RESEARCH ARTICLE

α-Synuclein in Plasma-Derived Extracellular Vesicles Is a Potential Biomarker of Parkinson's Disease

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ABSTRACT: Background: Extracellular vesicles are small vesicles that are released from many cells, including neurons. α-Synuclein has recently been described in extracellular vesicles derived from the central nervous system and may contribute to the spreading of disease pathology in α-synuclein-related neurodegeneration.
Objectives: We aimed to examine the potential diagnostic value of α-synuclein in plasma extracellular vesicles from patients with Parkinson's disease (PD).
Methods: Preanalytical variables were studied to establish an optimized assay for preparation of plasma extracellular vesicles and detection of extracellular vesicle–derived α-synuclein. Plasma samples were obtained from 2 independent cohorts. The Tübingen cohort contained 96 patients with PD, 50 patients with dementia with Lewy bodies, 50 patients with progressive supranuclear palsy (PSP), and 42 healthy controls; the Kassel cohort included 47 patients with PD, 43 patients with dementia with Lewy bodies, and 36 controls with secondary parkinsonian syndromes. Extracellular vesicles were prepared from total plasma by size exclusion chromatography and quantified by nanoparticle tracking analysis, α-synuclein content was measured by an electrochemiluminescence assay.
Results: α-Synuclein concentration in plasma extracellular vesicles provided the best discrimination between PD, dementia with Lewy bodies, PSP, and healthy controls, with an area under the curve of 0.804 (PD vs dementia with Lewy bodies), 0.815 (PD vs. PSP), and 0.769 (PD vs healthy controls) in the Tübingen cohort. Results were validated in the Kassel cohort.
Conclusions: The concentration of α-synuclein in plasma extracellular vesicles may serve as a potential diagnostic biomarker for PD. © 2021 The Authors. *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society

Key Words: biomarker; plasma; extracellular vesicles; α-synuclein; Parkinson's disease

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Parkinson's disease (PD) and dementia with Lewy bodies (DLB) are neurodegenerative diseases characterized by pathological aggregation of α-synuclein in intracellular deposits termed Lewy bodies.[1] In contrast to PD and DLB, the neuropathological hallmark of progressive supranuclear palsy (PSP) is intracellular aggregates of microtubule-associated protein tau.[2] Because of overlap of symptoms and lack of disease-specific biomarkers, the differential diagnosis between PD and the atypical parkinsonian disorders DLB and PSP can be difficult, as reflected by high rates of misdiagnosis, especially in early disease stages.[3-5] So far, diagnostic biomarkers in PD include structural and functional imaging and, on a still-experimental level, the analysis of cerebrospinal fluid (CSF) α-synuclein, which is decreased in PD.[6] Less invasive biomarkers are preferable to CSF markers, and several studies have evaluated the diagnostic value of α-Synuclein in saliva and blood,[7-10] but data on the diagnostic potential of blood α-synuclein have been conflicting so far.[11-14] Biomarkers should ideally reflect major aspects of the disease's underlying pathology. In PD, α-synuclein pathology spreads between interconnected neurons by mechanisms that are still not fully understood.[15] One possible route is the transfer of α-synuclein with extracellular vesicles (EVs).[16,17] EVs are small vesicles 40–180 nm in diameter that are released by many cell types and serve various functions, including clearance of superfluous or toxic cellular content and cell–cell communication.[18,19] Based on their biogenesis, EVs are termed exosomes when derived from the endosomal system and microvesicles when secreted by direct budding from the plasma membrane. α-Synuclein is released together with EVs in vitro and in vivo.[20-22] Although EV-associated α-synuclein only accounts for a minor proportion of total extracellular α-synuclein, CSF EVs from patients with PD or DLB contain seeding-competent α-synuclein that can induce aggregation of soluble α-synuclein in target cells.[23] EVs with neuronal origin have been isolated from blood by immunocapture with an antibody against the neuronal protein L1CAM.[24] L1CAM EVs contained α-synuclein and distinguished PD from healthy controls with a sensitivity of 71.2%, a specificity of 50.0%, and an area under the curve (AUC) of 0.66,[24] and similar results have been reported by other groups.[25,26]

In this study, we explored the diagnostic potential of plasma EV-associated α-synuclein without prior enrichment for brain-derived EVs for the differential diagnosis of PD versus DLB as another α-synuclein-related neurodegenerative disease, PSP as a non-α-synuclein-related atypical parkinsonian disease, healthy controls (HCs), and neurological controls (NCs).

Materials and Methods

Cohorts

All samples were obtained in accordance with the ethical standards of the 1964 Declaration of Helsinki.

Patients with PD were diagnosed according to UK Brain Bank criteria, patients with DLB fulfilled McKeith consensus criteria,[27] and patients with PSP fulfilled the National Institute of Neurological Disorders and Stroke–Society for Progressive Supranuclear Palsy criteria for possible or probable disease.[28]

The Cross-Sectional Tübingen Cohort

Institutional review board (IRB) approval was obtained by the local ethical board of the Medical Faculty, University of Tübingen, Germany, IRB 390/2013BO2 and 258/2016BO2. Ethylenediaminetetraacetic acid (EDTA) plasma was obtained and processed according to a standardized operation protocol.[29] In brief, plasma was centrifuged for 10 minutes at 4°C at 2000g and aliquoted and stored at −80°C within 60 minutes after collection. Healthy controls included mostly spouses of PD patients taking part in observational studies. None of the neurological control patients suffered from Parkinson's disease or dementia (Table 1).

