

Blood-Based α -Synuclein Seeding Activity: A Dual Plasma and Erythrocyte RT-QuIC Assay for the Enhanced Diagnosis and Clinical Staging of Multiple System Atrophy

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Abstract

Background

Multiple system atrophy (MSA) is a devastating synucleinopathy with a clinical presentation that often overlaps with Parkinson's disease (PD), leading to diagnostic challenges. Although the ultrasensitive real-time quaking-induced conversion (RT-QuIC) assay for α -synuclein (α Syn) is promising, its sensitivity when applied to cerebrospinal fluid (CSF) for diagnosing MSA remains suboptimal, and CSF collection is invasive. We hypothesize that the less-invasive erythrocyte sample, which contains over 99% of blood α Syn, harbors prion-like α Syn-seeding activity, thereby offering a potential diagnostic biomarker for MSA.

Methods

We developed and applied two complementary real-time quaking-induced conversion (RT-QuIC) assays to detect α Syn-seeding activity in plasma and erythrocytes from clinically-established patients with MSA and PD, and healthy controls. The RT-QuIC end-products were characterized using proteinase K (PK) digestion, filter-trap assays (FTA), sucrose gradient ultracentrifugation, and transmission electron microscopy (TEM). Diagnostic performance was evaluated via receiver operating characteristic (ROC) analysis, and correlations with clinical scales were assessed.

Results

The plasma RT-QuIC assay demonstrated high specificity (91.3%) but moderate sensitivity (61.4%) for MSA, while the erythrocyte assay showed a sensitivity of 70.0% for MSA and 70.6% for PD with a specificity of 85.7%. Combining both assays yielded a synergistic improvement, achieving an area under the curve (AUC) of 0.93 for MSA. Physicochemical characterization confirmed the bona fide generation of PK-resistant, fibrillar α Syn aggregates in positive reactions. Notably, plasma seeding activity correlated significantly with motor severity (UMSARS), autonomic dysfunction (SCOPA-AUT), and neuropsychological burden (PHQ-9, GAD-7) in MSA patients, whereas erythrocyte seeding activity did not.

Conclusions

This study is the first to establish erythrocytes as a robust, accessible matrix for detecting α Syn-seeding activity in MSA and PD. A combined blood-based approach using both plasma and erythrocytes significantly enhances diagnostic accuracy, potentially rivaling CSF-based assays. This dual-assay strategy represents a transformative, minimally invasive tool for the differential diagnosis and clinical management of synucleinopathies.

INTRODUCTION

Multiple system atrophy (MSA) is a rare and fatal neurodegenerative disorder classified as a synucleinopathy, alongside Parkinson's disease (PD), PD dementia (PDD), and dementia with Lewy bodies (DLB) [1, 2]. Clinically, MSA is characterized by a variable combination of autonomic dysfunction, parkinsonism, and cerebellar ataxia, leading to its subclassification into two predominant motor subtypes: MSA with predominant parkinsonism (MSA-P) and MSA with predominant cerebellar ataxia (MSA-C)[3, 4]. Neuropathologically, MSA is uniquely defined by the accumulation of misfolded α -synuclein (α Syn) in glial cytoplasmic inclusions (GCIs) within oligodendrocytes, a feature that distinguishes it from PD [5].

α Syn is a presynaptic protein highly expressed in the central nervous system but also detectable in peripheral tissues. Notably, erythrocytes serve as the primary peripheral reservoir, harboring over 99% of α Syn found in whole blood [6–8]. Converging evidence indicates that erythrocytic α Syn levels are elevated not only in PD but also in MSA and idiopathic rapid eye movement (REM) sleep behavior disorder (iRBD), suggesting a shared peripheral dysregulation [8–12]. Beyond serving as a passive biomarker, erythrocyte-derived α Syn is increasingly recognized as a potentially active contributor to disease pathogenesis. Erythrocytes laden with α Syn, and their secreted extracellular vesicles, can cross the blood-brain barrier and interact with glial and gastrointestinal cells, thereby potentially seeding pathology and propagating neuroinflammation [13–16].

