84.9 AU). Detailed kinetic parameters of the iRBD patients are presented in Table S4.

Clinical Characteristics of the TREND Cohort

Detailed clinical characteristics of each PD converter can be observed in Figure S5A.

Prodromal Scores

Posttest probability of prodromal PD was below the threshold of 50% for all controls (mean ± SD, range: 3.5 ± 6.3%, 0.05%–21.8%). Among incident PD patients, 5 of 12 (41.7%) had possible and 1 of 12 (8.3%) had probable prodromal PD (>80% posttest probability in the individual with polysomnographic-proven iRBD) at the first time point at which a positive α-syn SAA was detected in the prodromal phase. Probability values of PD converters (23.3 ± 31.8%, 0.28%–95.9%) at baseline were significantly higher compared to controls ($P = 0.004$).

(Mild) Motor Symptoms

Mean MDS-UPDRS-III score after clinical diagnosis of the 12 PD converters was 10 points (SD: 6 points); 11 of 12 patients showed mild motor signs at one or several time points before conversion, with over-time fluctuating MDS-UPDRS-III scores ranging from 1 to 9 points. A total of 45 corresponding MDS-UPDRS-III sum scores and F_{max} values were available. MDS-UPDRS-III scores correlated significantly with F_{max} using all available assessments before and after conversion (rho = 0.719, $P < 0.001$). When correlating MDS-UPDRS-III and F_{max} intensity scores before and after conversion, respectively, a significant correlation after Bonferroni correction could be observed for MDS-UPDRS-III scores/F_{max} only before conversion (before conversion: rho = 0.432, $P = 0.012$; after conversion: rho = 0.614, $P = 0.034$).

Nonmotor Symptoms

(1) Hyposmia: 11 individuals fulfilled criteria for hyposmia at the time of (or directly after) conversion. Of these, 10 had hyposmia at one or more time points before conversion and at baseline. One individual showed no signs of hyposmia throughout the study. In total, 5 individuals presented hyposmia *before* showing a positive SAA. (2) RBD: in the 1 individual with a diagnosis of polysomnographic-proven iRBD at baseline (PD7), a positive SAA was observed 2 years *after* polysomnography-confirmed diagnosis of iRBD (F_{max} at BL: 1.51 AU, F_{max} after 10 years: 47.56 AU) and 8 years before diagnosis of PD. Seven individuals had normal scores of the RBDSQ, and 4 showed fluctuating results over time. (3) Depression: 4 individuals reported a history of lifetime depression, but none of the PD converters showed signs of current