The Cross-Sectional Kassel Cohort

Institutional review board approval was obtained by the local board of Hessen, Germany, IRB 09/07/04 and 26/07/02. A detailed description of the cohort is available in Mollenhauer et al[6] and the Supporting Information. Plasma was collected into EDTA plasma tubes, centrifuged at 20°C for 10 minutes (2500g), and stored within 30 minutes at −80°C. Neurological controls included mostly patients with secondary parkinsonian syndromes. None of the neurological control patients suffered from Parkinson's disease or dementia (Table 2).

Purification of Extracellular Vesicles from Plasma

Plasma samples (500 μL) were thawed on ice and processed according to a serial centrifugation protocol established to preclear fluids from cell debris and extracellular vesicles in a size range of 1 μm. To this end, plasma was centrifuged at 4°C and 3500g, 2 times at 4500g (10 minutes each) and 1 time at 4°C and 10,000g for 30 minutes. The pellet (microvesicle fraction) was washed with 1 mL of 20 mM HEPES buffer (pH 7.4) at 4°C at 10,000g for 30 minutes and dissolved in 100 μL of 1× denaturing sample buffer for Western blotting. The supernatant was subsequently applied to a size exclusion column (qEVoriginal, 70 nm+; Izon Science Limited, Cambridge, MA) that had been equilibrated with 10 mL of 20 mM HEPES buffer (pH 7.4) following a modified protocol of Boing et al.[30] The sample was eluted with 20 mM HEPES buffer (pH 7.4) into 24 fractions of a 500-μL volume. The concentration of particles was measured by nanoparticle tracking analysis. The α-synuclein content of each fraction was determined by electrochemiluminescence

TABLE 1 *Sociodemographic and clinical characteristics of the Tübingen cohort (mean ± SD): whole group, n = 238; PD group, n = 96; DLB group, n = 50; PSP group, n = 50; HC group, n = 42*

	PD	DLB	PSP	HC	P
Age (years)	65.0 ± 11.8	70.8 ± 6.5	69.4 ± 5.7	61.6 ± 14.1	$P < 0.0001$
Sex (%)					
Male	53.7	68	44	45	$P = 0.0670$
Female	46.3	32	66	55.5	
Disease duration (years)	7.6 ± 5.6	2.6 ± 2.0	3.6 ± 3.0	nd	$P < 0.0001$
H&Y score	2.2 ± 0.7	nd	nd	nd	
MMSE	27.8 ± 2.5	18.2 ± 5.7	nd	nd	$P < 0.0001$
Plasma α-synuclein	8239 ± 523.9	8006 ± 778.7	9368 ± 957.6	6780 ± 607.3	ns
EV α-synuclein (pg)	12.660 ± 0.828	10.630 ± 1.152	8.244 ± 0.643	8.083 ± 0.540	$P < 0.0001$
Particles (/mL)	$3.24 \times 10^{10} \pm 2.29 \times 10^{9}$	$7.62 \times 10^{10} \pm 6.57 \times 10^{9}$	$6.20 \times 10^{10} \pm 5.96 \times 10^{9}$	$6.44 \times 10^{10} \pm 5.49 \times 10^{9}$	$P < 0.0001$
EV α-synuclein/ particles (pg/particle)	$6.10 \times 10^{-10} \pm 6.58 \times 10^{-11}$	$2.35 \times 10^{-10} \pm 4.68 \times 10^{-11}$	$2.13 \times 10^{-10} \pm 4.11 \times 10^{-11}$	$2.24 \times 10^{-10} \pm 2.92 \times 10^{-11}$	$P < 0.0001$
n	96	50	50	50	

nd, Not determined. Age, 1-way ANOVA, post hoc Tukey's multiple comparison test: PD vs HC ns, DLB vs HC, $P = 0.0002$; PSP vs HC, $P = 0.0002$; other comparisons not significant. Sex: post hoc Tukey's multiple comparison test. Disease duration: 1-way ANOVA, post hoc Tukey's multiple comparison test; PD vs DLB, $P < 0.0001$; PD vs PSP, $P = 0.0001$; other comparisons, not significant. MMSE: 2-tailed t test. All data given as mean ± SD. Plasma α-synuclein: mean ± SEM, Welch's ANOVA test, not significant. EV α-synuclein: normalized to 1 mL of plasma and given as mean ± SEM, Welch's ANOVA (PD vs PSP, $***P < 0.0003$; PD vs HC, $****P < 0.0001$; Dunnett's T3 multiple-comparisons test). Number of EVs in the plasma EV fraction: normalized to 1 mL of plasma and given as mean ± SEM, Welch's ANOVA (PD vs DLB, $****P < 0.0001$; PD vs PSP, $****P < 0.0001$; PD vs HC, $****P < 0.0001$; Dunnett's T3 multiple comparison test). Plasma EV α-synuclein concentration (pg/particle), mean ± SEM, Welch's ANOVA (PD vs DLB, $****P < 0.0001$; PD vs PSP, $****P < 0.0001$; PD vs HC, $****P < 0.0001$; Dunnett's T3 multiple-comparisons test, $****P < 0.0001$).

TABLE 2 *Sociodemographic and clinical characteristics of the Kassel cohort (mean ± SD): whole group, n = 126; PD group, n = 47l DLB group, n = 43; parkinsonian syndrome group, n = 36*