In contrast to peripheral findings, studies on cerebrospinal fluid (CSF) have yielded inconsistent results. While several reports describe a decrease in total CSF α Syn levels in MSA patients, often more pronounced than in PD [17–20], others have found no significant differences among synucleinopathies [21, 22]. Similarly, data on phosphorylated α Syn (p- α Syn) in CSF are conflicting, with one study showing elevated levels in neuropathologically-confirmed MSA [23], and another reporting increased p- α Syn in PD but decreased levels in MSA [24]. Plasma and serum measurements have also been discordant, with reports of both increased[25, 26] and unchanged[19] α Syn levels. This substantial heterogeneity in α Syn concentrations across CSF, blood, and other fluids has limited the diagnostic utility of conventional quantitative assays.

The α Syn seed amplification assay (SAA), encompassing real-time quaking-induced conversion (RT-QuIC) and protein misfolding cyclic amplification, offers a paradigm shift by leveraging the self-propagating properties of pathological α Syn conformers [27–29]. In PD and DLB, CSF α Syn-SAA demonstrates remarkable diagnostic performance, with sensitivities of up to 95% and specificities approaching 100% [27, 28, 30]. For MSA, however, CSF SAA sensitivity has varied widely across studies, ranging from 4.4% to 80% [29–32]. The assay has also been extended to diverse peripheral matrices, including skin, saliva, oral mucosa, olfactory mucosa, and blood. Among these, skin biopsies yield sensitivity and specificity comparable to CSF, whereas other matrices exhibit markedly lower performance [33–43]. Notably, although erythrocytes harbor the highest α Syn levels in blood [6–8],

whether α Syn in these cells is of prion-like seeding activity remains unclear. Moreover, the presence of such seeding activity in the plasma of MSA patients has yet to be explored.

In this study, we applied α Syn-SAA to establish a novel erythrocyte-based assay and also to probe plasma α Syn-seeding activity, aiming to identify a sensitive diagnostic signature for MSA. Both modalities demonstrate individual diagnostic value; crucially, their combined use yields improved accuracy, reinforcing their translational potential in routine clinical practice.

METHODS

Participants and specimen collection

The study was approved by the Institutional Review Boards of Xiangya Hospital Central South University and the First Affiliated Hospital of Nanchang University, and written informed consent was obtained from all participants. Plasma samples were collected from 69 controls and 101 MSA patients; erythrocyte samples, from 70 controls, 100 MSA patients, and 51 PD patients. A subset of 27 controls and 80 MSA patients provided both sample types. Patients were recruited from the neurology outpatient and inpatient services at Xiangya Hospital between 2023 and 2024. MSA was diagnosed according to the Movement Disorder Society criteria [5], and PD was diagnosed using the corresponding Clinical Diagnostic Criteria [44]. Controls were individuals without PD, MSA, or any other neurological disorder. Demographic characteristics and relevant clinical scale scores for all participants are summarized in Tables 1–3.

Table 1
Demographic characteristics of different groups in the plasma α Syn SAA analysis

Sex (male/female)	Control	MSA	<i>p</i>
	(n = 69)	(n = 101)	
	34/35	60/41	0.192
MSA-P/MSA-C	NA	47/54	NA
Age (years)	56.00(49.00, 67.50)	58.00(52.50, 65.50)	0.227
Age at onset (years)	NA	56.00(51.00, 63.00)	NA
Duration (years)	NA	2.00(1.00, 3.00)	NA
H&Y stage	NA	3.00(2.50, 3.00)	NA
UMSARS-I	NA	16.00(12.00, 19.00)	NA
UMSARS-II	NA	15.00(12.00, 21.75)	NA
UMSARS-III	NA	2.00(1.00, 3.00)	NA
SCOPA-AUT	NA	17.00(12.50, 23.50)	NA
Wexner	NA	6.00(2.00, 8.75)	NA
HRS	NA	24.00(24.00, 24.00)	NA
RBDSQ	NA	6.00(3.00, 9.00)	NA
PHQ-9 ^a	NA	7.00(1.00, 11.00)	NA
GAD-7 ^a	NA	5.00(0.75, 8.25)	NA
MMSE ^a	NA	26.00(24.00, 29.00)	NA

Data were presented as median (Q1, Q3).