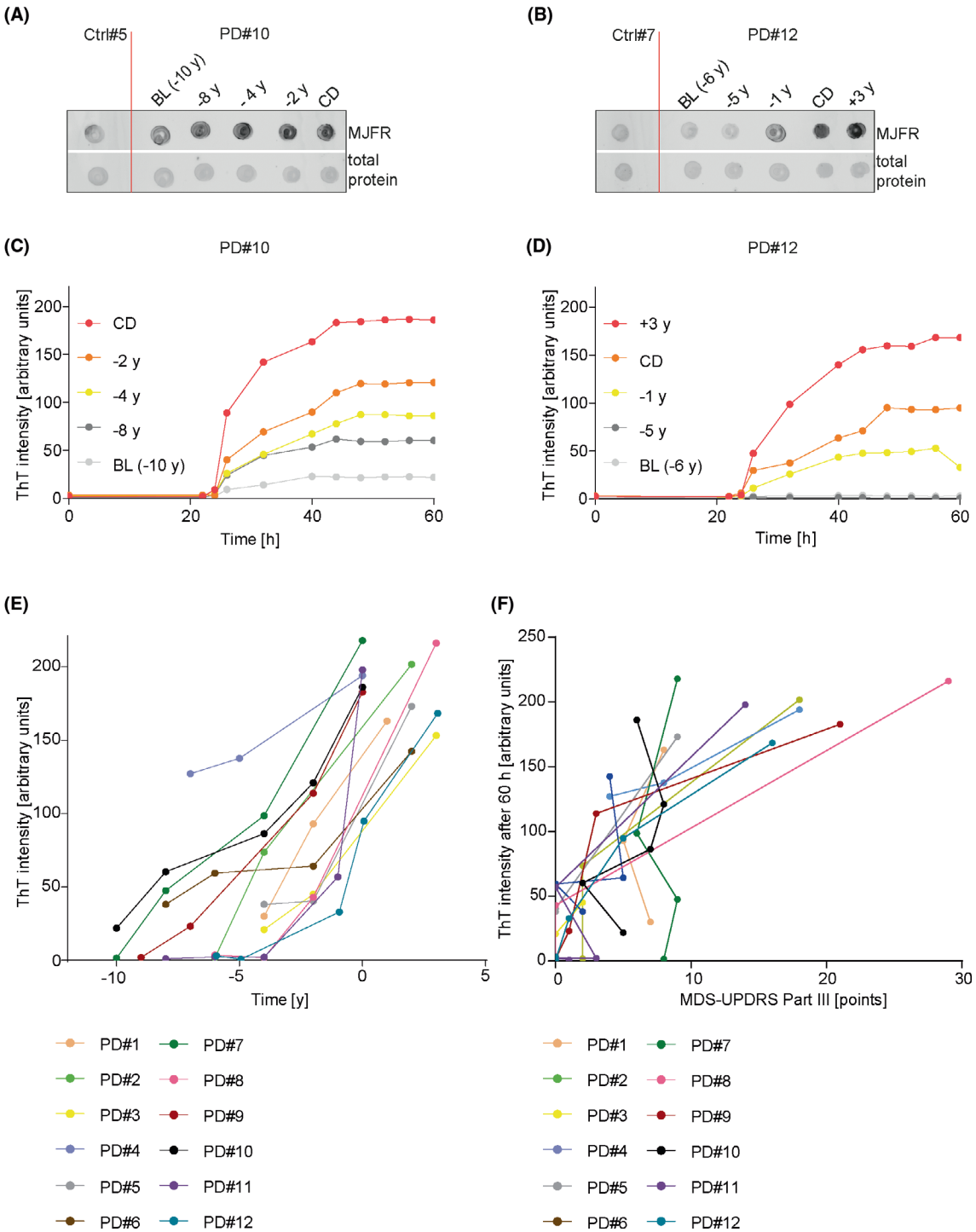

FIG. 1. Detection and amplification of pathological NE (neuronal extracellular)–derived α-syn (α-synuclein) in patients before and after clinical conversion to PD (Parkinson's disease). AU, arbitrary units, BL, baseline, CD, clinical diagnosis, y, years. Representative (**A, B**) MJFR-14-6-4-2-antibody dot blots and (**C, D**) α-Syn seed amplification assays before and after diagnosis of Parkinson's disease. (**E**) Endpoint ThT (Thioflavin T) values (after 60 h). (**F**) Overview of all ThT endpoints in AU and corresponding MDS-UPDRS-III (Movement Disorder Society Unified Parkinson's Disease Rating Scale, Part III) scores in points of all 12 PD patients analyzed at different time points. Different colors represent different patients. MDS-UPDRS-III showed a significant correlation with ThT signal intensity before and after conversion. [Color figure can be viewed at wileyonlinelibrary.com]

depression throughout the study. Three of these 4 individuals showed a positive SAA already at baseline (ThT signal ranging from 21.02 to 127.09 AU). (4) MCI: 8 individuals showed no signs of amnestic or nonamnestic cognitive impairment during the course of the study. One individual with nonamnestic MCI was detected at follow-up; in the remaining 3 individuals fluctuating results over time were assessed, with no clear development of amnestic

or nonamnestic cognitive impairment throughout the observational period. Only 1 individual showed signs of MCI (nonamnestic) *before* showing a positive SAA. Correlation analyses of all individual cognitive domains with F_{max} values (total of n = 45 available corresponding data) revealed no significant correlations between cognitive function and F_{max} intensities (for all assessments—attention domain: rho = 0.014, $P = 0.93$; memory domain: rho = 0.071, $P = 0.64$; language domain: rho = −0.031, $P = 0.84$; executive domain: rho = −0.060, $P = 0.69$; and visuospatial domain: rho = 0.003, $P = 0.99$).

Clinical Trajectories after Conversion

An overview of the development of clinical symptoms after conversion can be observed in Figure S5. All individuals diagnosed with PD showed a clear asymmetry of motor symptoms (six left sided, seven right sided). In 7 of these individuals, DAT-SPECT imaging was performed and showed PD-typical reduced dopamine transporter binding. At the time of conversion, 7 individuals were considered to have an akinetic-rigid subtype, 2 were rated as tremor-dominant, and 3 were rated as equivalent type. For 4 individuals, no follow-up data (recent diagnosis) were available at the time of analysis; for the remaining 8 individuals, follow-up data ranged from 2 to 5 years; all stayed with the diagnosis of sporadic PD (no signs of other synucleinopathies). None of them showed (mild) cognitive impairment in the CERAD assessment available after conversion to clinical PD.