	PD	DLB	Parkinsonian syndrome	
Age (years)	68.0 ± 10.3	70.6 ± 6.4	70.0 ± 7.2	$P = 0.664$
Sex (%)				
Male	60.6	62.8	72.2	$P = 0.386$
Female	39.4	37.2	27.8	
Disease duration (years)	5.9 ± 5.6	3.0 ± 1.8	4.5 ± 3.7	$P = 0.014$
H&Y score	3.3 ± 1.0	3.0 ± 0.7	3.4 ± 1.0	$P = 0.003$
MMSE	23.4 ± 5.5	20.3 ± 6.2	23.1 ± 6.9	$P = 0.047$
Plasma α-synuclein	4601 ± 461.2	5720 ± 670.7	4517 ± 850.0	ns
EV α-synuclein (pg)	8.1 ± 0.874	7.1 ± 0.437	8.2 ± 0.802	ns
Particles (/mL)	$1.90 \times 10^{10} \pm 2.64 \times 10^{9}$	$3.47 \times 10^{10} \pm 3.03 \times 10^{9}$	$2.57 \times 10^{10} \pm 2.71 \times 10^{9}$	$P < 0.001$
EV α-synuclein/ particles (pg/ particle)	$9.49 \times 10^{-10} \pm 1.99 \times 10^{-10}$	$2.89 \times 10^{-10} \pm 3.67 \times 10^{-11}$	$4.08 \times 10^{-10} \pm 5.44 \times 10^{-11}$	$P = 0.0003$
n	47	43	36	

Disease duration: post hoc Tukey's multiple-comparisons test: PD vs DLB, $P = 0.019$. Other comparisons not significant. H&Y score: PD vs DLB, $P = 0.005$; DLB vs parkinsonian syndrome, $P = 0.003$; other comparisons not significant. Diagnoses of patients included PSP (n = 13), possible PSP (n = 6), secondary parkinsonian syndrome (n = 5), parkinsonian syndrome induced by neuroleptics (n = 1), vascular parkinsonian syndrome (n = 4), hypokinetic syndrome of unknown etiology (n = 3), atypical parkinsonian syndrome not further specified (n = 2). One-way ANOVA, mean ± SD. Plasma α-synuclein: mean ± SEM, Welch's ANOVA, not significant. EV α-synuclein: normalized to 1 mL of plasma volume, mean ± SEM, Welch's ANOVA test, not significant. Number of particles in the plasma EV preparation: normalized to 1 mL of plasma; mean ± SEM, Welch's ANOVA (PD vs DLB, ★★★$P = 0.0006$; PD vs parkinsonian syndrome ns, $P = 0.22$; DLB vs parkinsonian syndrome ns, $P = 0.09$; Dunnett's T3 multiple-comparisons test). Plasma EV α-synuclein per particles: mean ± SEM, Welch's ANOVA (PD vs DLB, ★★$P = 0.006$; PD vs parkinsonian syndrome, ★$P = 0.033$; DLB vs. parkinsonian syndrome ns, $P = 0.21$; Dunnett's T3 multiple-comparison test).

analysis (Fig. 1A). Fractions 7–10 containing the highest concentrations of EVs and α-synuclein were pooled. A volume of 4 μL was removed for nanoparticle tracking analysis prior to concentrating pooled fractions 7–10 to 90 μL by centrifugation at 4000g at 4°C in an Amicon Ultra centrifugal filter with a 3-kD cutoff (Merck Millipore, Darmstadt, Germany). Ten microliters of 10% CHAPS lysis buffer (in 20 mM HEPES) was added, and samples were stored at −20°C until α-synuclein quantification.

Nanoparticle Tracking Analysis

EV concentration was analyzed from 4 μL of pooled fractions 7–10 diluted 1:125 in 0.025% HEPES-Tween using a NanoSight NS500 instrument equipped with a 532-nm laser (NanoSight; Malvern Instruments Ltd, Malvern, UK). For additional details, please refer to the Supporting Information.

Electrochemiluminescence Assay for α-Synuclein Quantification

Quantification of α-synuclein in cell- and plasma-derived EVs was performed as described before[22,23,31] and in the Supporting Information.

Data and Statistical Analysis

Data were measured by an experimenter blinded to the diagnosis. Statistical analysis was performed using GraphPad Prism 8 asoftware. Group differences were analyzed by Welch's analysis of variance (ANOVA) test, followed by Dunnett's T3 post hoc test for multiple comparisons or by 1-way ANOVA and post hoc Tukey's test. Correlation analysis was performed using Pearson's correlation. Receiver operating characteristic (ROC) curves were used to evaluate sensitivity and specificity relationships to determine the diagnostic performance of the diagnostic tests. Analysis of covariance (ANCOVA) analysis was performed using SPSS version 23.

Additional methods are described in the Supporting Information.