^a Only patients who can determine clinical scales of disease severity are counted.

α Syn, α -synuclein; GAD-7, Generalized Anxiety Disorder-7; H&Y stage, Hoehn and Yahr stage; HRS, Hyposmia rating scale; MMSE, Mini-mental State Examination; MSA, Multiple system atrophy; NA, not applicable; PHQ-9, Patient Health Questionnaire-9; RBDSQ, REM Sleep Behavior Disorder Screening Questionnaire; SAA, seed amplification assay; SCOPA-AUT, The Scales for Outcomes in PD-Autonomic; UMSARS-I, Unified Multiple System Atrophy Rating Scale Part I; UMSARS-II, Unified Multiple System Atrophy Rating Scale Part II; UMSARS-III, Unified Multiple System Atrophy Rating Scale Part III; Wexner, Wexner continence grading scale.

Table 3
Demographic characteristics for the combined analysis

Sex (male/female)	Control	MSA
	(n = 27)	(n = 80)
	19/8	50/30
MSA-P/MSA-C	NA	33/47
Age (years)	50.00(44.00, 57.00)	57.50(52.00, 66.75)
Age at onset (years)	NA	55.00(51.00, 63.75)
Duration (years)	NA	2.00(1.00, 3.00)
H&Y stage	NA	3.00(2.50, 3.00)
UMSARS-I	NA	16.00(12.00, 19.00)
UMSARS-II	NA	14.00(11.00, 20.00)
UMSARS-III	NA	2.00(1.00, 3.00)
SCOPA-AUT	NA	17.00(12.00, 22.00)
Wexner	NA	5.00(2.00, 8.00)
HRS	NA	24.00(24.00, 24.00)
RBDSQ	NA	5.00(3.00, 8.00)
PHQ-9 ^a	NA	6.00(1.00, 10.00)
GAD-7 ^a	NA	4.00(0.00, 8.00)
MMSE ^a	NA	26.50(24.00, 29.00)
Plasma αSyn SAA results (-/+)	25/2	36/44
Plasma αSyn SAA	6.00(4.00, 19.00)	47.00(24.50, 56.50)

Data were presented as median (Q1, Q3).

^a Only patients who can determine clinical scales of disease severity are counted.

αSyn, α-synuclein; GAD-7, Generalized Anxiety Disorder-7; H&Y stage, Hoehn and Yahr stage; HRS, Hyposmia rating scale; MMSE, Mini-mental State Examination; MSA, Multiple system atrophy; NA, not applicable; PHQ-9, Patient Health Questionnaire-9; RBDSQ, REM Sleep Behavior Disorder Screening Questionnaire; SAA, seed amplification assay; SCOPA-AUT, The Scales for Outcomes in PD-Autonomic; UMSARS-I, Unified Multiple System Atrophy Rating Scale Part I; UMSARS-II, Unified Multiple System Atrophy Rating Scale Part II; UMSARS-III, Unified Multiple System Atrophy Rating Scale Part III; Wexner, Wexner continence grading scale.