Clinical Characteristics of the iRBD Cohort

Comparing iRBD individuals with a positive versus negative SAA, no significant statistical differences were observed for age, gender, or disease duration (age—positive SAA: 69 ± 8 years, negative SAA: 65 ± 7 years, $P = 0.35$; male sex—positive SAA: 6, 100%, negative SAA: 12, 86%, $P = 0.56$; disease duration—positive SAA: 14, 4–30 years, negative SAA: 6, 2–16 years, $P = 0.29$). Additionally, no significant differences in the MDS probability scores (positive SAA: 82.7 ± 38.9%, negative SAA: 67.2 ± 33.6%, $P = 0.38$) were observed. Of 20 iRBD individuals, DaTscan imaging was performed in 11, with 5 of 11 having a positive test result (reduced striatal DaT binding). DaTscan results were comparable between individuals with a positive and negative SAA (positive SAA: 1 of 3, 33.3% with positive DaTscan; negative SAA: 4 of 8, 50% with positive DaTscan).

(Mild) Motor Symptoms

Of all individuals of the iRBD cohort, n = 11 showed mild motor symptoms at the time of blood collection. Comparing iRBD individuals with a positive versus negative SAA, no significant differences in MDS-UPDRS-III scores were observed (positive SAA: 5, 2–20 points; negative SAA: 5, 0–13 points, $P = 0.60$). No significant correlation between MDS-UPDRS-III scores and F_{max} was observed when all iRBD individuals were included (rho = 0.199, $P = 0.40$). In the subgroup of SAA-positive iRBD individuals, MDS-UPDRS-III scores correlated significantly with F_{max} (rho = 0.928, $P = 0.008$).

Nonmotor Symptoms

Individuals with iRBD and a positive SAA showed no differences in hyposmia or cognition when compared with individuals with a negative SAA (positive SAA: 3 hyposmic, 50%, negative SAA: 8 hyposmic, 57%, $P = 0.99$; MOCA score positive SAA: 28, 23–30 points, negative SAA: 28, 26–30 points, $P = 0.90$).

Discussion

This proof-of-concept study suggests that it is possible to detect pathological α-syn conformers and increasing seeding activity in NEs derived from blood up to 10 years before the clinical diagnosis of PD. Our findings show the high potential of this NE-derived α-syn SAA as a new prodromal biomarker.

Currently, no established blood-based test exists to define the diagnosis of PD neither in early or prodromal disease stages nor after clinical conversion to PD. Several studies focused on the measurement of total α-syn concentrations in blood serum or plasma, but initially inconsistent findings influenced by, for example, α-syn from red blood cells have been described.[40-45] In recent studies measurement of total α-syn in NEs (L1CAM/NCAM-L1-positive EVs) extracted from blood showed high potential to distinguish clinical PD patients[46-49] and most recently iRBD patients from healthy controls.[50] However, these measurements of total α-syn levels cannot differentiate between physiological and pathological forms of α-syn. Additionally, Okuzumi and colleagues were recently able to detect *pathological* α-syn seeds in serum samples of individuals with synucleinopathies using a combination of SAA and immunoprecipitation. They observed a positive seeding in 259 of 275 clinical PD patients and 4 of 9 individuals with iRBD.[21] In the present study, we combined both principles, the extraction of EV/NEs and the SAA technique, to detect pathological α-syn conformers in the blood and could confirm a high sensitivity in clinical PD,[31] showing a positive α-syn seeding in all PD patients after clinical conversion and in none of the analyzed healthy controls.

Moreover, this proof-of-concept study widens the use of this blood-based biomarker to the prodromal phase of PD. Apart from the aforementioned blood-based studies, a variety of tissue- and CSF-based studies addressed the

detection of α-syn biomarkers in the prodromal stage of α-synucleinopathies, focusing mainly on individuals with iRBD. Iranzo and colleagues demonstrated that 90% (47 of 52) of iRBD patients showed positive α-syn seeding in a CSF-derived SAA.[15] During follow-up, 62% of the iRBD patients were diagnosed with PD or dementia with Lewy bodies, of whom 97% had been positive in the α-syn SAA at baseline. Moreover, Siderowf and colleagues recently showed in CSF samples of the Parkinson's Progression Markers Initiative cohort that 86% of the individuals with iRBD (28 of 33) or hyposmia (16 of 18) had positive α-syn seeding activity at baseline.[27] However, longitudinal data have not yet been reported. In addition, cross-sectional studies on tissue SAAs revealed positive seeding for 44% (28 of 63) of iRBD patients when analyzing olfactory mucosa and up to 97% (37 of 38) in skin samples.[17,29,51]