Results

Purification of Plasma EVs

We first established a protocol for purification of plasma EVs and subsequent quantification of EV-associated α-synuclein. Ultracentrifugation (UC) protocols bear several disadvantages such as cosedimentation of lipoprotein particles and immune complexes together with plasma EVs. In addition, UC is not readily
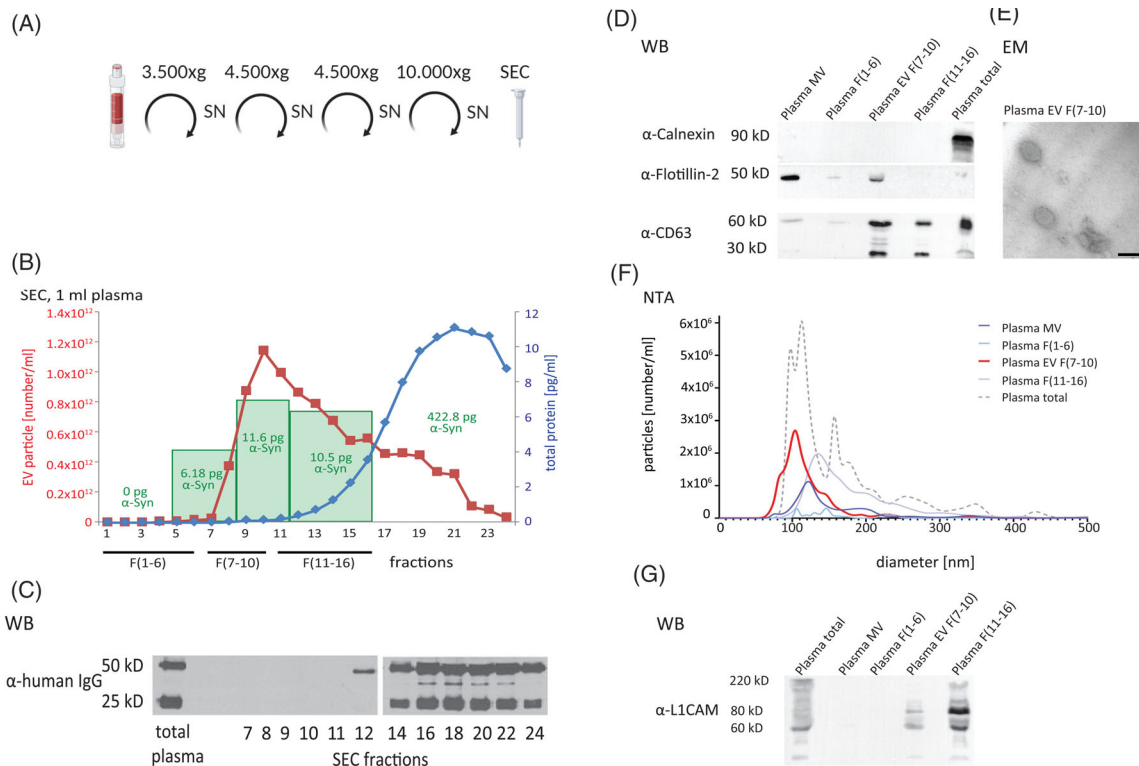
FIG. 1. Purification of plasma EVs. (**A**) Schematic overview on serial centrifugation of plasma to remove cellular debris and vesicles in the size range of ∼1 μm prior to loading the precleared plasma on a size exclusion column (SEC). (**B**) Eluate was collected in 24 fractions. Protein (blue) and particle (red) concentrations were measured in each fraction. α-Synuclein content (green) was determined in pooled fractions. (**C**) Western blot analysis of plasma and selected SEC fractions probed with an antibody against human IgG. (**D**) Western blot analysis of (lanes from left to right) plasma microvesicles (MVs), plasma, pooled SEC fractions 1–6, 7–10, and 11–16 with antibodies directed against the EV marker proteins flotillin-2 and CD63 as well as the ER marker calnexin to rule out microsomal contamination of the preparation. (**E**) Electron microscopy image of plasma EVs visualized using negative staining. Scale bar: 200 nm. (**F**) Nanoparticle tracking analysis (NTA) quantification showing size distribution and particle concentration of different plasma fractions: microvesicles (MVs), pooled SEC fractions 1–6, 7–10, 11–16, and total plasma. (**G**) Western blot analysis (lanes from left to right) of plasma total, plasma microvesicles (MVs), pooled SEC fractions 1–6, and fractions 7–10 and 11–16, with antibody directed against L1CAM. [Color figure can be viewed at wileyonlinelibrary.com]

accessible to all diagnostic laboratories. A size exclusion column (SEC) is a robust and reproducible isolation method that leads to high yield of physically and functionally intact EVs.[32,33] Therefore, we employed commercially available SEC columns, which were developed and optimized for the purification of EVs from small sample volumes. Plasma samples were centrifuged at 3500*g*, 2 × 4500*g*, followed by 1 × 10,000*g* centrifugation step to remove cellular debris, microsomes, and EVs in the 1-μm diameter range. Columns were then loaded with 0.5 mL of precleared plasma and samples were eluted into 24 fractions (Fig. 1A). Using nanoparticle tracking analysis (NTA), we determined the distribution of EVs in each fraction. In parallel, we measured α-synuclein content of pooled fractions 1–4, 5–8, 9–11, 12–16, and 17–24. As shown in Figure 1B, the downward slope of the EV peak overlaps with the increasing total protein concentration per fraction as measured by nanodrop analysis. Total protein content started to rise with fraction 12, and this was paralleled by the appearance of IgG as shown in the Western blot analysis (Fig. 1C). Based on these data, we pooled fractions 7–10 as EV-containing fractions without

contamination of free, non-EV-associated protein. To further test for the presence of EV markers (flotillin-2, CD63), CNS EV marker L1CAM and the absence of microsomal contamination (calnexin),[34] we performed Western blotting (Fig. 1D,G), EM (Fig. 1E), and NTA (Fig. 1F) analysis of the different SEC fractions and subsequently referred to the pooled fractions 7–10 as the plasma EV fraction.

To test for unspecific coelution of free α-synuclein with the plasma EV fraction, we prepared fractions 7–10 from plasma after EV depletion by ultracentrifugation (Supporting Information and Supporting Information Fig. S4). In addition we performed spiking experiments (Supporting Information and Supporting Information Fig. S5).

Preanalytical Variables

Previous studies suggested that different anticoagulants could have a strong impact on measurable plasma EV concentrations.[35] We therefore tested the influence of different preanalytical variables, anticoagulant, and

sample-processing temperatures on plasma EV concentrations, EV preparation yields, and EV α-synuclein content. For the complete analysis, please see the accompanying Supporting Information and Supporting Figure S1. Because citrate-based anticoagulants were consistently associated with lower α-synuclein measurements compared with heparin and EDTA plasma and as processing of EDTA and heparin plasma at room temperature resulted in a nonsignificant increase of α-synuclein measurements compared with 4°C, we decided to use EDTA plasma processed at 4°C for all further analyses.

Perianalytical Variables

For analysis of matrix effects and interrater and interday variability, please refer to the accompanying Supporting Information and Supporting Figure S2.