Sex (male/female)	Control	MSA
	(n = 27)	(n = 80)
	19/8	50/30
ThT Fluorescence (%)		
Plasma α Syn SAA	53.25(53.25, 53.25)	50.25(39.00, 53.25)
Lag Phase (h)		
Erythrocyte α Syn SAA	20/7	21/59
results (-/+)		
Erythrocyte α Syn SAA	13.00(6.00, 20.00)	29.00(19.25, 45.00)
ThT Fluorescence (%)		
Erythrocyte α Syn SAA	119.80(110.50, 119.80)	92.00(74.31, 104.70)
Lag Phase (h)		
Data were presented as median (Q1, Q3).		
^a Only patients who can determine clinical scales of disease severity are counted.		
α Syn, α -synuclein; GAD-7, Generalized Anxiety Disorder-7; H&Y stage, Hoehn and Yahr stage; HRS, Hyposmia rating scale; MMSE, Mini-mental State Examination; MSA, Multiple system atrophy; NA, not applicable; PHQ-9, Patient Health Questionnaire-9; RBDSQ, REM Sleep Behavior Disorder Screening Questionnaire; SAA, seed amplification assay; SCOPA-AUT, The Scales for Outcomes in PD-Autonomic; UMSARS-I, Unified Multiple System Atrophy Rating Scale Part I; UMSARS-II, Unified Multiple System Atrophy Rating Scale Part II; UMSARS-III, Unified Multiple System Atrophy Rating Scale Part III; Wexner, Wexner continence grading scale.		

Blood samples collected in EDTA-K₂ tubes were processed according to the following protocol. To obtain plasma, whole blood was centrifuged at 2,000 × g for 10 min at 4°C; the supernatant was collected, aliquoted, and stored at – 80°C for subsequent analyses. Erythrocytes were purified from a separate blood aliquot using Percoll density gradient centrifugation (Cytiva, Cat# 17089101, USA). Briefly, the aliquot was centrifuged at 350 × g for 10 min at 4°C, and the resulting cell pellet was resuspended 1:1 with PBS. This suspension was carefully layered onto a 70% Percoll cushion and centrifuged at 2,000 rpm for 18 min at 4°C. The bottom erythrocyte layer was then collected, washed with PBS (2,000 rpm, 5 min, 4°C), aliquoted, and stored at – 80°C for future use.

Expression and purification of recombinant human α Syn for RT-QulC assays

Recombinant human wild-type α Syn, used as the substrate in the RT-QulC assay, was prepared as previously described[45]. In brief, a stock of *E. coli* was inoculated into 4 mL of Terrific Broth supplemented with antibiotics and cultured for 6 h at 37°C. This starter culture was then scaled up to 4 L

of Terrific Broth and grown overnight at 37°C with constant shaking at 300 rpm. Cells were centrifuged at $6,000 \times g$ for 10 min at 4°C. The resulting pellet was resuspended in 750 mM NaCl, 10 mM Tris-HCl, pH 7.6, and 1 mM EDTA containing a protease inhibitor and lysed by sonication. After lysis, the homogenate was boiled for 15 min and centrifuged at $10,000 \times g$ for 20 min at 4°C. The supernatant was dialyzed overnight at 4°C in 10 mM Tris-HCl (pH 7.6) with 50 mM NaCl and 1 mM EDTA, and subsequently concentrated using a 3 kDa molecular weight cutoff (MWCO) filter. The concentrated protein was first purified by size-exclusion chromatography on a Superdex-200 column. Fractions enriched in α Syn were pooled, dialyzed against 10 mM Tris-HCl (pH 7.6) containing 25 mM NaCl and 1 mM EDTA, and then subjected to anion-exchange chromatography on a HiTrap Q HP column, with elution performed using a linear salt gradient (25–1,000 mM NaCl). The final product was dialyzed in 10 mM Tris-HCl (pH 7.6) with 50 mM NaCl, adjusted to 0.8–1.0 mg/mL, aliquoted, and stored at – 80°C until use. Protein identity and purity were confirmed by Coomassie Blue staining and western blotting probing with the anti- α Syn antibody MJFR-1 (Abcam, Cat# ab138501, Cambridge, UK).