In contrast to these studies, the present analysis stands out as it uses an easy-to-obtain, blood-derived α-syn biomarker. In our longitudinal approach, we could find positive α-syn seeding in all prodromal individuals before conversion to PD, with a positive SAA between 1 and 10 years before conversion in a cohort recruited from the general population with different prodromal features. Additionally, similar to the findings of Okuzumi and colleagues (44% positive seeding in iRBD patients), 30% of the iRBD patient cohort showed a positive SAA in a cross-sectional analysis. In this respect it is important to discuss why CSF or skin SAAs for iRBD show considerably higher rates of seeding positive individuals, when compared to the blood assays. Okuzumi and colleagues already postulated that longer periods of α-syn deposition might be important to detect seeding in the blood, as they found a strong positive correlation between disease duration and forming rate of α-syn seeds in clinical PD.[21] Although we could not find a significant difference in iRBD disease duration between individuals with and without a positive SAA, we did find a trend (median of 14 years of disease duration in SAA-positive iRBD patients, median of 6 years in SAA-negative iRBD patients). Moreover, it has been postulated that iRBD patients represent a specific prodromal ("body-first") subtype, in which α-syn pathology spreads from the periphery to the brain.[52] It might be possible that seeding derived from neuronal vesicles in blood occurs later due to a still less-extended pathology in the nigrostriatal system, whereas other parts of the body, such as the skin, already show higher concentrations of pathological α-syn conformers. Taken together, further longitudinal studies are urgently needed to elucidate whether a positive seeding in the blood may be associated with a closer conversion to clinical PD, and in which chronological sequence different biofluids and tissues becomes involved.

The blood assessments presented here followed a two-step analysis technique. First, an immunoblot of the soluble NE fraction under native conditions was employed using the conformation-specific α-syn MJFR-14-6-4-2-antibody raised against oligomeric and fibrillary α-syn conformers. Qualitatively increased signal intensities of the MJFR-14-6-4-2-antibody indicating the presence of pathological α-syn forms were observed in all PD patients after clinical conversion and in only 1 of the control samples. In prodromal PD samples, MJFR-14-6-4-2-antibody signal intensities were detected between 1 and 10 years before conversion. Second, an α-syn SAA protocol was applied to form amyloid α-syn conformers, using the soluble protein fractions of NEs; 1 to 10 years before clinical diagnosis, a positive seeding could be observed in all individuals later diagnosed with PD. Moreover, this study is the first to suggest that this blood-based α-syn SAA might add a quantitative value, as seeding activity (measured by F_{max}) continuously increased during the prodromal phase until clinical conversion. In contrast, all healthy controls showed a negative SAA. The observation that 1 individual from the control cohort had a positive MJFR-14-6-4-2-antibody signal in the immunoblot and no ThT increase in the α-syn amplification protocol was visible indicates less specificity of the immunoblot assay compared to the α-syn SAA.

The analysis of clinical data on individuals from the TREND study showed that the α-syn seeding activity in PD patients correlated with motor impairment as assessed by the MDS-UPDRS-III. However, the SAA was superior to the motor assessment in detecting disease progression in the prodromal phase, as it demonstrated increasing seeding activities over the years, whereas MDS-UPDRS-III assessment showed fluctuating signs of mild motor impairment in the prodromal phase. Still, most interestingly, some individuals showed non-motor symptoms (hyposmia or iRBD) before an increase in seeding activity could be detected. In the cross-sectional analysis of the iRBD cohort, no differences regarding mild motor or non-motor symptom manifestation could be observed in individuals with a positive versus negative SAA. In the small group of individuals with positive SAA, the MDS-UPDRS-III scores correlated significantly with F_{max}. However, again, the cross-sectional character of this cohort needs to be acknowledged. A longitudinal follow-up until the development of motor PD (in larger cohorts) will be necessary to give a more reliable statement regarding the manifestation of motor- and non-motor symptoms and their correlation to the SAA.