Characterization of Plasma EV α-Synuclein in the Tübingen Cohort

We next characterized the potential of plasma EV-associated α-synuclein as a diagnostic biomarker in α-synuclein-related neurodegeneration, namely, PD (n = 94) and DLB (n = 48). As controls, we chose PSP (n = 49) as a non-α-synuclein-related neurodegenerative disease with parkinsonian syndrome as well as healthy controls (HCs, n = 50). EV α-synuclein was highest in PD (1.19-fold higher compared with DLB, 1.54-fold higher compared with PSP, and 1.57-fold higher compared with HC (EV α-synuclein: PD, 12.660 ± 0.828 pg; DLB, 10.630 ± 1.152 pg; PSP, 8.244 ± 0.643 pg; HC, 8.083 ± 0.540 pg; all data normalized to 1 mL of plasma and given as mean $\pm$ SEM, Welch's ANOVA, W = 8.245, $P < 0.0001$; PD vs PSP, ***$P < 0.0003$; PD vs HC, ****$P < 0.0001$; Dunnett's T3 multiple-comparisons test; Fig. 2A). EV α-synuclein correlated significantly with plasma α-synuclein concentrations in all diagnostic groups (Supporting Table S1).

The number of EVs in the plasma EV fraction was significantly lower in the PD group compared with the DLB, PSP, and HC groups, with an AUC of 0.833 for PD versus DLB, 0.767 for PD versus PSP, and 0.805 for PD versus HC (PD, $3.24 \times 10^{10} \pm 2.29 \times 10^9$ particles; DLB, $7.62 \times 10^{10} \pm 6.57 \times 10^9$ particles; PSP, $6.20 \times 10^{10} \pm 5.96 \times 10^9$ particles; HC, $6.44 \times 10^{10} \pm 5.49 \times 10^9$ particles; all data normalized to 1 mL of plasma and given as mean $\pm$ SEM, Welch's ANOVA, W = 24.34, $P < 0.0001$; PD vs DLB, ****$P < 0.0001$; PD vs PSP, ****$P < 0.0001$; PD vs HC, ****$P < 0.0001$; Dunnett's T3 multiple-comparison test; Fig. 2B). Therefore, we normalized α-synuclein protein levels measured in the EV preparation to the number of EVs determined by NTA. The plasma EV α-synuclein concentration (ratio of EV α-synuclein to EV particle number) was significantly higher in PD compared with DLB (2.60-fold), PSP (2.86-fold), and HC (2.72-fold) groups, whereas no difference was detected for

DLB versus PSP or HC (PD, $6.10 \times 10^{-10} \pm 6.58 \times 10^{-11}$ pg/particle; DLB, $2.35 \times 10^{-10} \pm 4.68 \times 10^{-11}$ pg/particle; PSP, $2.13 \times 10^{-10} \pm 4.11 \times 10^{-11}$ pg/particle; HC, $2.24 \times 10^{-10} \pm 2.92 \times 10^{-11}$ pg/particle; all data are given as mean $\pm$ SEM, Welch's ANOVA, W = 10.35, $P < 0.0001$; PD vs DLB, ****$P < 0.0001$; PD vs PSP, ****$P < 0.0001$; PD vs HC, ****$P < 0.0001$; Dunnett's T3 multiple-comparison test, ****$P < 0.0001$; Fig. 2C). These data indicate that the α-synuclein concentration in EVs is significantly higher in PD compared with all other diagnostic groups. ROC analysis revealed an area under the curve (AUC) of 0.804 for the distinction between PD and DLB with 77.08% sensitivity and 73.40% specificity and an AUC of 0.815 for the distinction between PD and PSP with 81.63% sensitivity and 69.15% specificity. The concentration of α-synuclein in plasma EVs distinguished PD from HC with an AUC of 0.769 and with 83.33% sensitivity and 60.64% specificity (Fig. 2D). In the PD group, the concentration of α-synuclein in plasma EVs did not correlate with sex (Pearson's $r = -0.034$, $P = 0.750$), age (Pearson's $r = -0.163$, $P = 0.118$), age at disease onset (Pearson's $r = -0.130$, $P = 0.213$), disease duration (Pearson's $r = 0.058$, $P = 0.580$), or Mini–Mental State Examination (Pearson's $r = 0.071$, $P = 0.510$). A correlation with Mini–Mental State Examination (MMSE) was also not found in the DLB group (Pearson's $r = -0.0824$, $P = 0.600$). Because age differed significantly between the diagnostic groups, we performed ANCOVA to compare differences of (log-transformed) plasma EV α-synuclein concentrations between groups while controlling for age (corrected model: $F = 19.875$, $P < 0.005$; age: $F = 1.686$, $P = 0.195$). Post hoc Bonferroni analysis still demonstrated significant differences between PD and all other diagnostic groups. We therefore conclude that age bears no significant impact on plasma EV α-synuclein concentrations.

A significant inverse correlation was observed with UPDRS III (Pearson's $r = -0.266$, $P = 0.021$) and Hoehn and Yahr (H&Y) score (Pearson's $r = -0.259$, $P = 0.013$) in the PD group. Kruskal–Wallis test analysis of plasma EV α-synuclein concentrations revealed a significant difference between H&Y stages 1 and 3 (H&Y stage 1, $1.063 \times 10^{-9} \pm 3.037 \times 10^{-10}$ pg/particle; H&Y stage 3, $4.126 \times 10^{-10} \pm 8.989 \times 10^{-11}$ pg/particle; Kruskal–Wallis test, $P = 0.034$; H&Y stage 1 vs H&Y stage 3, *$P = 0.027$; Dunn's multiple-comparisons test; Fig. S3).