Direct detection of plasma α Syn-seeding activity via RT-QulC

The plasma α Syn RT-QulC assay was modified according to a previously reported method[39, 40, 45]. Briefly, the final RT-QulC reaction buffer contained 100 mM (2- [N,N-Bis (2-hydroxyethyl) amino] ethanesulfonic acid) (BES) (pH 7.2), 70 mM NaCl, 20 μ M Thioflavin T (ThT), and 0.2 mg/mL recombinant α Syn. In a 96-well plate (Thermo Fisher Scientific, USA), each well contained 195 μ L of reaction buffer and 5 μ L of diluted plasma as the seeds. The plate was incubated in a FLUOstar Omega microplate reader (BMG Labtech, Germany) at 37°C for 53 h 15 min with continuous shaking (double orbital mode, 400 rpm). ThT fluorescence was measured every 45 min (448 nm excitation and 482 nm emission).

Each sample was tested in triplicate. For data analysis, the mean ThT fluorescence of the three replicate wells was calculated and normalized to the percentage of the maximum fluorescence reading. Based on receiver operating characteristic (ROC) curve analysis, the threshold was set at 42.5%. A well was considered positive if its endpoint fluorescence exceeded this threshold, and a sample was considered positive if at least two of its three replicate wells were positive. The kinetic parameters of the α Syn RT-QulC included the endpoint ThT fluorescence and the lag time. Endpoint ThT fluorescence was defined as the normalized fluorescence reading at the end of the incubation, while lag time was defined as the time required for the fluorescence of a sample well to reach the threshold.

Detection of erythrocytic α Syn-seeding activity via immunoprecipitation-enhanced RT-QulC

The RT-QulC assay for erythrocytic α Syn-seeding activity was adapted from a previously described protocol[37, 39] with modifications to incorporate immunoprecipitation (IP) enrichment. For the IP step, 495 μ L of lysis buffer (0.5% sodium deoxycholate, 0.5% NP-40, 100 mM NaCl, 10 mM EDTA, and 10 mM Tris-HCl, pH 7.4) was combined with 1.7 μ g of MJFR-1 (Abcam, Cat# ab138501, Cambridge, UK) and 30

μ L of protein A/G magnetic beads (Thermo Fisher Scientific, Cat# 88803, USA), and incubated with rotation at 4°C for 24 h. Subsequently, 5 μ L of erythrocyte lysate was added to antibody-conjugated beads mixture and incubated overnight at 4°C with rotation. Bound proteins were then eluted using an elution buffer (Thermo Fisher Scientific, USA), and the eluate was adjusted to pH 7.5 to serve as the α Syn seed source for the RT-QulC assay.

The reaction buffer for the RT-QulC assay of the erythrocytic α Syn contained 100 mM phosphate buffer (pH 8.0), 140 mM NaCl, 20 μ M ThT, and 0.1 mg/mL recombinant α Syn. Each well of a 96-well plate (Thermo Fisher Scientific, USA) was loaded with 95 μ L of reaction buffer and two silica beads (800 μ m, OPS Diagnostics, USA), followed by the addition of 5 μ L of the diluted IP-enriched sample. After sealing the plate with a transparent membrane, the plate was incubated in a FLUOstar Omega microplate reader (BMG Labtech, Germany) at 37°C for 119 h 45 min, with a cycle consisting of 1 min of shaking at 200 rpm followed by 14 min of rest. ThT fluorescence was measured every 15 min (excitation at 448 nm and emission at 482 nm). Each sample was tested in triplicate. The positivity threshold was defined as 17.78%, determined by ROC curve analysis, which corresponded to the mean fluorescence of all negative controls plus one standard deviation (SD).

Proteinase K digestion assay

Proteinase K (PK) resistance is widely used to assess whether a protein has undergone a conformational switch from a detergent-soluble state to detergent-insoluble misfolded aggregates[45, 46]. To evaluate the PK resistance of α Syn in RT-QulC end-products across the different groups, the end-products were incubated with varying concentrations of PK at 37°C for 1 h under continuous shaking (350 rpm). Digestion was terminated by adding a protease inhibitor cocktail (Abcam, Cambridge, UK). Subsequently, the samples were mixed with 5 $\times$ sample loading buffer, heated to 100°C for 10 min, and analyzed by Western blotting as described below.