This study has several limitations. Thus far, we can only hypothesize that the presented parameters of a blood-based SAA might add a quantitative value by showing increasing ThT signal intensities over time. Additional studies will need to advocate or discard this hypothesis, considering among others different structures or strains of α-syn. Moreover, further biochemical analyses are needed to determine the remaining unclear aspects of the EV/NE extraction. This includes (1) the

confirmation of the localization of α-syn conformers (outside or inside of EVs); (2) analyses to clarify the purity of EV preparations (as calnexin has also been discussed as a contamination marker); and (3) the confirmation of NCAM-L1 as a specific surface marker of NEs, as this has been a matter of discussion.[53] Although we could previously confirm the extraction of NEs using immunoblotting, TEM, and dynamic light scattering,[31] further analyses are warranted to confirm the extraction of NEs using this protocol. Finally, it should be mentioned that a possible contamination with free-circulating aggregates cannot be completely excluded (detection of the potential contamination marker calnexin in Western blot). Nevertheless, the procedure used here included the NCAM-L1 precipitation as it is commonly used,[46,50] and neuronal proteins were enriched after NE isolation.

Moreover, according to strict clinical criteria and the necessity of sufficient blood (at least 2 mL for the extraction of NEs is necessary), only 12 patients and 13 controls of the extensively characterized TREND cohort could be included. Therefore, the SAA was performed in only one replicate. However, it has to be acknowledged that to find 20 clinical converters to PD (which does not include only individuals with iRBD), 1201 individuals were recruited from the general population and followed up for more than 10 years within the TREND study, resulting in the limited blood material of 12 clinical converters presented here. These circumstances illustrate the high value and rarity of this kind of longitudinally collected blood samples. Of course, the high correspondence with clinical diagnosis of the α-syn SAA in prodromal PD needs to be confirmed in larger cohorts (including specific longitudinally assessed iRBD cohorts), and its specificity compared to other α-synucleinopathies needs to be determined. Regarding the iRBD cohort, it is striking that the range of MDS-UPDRS-III scores goes up to 20 points; however, none of these individuals showed a detectable combination of bradykinesia and rigor or resting tremor defining the clinical stage of PD. Additionally, it has to be acknowledged that the cohort comprised only individuals later diagnosed with (sporadic) PD with limited follow-up data up to 5 years. Thus far, there were no clinical signs that these individuals suffered from other α-synucleinopathies. However, in the future it would be of particular interest to compare changes in α-syn aggregation measured using SAA during the prodromal and clinical phases of different α-synucleinopathies. Finally, the sophisticated analyses used in this proof-of-concept study need to be optimized before this approach can be rolled out to larger populations and potentially in the long run to clinical care.

Conclusion

This study is the first to show that α-syn seeding activity in blood can be detected up to 10 years before the clinical diagnosis of PD in longitudinally followed individuals. Due to the still limited number of blood samples analyzed in this proof-of-concept study, these results should be considered as preliminary. However, we believe that this blood-based SAA holds great potential as a future diagnostic biomarker for the prodromal phase of PD, paving the way for early intervention approaches. A future concept for clinical studies as well as clinical care might include a screening for prodromal symptoms such as hyposmia or iRBD, followed by α-syn biomarker assessment as described here.

Further prospective studies in larger prodromal PD cohorts are necessary to confirm sensitivity, to compare seeding activity in potential subtypes of (prodromal) PD, and to further elucidate correlations with prodromal or risk markers as well as the development of other α-synucleinopathies. ■

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Data Availability Statement

Data are not available on request due to privacy/ethical restrictions.

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Supporting Data

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(1) Research project: A. Conception, B. Organization, C. Execution; (2) Statistical Analysis: A. Design, B. Execution, C. Review and Critique; 3. Manuscript: A. Writing of the first draft, B. Review and Critique. A.K.: 1A, 1B, 1C, 2C, 3A, 3B. E.S.: 1A, 1B, 2A, 2B, 2C, 3A, 3B. J.B.: 1C, 3B. M.S.: 1A, 1B, 1C, 3B. S.R.: 1A, 1B, 1C, 3B. C.S.: 1B, 3B. B.R.: 1C, 3B. A.T.: 1B, 1C, 3B. J.W.: 2A, 2B, 2C, 3B. R.L.: 1B, 3B. S.H.: 1B, 2C, 3B. W.X.: 1C, 3B. G.W.E. 1B, 3B. W.M.: 1B, 3B. U.S.: 1A, 1B, 1C, 3B. D.B. 1A, 1B, 3B.

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