Characterization of Plasma EV α-Synuclein in the Validation Cohort

We next tested the diagnostic potential of the plasma EV α-synuclein concentration in a second independent cohort, the Kassel cohort. In contrast to the Tübingen cohort, the Kassel cohort did not include healthy controls. As a control group that better resembles a real-life diagnostic scenario, we included patients with a variety of parkinsonian syndromes, such as PSP (52.7%) and
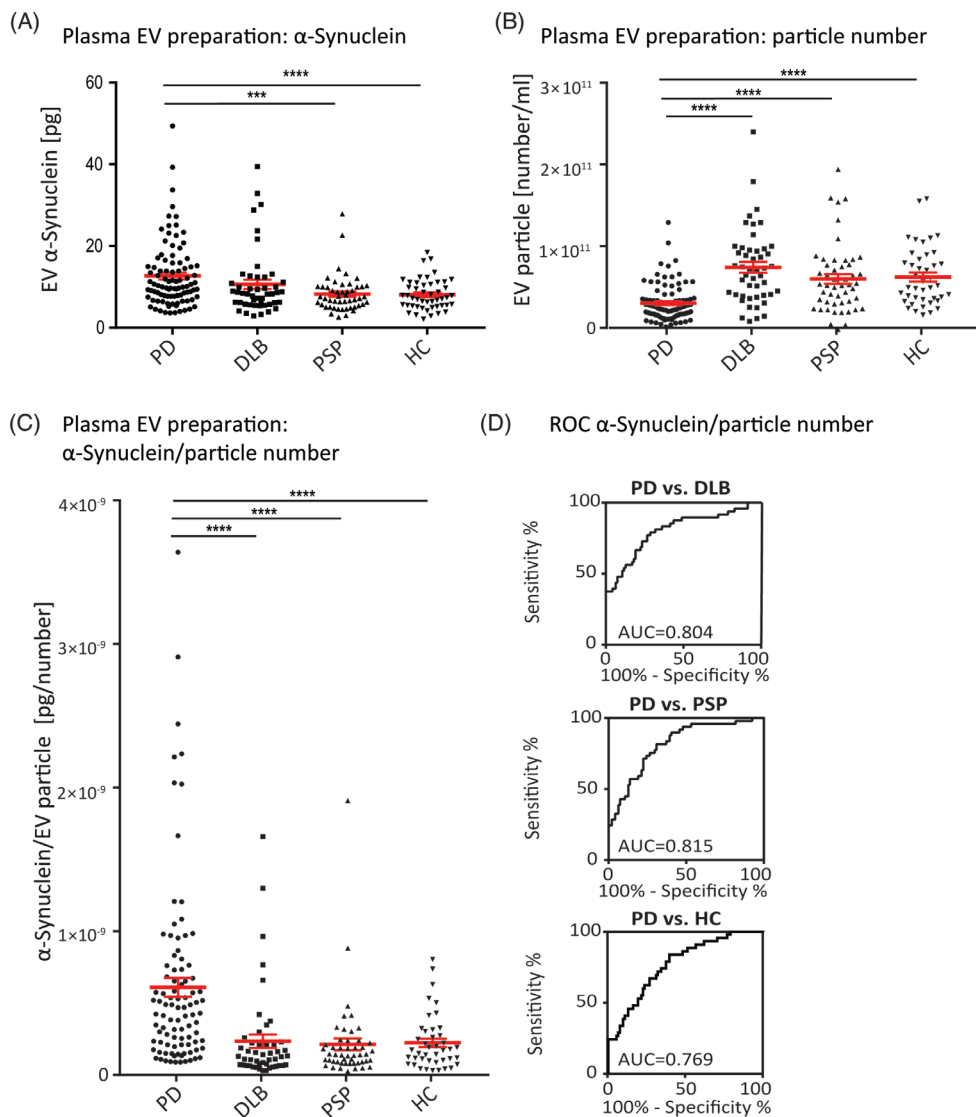
FIG. 2. Characterization of plasma EV α-synuclein in the Tübingen cohort. (**A**) EV α-synuclein content was determined by electrochemiluminescence analysis and normalized to 1 mL of plasma preparation volume. PD, Parkinson's disease; DLB, dementia with Lewy bodies; PSP, progressive supranuclear palsy; HC, healthy control. Red: mean ± SEM. ***$P < 0.0003$, ****$P < 0.0001$. (**B**) EV particle concentration was quantified by NTA from the plasma EV preparation and normalized to 1 mL of plasma preparation volume. PD, Parkinson's disease; DLB, dementia with Lewy bodies; PSP, progressive supranuclear palsy; HC, healthy control. Red: mean ± SEM. ****$P = 0.0001$. (**C**) Ratio of EV α-synuclein to the number of EV particles in the preparation (EV α-synuclein concentration). PD, Parkinson's disease; DLB, dementia with Lewy bodies; PSP, progressive supranuclear palsy; HC, healthy control. Red: mean ± SEM. ****$P = 0.0001$. (**D**) Receiver operating curve (ROC) of the EV α-synuclein concentration to distinguish PD from DLB (upper), from PSP (middle), and from HC (lower). AUC, area under the curve. [Color figure can be viewed at wileyonlinelibrary.com]

secondary, vascular, or atypical parkinsonian syndromes (47.2%); see Table 2. Similar to our findings in the Tübingen cohort, the number of particles in the plasma EV preparation was lower in the PD group than the DLB and parkinsonian syndromes groups (PD, $1.90 \times 10^{10} \pm 2.64 \times 10^{9}$ particles; DLB, $3.47 \times 10^{10} \pm 3.03 \times 10^{9}$ particles; parkinsonian syndromes, $2.57 \times 10^{10} \pm 2.71 \times 10^{9}$ particles; data are normalized to 1 mL of plasma; all data given as mean ± SEM, Welch's ANOVA, W = 7.54, $P < 0.001$; PD, n = 46; DLB, n = 43; parkinsonian syndromes, n = 34; PD vs DLB, ***$P = 0.0006$; PD vs parkinsonian syndromes ns, $P = 0.22$; DLB vs parkinsonian syndromes ns,