Sucrose step gradient ultracentrifugation

The α Syn RT-QulC end-products were diluted with 20% Sarcosyl to a final concentration of 1% and layered onto a 10%–60% discontinuous sucrose gradient. Following ultracentrifugation at 200,000 $\times$ g for 1 h at 4°C, as previously described[45], twelve fractions were sequentially collected from the top to the bottom of the gradient. Each fraction was precipitated with ice-cold methanol at – 20°C for 4 h. After centrifugation at 14,000 $\times$ g for 30 min at 4°C, the supernatants were discarded, and the pellets were resuspended in 1 $\times$ sample loading buffer, heated to 100°C for 10 min, and analyzed by Western blotting as described below.

Western blot analysis

Proteins were separated by SDS-PAGE using home-made 10% or 12.5% gels and subsequently transferred to PVDF membranes (Millipore Sigma, Carlsbad, CA, USA). The membranes were incubated overnight at 4°C with the primary antibody MJFR-1 (Abcam, Cat# ab138501, Cambridge, UK) at a 1:2,000 dilution. After washing, the membranes were incubated with an HRP-conjugated donkey anti-rabbit secondary antibody at a 1:10,000 dilution. Immunoreactive bands were visualized by chemiluminescence and quantified using ImageJ software (NIH, USA), with images acquired on a Bio-Rad imaging system.

Filter trap assay

Filter trap assays (FTA) were conducted as previously described[45, 47]. An aliquot of 10 µL of the αSyn RT-QulC end-products was denatured in 190 µL of 2% SDS and applied to a cellulose acetate membrane under vacuum using a Bio-Dot SF microfiltration apparatus (Bio-Rad, USA). The membrane was then probed with a conformation-specific primary antibody directed against αSyn aggregates, MJFR-14-6-4-2 (Abcam, Cat# ab209538, Cambridge, UK), at a 1:2,000 dilution, followed by incubation with a donkey anti-rabbit HRP-conjugated secondary antibody at a 1:10,000 dilution. Immunoreactive signals were visualized by chemiluminescence as described above.

Transmission electron microscopy

Transmission electron microscopy (TEM) was performed as described previously[45, 47]. Aliquots of the αSyn RT-QulC end-products were loaded onto 200-mesh copper grids coated with a carbon support film and incubated for 45 s. Excess liquid was removed by blotting with filter paper, and the grids were then negatively stained. After air-drying, the grids were examined using a transmission electron microscope (Thermo Fisher Tecnai Spirit) operating at 120 kV and equipped with an EMSIS Veleta camera (2 k × 2 k).

Statistical analysis

Statistical analyses were performed using SPSS 26.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 9.5 (GraphPad Software, La Jolla, CA, USA). Continuous variables with a normal distribution are summarized as mean ± SD, whereas those with a non-normal distribution are summarized as median (Q1, Q3, denoting the first and third quartiles, respectively) with interquartile range (IQR). The Shapiro–Wilk test was used to assess normality of demographic characteristics, clinical data, and αSyn RT-QulC kinetic parameters. Group comparisons were performed using Student's t-test or analysis of variance (ANOVA) for normally distributed data, and the Mann–Whitney U-test or Kruskal–Wallis test for non-normally distributed data. Post hoc multiple comparisons were conducted with Dunn's test, applying Bonferroni correction for p-value adjustment. ROC analysis was performed to evaluate the diagnostic performance of αSyn RT-QulC kinetic parameters in discriminating synucleinopathies from controls; the area under the curve (AUC), sensitivity, and specificity were calculated to determine their accuracy and efficacy as diagnostic biomarkers. Spearman's rank correlation was used to assess associations

between α Syn RT-QulC kinetic parameters and clinical scores. All statistical tests were two-sided, and $p < 0.05$ was considered statistically significant.

RESULTS

Demographic characteristics of the study cohorts

For the plasma cohort, 69 controls and 101 patients with MSA were enrolled. Their median ages were 56.00 years (IQR: 49.00–67.50) and 58.00 years (IQR: 52.50–65.50), respectively, with no significant difference ($p = 0.227 > 0.05$). The proportion of males was also comparable between the control (34/69, 49.3%) and MSA (60/101, 59.4%) groups ($p = 0.192 > 0.05$) (Table 1).

For the erythrocyte cohort, 70 controls, 100 MSA patients, and 51 PD patients were included. Their median ages were 51.50 years (IQR: 48.00–59.00), 57.00 years (IQR: 52.00–65.00), and 62.00 years (IQR: 60.00–70.00), respectively. Controls were significantly younger than both MSA ($p = 0.009 < 0.01$) and PD patients ($p < 0.001$), while PD patients were older than MSA patients ($p = 0.001$). Sex distribution did not differ significantly among the three groups ($p = 0.594$), with 45 (64.3%), 65 (65.0%), and 29 (56.9%) males in the control, MSA, and PD groups, respectively (Table 2). Additionally, a subset of the erythrocyte cohort, 27 controls and 80 MSA patients, also underwent α Syn RT-QulC analysis using both erythrocyte and plasma samples (Table 3).

Table 2
Demographic characteristics of different groups in the erythrocyte α Syn SAA analysis

Sex (male/female)	Control (n = 70)	MSA (n = 100)	PD (n = 51)	p
	45/25	65/35	29/22	0.594
MSA-P/MSA-C	NA	38/62	NA	NA
Age (years)	51.50(48.00, 59.00)	57.00(52.00, 65.00)	62.00(60.00, 70.00)	< 0.001
Age at onset (years)	NA	55.00(50.25, 61.50)	58.00(51.00, 65.00)	0.192
Duration (years)	NA	2.00(1.00, 3.00)	5.00(3.00, 10.00)	< 0.001
H&Y stage	NA	3.00(2.50, 3.00)	2.50(2.00, 3.00)	0.003
UMSARS-I	NA	16.00(11.00, 18.25)	NA	NA
UMSARS-II	NA	14.00(10.00, 20.00)	NA	NA
UMSARS-III	NA	2.00(1.00, 3.00)	NA	NA
UPDRS-III ^a	NA	NA	35.00(23.00, 40.50)	NA
SCOPA-AUT	NA	17.00(12.00, 22.00)	NA	NA
Wexner	NA	5.00(1.00, 8.00)	NA	NA
HRS	NA	24.00(24.00, 24.00)	NA	NA
RBDSQ	NA	5.00(3.00, 8.00)	NA	NA
PHQ-9 ^a	NA	6.00(1.00, 9.00)	NA	NA

Data were presented as median (Q1, Q3).

^a Only patients who can determine clinical scales of disease severity are counted.

α Syn, α -synuclein; GAD-7, Generalized Anxiety Disorder-7; H&Y stage, Hoehn and Yahr stage; HRS, Hyposmia rating scale; MMSE, Mini-mental State Examination; MSA, Multiple system atrophy; NA, not applicable; PD, Parkinson's disease; PHQ-9, Patient Health Questionnaire-9; RBDSQ, REM Sleep Behavior Disorder Screening Questionnaire; SAA, seed amplification assay; SCOPA-AUT, The Scales for Outcomes in PD-Autonomic; UMSARS-I, Unified Multiple System Atrophy Rating Scale Part I; UMSARS-II, Unified Multiple System Atrophy Rating Scale Part II; UMSARS-III, Unified Multiple System Atrophy Rating Scale Part III; UPDRS-III, The Movement Disorder Society-Sponsored Revision of the Unified Parkinson's Disease Rating Scale Part III; Wexner, Wexner continence grading scale.