$P = 0.09$; Dunnett's T3 multiple-comparisons test; Fig. 3A). Plasma EV α-synuclein concentrations were significantly higher in the PD group compared with the DLB group, 3.28-fold, and the group of patients with parkinsonian syndromes, 2.33-fold (PD, $9.49 \times 10^{-10} \pm 1.99 \times 10^{-10}$ pg/particle; DLB, $2.89 \times 10^{-10} \pm 3.67 \times 10^{-11}$ pg/particle; parkinsonian syndromes, $4.08 \times 10^{-10} \pm 5.44 \times 10^{-11}$ pg/ particle; all data given as mean ± SEM, Welch's ANOVA, W = 7.54, $P = 0.0003$; PD, n = 46; DLB, n = 43; parkinsonian syndromes, n = 34; PD vs DLB, **$P = 0.006$; PD vs parkinsonian syndromes, *$P = 0.033$; DLB vs parkinsonian syndromes ns, $P = 0.21$; Fig. 3B). Similar
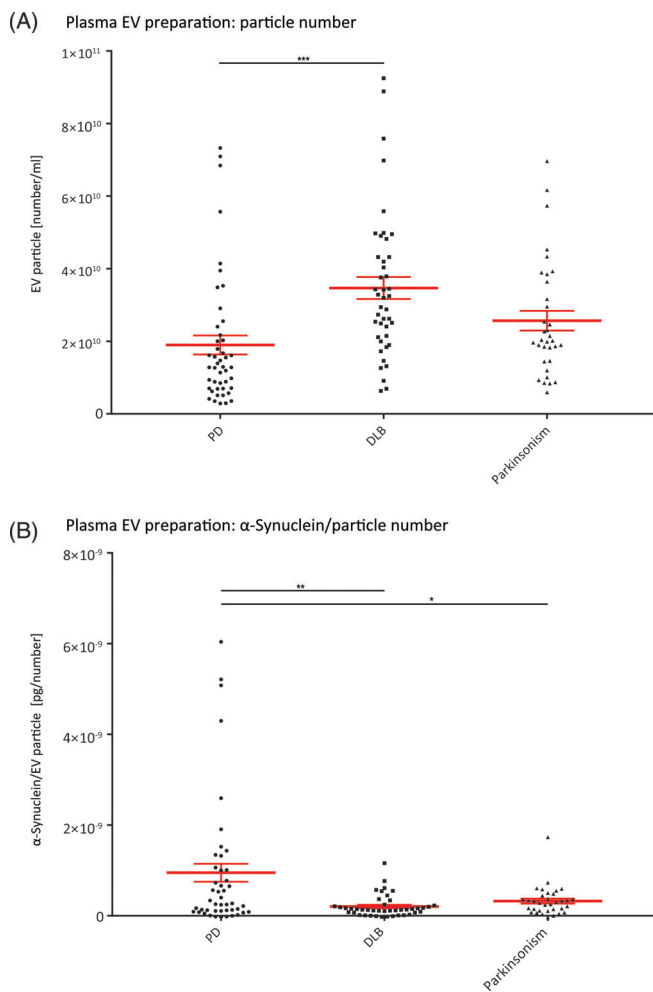
FIG. 3. Characterization of plasma EV α-synuclein in the validation cohort. (**A**) EV particle concentration in the preparation, normalized to 1 mL of starting plasma volume. PD, Parkinson's disease; DLB, dementia with Lewy bodies; parkinsonism, parkinsonian syndromes. Red: mean ± SEM. ***P = 0.0006. (**B**) Plasma EV α-synuclein concentration. PD, Parkinson's disease; DLB, dementia with Lewy bodies. Red: mean ± SEM. **PD versus DLB, P = 0.006; **PD versus parkinsonism P = 0.033. [Color figure can be viewed at wileyonlinelibrary.com]

to the Tübingen cohort, plasma EV α-synuclein concentrations did not correlate with sex (Pearson's r = 0.025, P = 0.870), age (Pearson's r = −0,097, P = 0.522), disease duration (Pearson's r = 0.159, P = 0.303), clock-drawing test (Pearson's r = −0.043, P = 0.813), or MMSE (Pearson's r = −0.097, P = 0.569). In contrast to our findings from the Tübingen cohort, we did not detect a significant correlation with motor score (H&Y, Pearson's r = −0.121, P = 0.483) or of EV α-synuclein with plasma α-synuclein level (Supporting Table S1).

Discussion

In this study, we established a precise protocol for preparation of plasma EVs and quantification of EV-associated α-synuclein and investigated whether plasma EV-associated α-synuclein may serve as a diagnostic biomarker in PD. We found that EV number is significantly lower in plasma from PD patients but contain higher concentrations of α-synuclein compared with 3 different control groups: (1) DLB as another α-synuclein-related neurodegenerative disease, (2) PSP as a neurodegenerative disease with parkinsonian syndrome unrelated to α-synuclein pathology, and (3) a healthy control group. We confirmed these findings in a second independent cohort of patient groups with PD and DLB and a group with different secondary parkinsonian syndromes to resemble real-life differential-diagnostic conditions.

EVs have been implicated in the transfer of α-synuclein aggregates from diseased to healthy neurons, thus contributing to the spreading of PD pathology. This hypothesis is supported by several reports demonstrating that CSF, plasma or brain-derived EVs from patients with PD and/or DLB can confer toxicity[21] and induce the aggregation of intracellular α-synuclein in vitro and in vivo.[36-38] High EV α-synuclein concentrations could favor α-synuclein aggregation and provide the basis for a more efficient transfer of α-synuclein and its seeding-competent aggregates to target cells. Although EVs can cross the blood–brain barrier, no evidence supports a role for peripheral EVs contributing to CNS α-synuclein pathology. However, increased peripheral EV α-synuclein concentrations could reflect upregulation of α-synuclein sorting to EVs. Recent evidence suggests that EV-associated release of α-synuclein may serve as a clearance pathway to remove toxic α-synuclein from cells when other degradative pathways such as autophagy fail.[21,39] Interestingly, plasma EV concentrations in PD were significantly decreased compared with all other diagnostic groups. As the majority of plasma EVs stem from cells outside the CNS, our data could hint toward general impairment of EV secretion in PD. Decreased clearance via this pathway may contribute to PD pathology by favoring intracellular accumulation of α-synuclein followed by aggregation and cytotoxicity. The observed increase in EV α-synuclein concentrations in PD may reflect upregulated EV α-synuclein sorting to compensate for clearance deficits. Consistent with this hypothesis, we observed a negative correlation between plasma EV α-synuclein concentrations and motor function in the Tübingen cohort with higher EV concentrations of α-synuclein in early disease stages. Progressive failure of compensatory EV-mediated α-synuclein clearance could then lead to clinical disease progression and be paralleled by decreasing EV α-synuclein concentrations. The inverse correlation between plasma EV α-synuclein concentration and motor function failed to reach significance in the independent Kassel replication cohort. However, in the Kassel cohort, the PD group included

only 1 participant with an H&Y score of 1, whereas the majority reached H&Y scores of 3 or 4. Thus, a significant correlation may have been missed because of the lack of less severely affected patients.

The more severe motor impairment in the Kassel cohort PD group may also explain the less pronounced differences between the plasma EV α-synuclein concentrations and other groups compared with the Tübingen cohort, in which the diagnostic accuracy was higher. Another reason could be the lack of a neurologically healthy control group in the Kassel cohort. Instead, we chose a group of patients with parkinsonian syndromes because of diverse etiologies, including PSP, vascular, neuroleptic drug-induced or other atypical parkinsonian syndromes unrelated to α-synuclein pathology. Although this group bears the risk of misdiagnosis, for example, of falsely negative included PD patients, it is better suited to reflect the challenges of routine clinical practice, which requires distinguishing PD from other parkinsonian syndromes.

There is an urgent need for noninvasive fluid biomarkers in PD, preferably from blood, especially in early disease stages, when a clinical diagnosis is associated with the highest rate of misdiagnosis.[40] So far, the quantification of plasma α-synuclein as a PD biomarker has yielded inconsistent results in previous studies.[11,41-43] This could be caused by high concentrations of α-synuclein in erythrocytes. Thus, hemolysis or contamination of plasma with erythrocytes may act as confounders when detecting plasma α-synuclein, but these confounders may have less impact on EV-associated α-synuclein. Here, we thoroughly tested for pre- and perianalytical variables to optimize our assay. SEC isolation ensures the absence of contamination with protein complexes and abundant plasma proteins such as albumin and IgG and allows for reliable quantification of the preparation yield by NTA. This is reflected by a low interrater and interassay variability of our assay together with a high diagnostic accuracy in the Tübingen cohort.

It is technically possible to enrich for a neuron-derived EV subpopulation from plasma using immunoprecipitation with neuronal cell adhesion molecule L1CAM-directed antibodies. Previous work reported increased α-synuclein in L1CAM EVs prepared from PD versus controls or other parkinsonian syndromes.[24,25,44] Our results suggest that isolation of the entire population of plasma EVs (including L1CAM EVs) may be a promising alternative approach. The majority of plasma α-synuclein and therefore most likely also of plasma EV-associated α-synuclein is derived from erythrocytes.[45] Only 1 other study has tested the concentration of total plasma EV α-synuclein as a potential biomarker.[46] It included 39 PD patients and 33 controls but no replication cohort, whereas our study sample contained 143 PD patients and altogether 221 different control samples. Cerri et al reported increased EV α-synuclein concentrations in PD, but no data were given on AUC values and pre- or perianalytical assay parameters, which were extensively investigated in our study. Of note, in this study, plasma EV concentrations did not differ between PD and controls, which may be caused by the different EV preparation protocol (UC as opposed to SEC) used by Cerri et al.

The differences in plasma EV α-synuclein concentrations between PD and controls could indicate systemic involvement in PD pathology, which is further supported by findings of increased α-synuclein in erythrocyte plasma membranes in PD[47] and higher concentrations of oligomeric α-synuclein in red blood cells from PD patients compared with controls.[48] In addition, erythrocyte-derived EVs from patients with PD were shown to cross the blood–brain barrier in mice and elicit a microglial proinflammatory response that was significantly higher compared with erythrocyte-derived EVs from a healthy control group.[49] Another line of evidence for systemic involvement in PD stems from findings of α-synuclein pathology in submandibular glands,[50] saliva,[51,52] and peripheral nerve fibers of the skin.[53-55]

It is not clear why DLB, another α-synuclein-related neurodegenerative disease, does not show increased EV α-synuclein concentrations similar to PD. Aggregation of α-synuclein is a pathological hallmark of DLB and PD; however, both diseases are distinct in terms of clinical phenotypes, affected brain regions, vulnerable neuron populations, and copathologies, such as Tau and amyloid-β in DLB.[56] Aggregates in α-synuclein-related neurodegenerative diseases differ by their posttranslational modifications and conformational state, which may influence their release via EVs.[22,57-62] The differences observed in plasma EV α-synuclein concentrations may thus represent another PD-specific property of α-synuclein.

Conclusions

Plasma EV α-synuclein may serve as a potential minimally invasive biomarker for PD. Further studies with larger numbers of patients in different disease stages are needed to further validate these findings and to better evaluate their potential correlation with motor impairment and disease progression. ●

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Supporting Data

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