Sex (male/female)	Control (n = 70)	MSA (n = 100)	PD (n = 51)	<i>p</i>
	45/25	65/35	29/22	0.594
GAD-7 ^a	NA	3.00(0.00, 7.00)	NA	NA
MMSE ^a	NA	27.00(24.50, 29.00)	27.00(23.50, 29.00)	0.748
Data were presented as median (Q1, Q3).				
^a Only patients who can determine clinical scales of disease severity are counted.				
αSyn, α-synuclein; GAD-7, Generalized Anxiety Disorder-7; H&Y stage, Hoehn and Yahr stage; HRS, Hyposmia rating scale; MMSE, Mini-mental State Examination; MSA, Multiple system atrophy; NA, not applicable; PD, Parkinson's disease; PHQ-9, Patient Health Questionnaire-9; RBDSQ, REM Sleep Behavior Disorder Screening Questionnaire; SAA, seed amplification assay; SCOPA-AUT, The Scales for Outcomes in PD-Autonomic; UMSARS-I, Unified Multiple System Atrophy Rating Scale Part I; UMSARS-II, Unified Multiple System Atrophy Rating Scale Part II; UMSARS-III, Unified Multiple System Atrophy Rating Scale Part III; UPDRS-III, The Movement Disorder Society-Sponsored Revision of the Unified Parkinson's Disease Rating Scale Part III; Wexner, Wexner continence grading scale.				

Plasma αSyn-seeding activity distinguishes MSA patients from controls with high specificity and varies by clinical subtype

Plasma αSyn SAAs were positive in 62 of 101 MSA patients (sensitivity, 61.39%) and negative in 63 of 69 control participants (specificity, 91.30%; Table 4). Consistent with these binary outcomes, MSA patients exhibited significantly elevated ThT fluorescence ($p < 0.001$) and significantly shortened lag times ($p < 0.001$) compared with controls (Fig. 1a–c). ROC curve analysis confirmed that both ThT fluorescence and lag time effectively discriminated MSA patients from controls, with ThT fluorescence demonstrating superior discriminatory performance (AUC = 0.86, 95% CI: 0.80–0.91) relative to lag time (AUC = 0.76, 95% CI: 0.69–0.83; Fig. 1d). When comparing seeding characteristics across MSA subtypes, MSA-P exhibited significantly shorter lag times than MSA-C ($p = 0.020 < 0.05$), whereas no significant difference in ThT fluorescence intensity was observed between the two groups ($p = 0.106$; Fig. 1e, f).

Table 4
Plasma αSyn SAA results

Diagnosis	n	Plasma αSyn SAA results +/-	Sensitivity	Specificity
Control	69	6/63		91.30%
MSA	101	62/39	61.39%	

Erythrocyte αSyn-seeding activity discriminates synucleinopathies from controls with high accuracy and shows superior performance in PD

Erythrocyte αSyn SAAs yielded positive results in 70 of 100 MSA patients (sensitivity, 70.00%) and in 36 of 51 PD patients (sensitivity, 70.59%), while remaining negative in 60 of 70 healthy controls (specificity, 85.71%; Table 5). Consistent with these binary classifications, patients with synucleinopathies (both MSA and PD) exhibited significantly elevated ThT fluorescence ($p < 0.001$) and significantly shortened lag times ($p < 0.001$) compared with controls (Fig. 2a–c). However, no significant differences in either ThT fluorescence ($p = 0.643 > 0.05$) or lag time ($p = 0.632 > 0.05$) were observed between MSA and PD patients (Fig. 2b, c), nor were any significant differences detected across the MSA-C, MSA-P, and PD subgroups for either metric ($p = 0.118 > 0.05$ and $p = 0.560 > 0.05$, respectively; Supplementary Fig. 1a, b).

Table 5 Erythrocyte αSyn SAA results				
Diagnosis	n	Erythrocyte αSyn SAA results +/-	Sensitivity	Specificity
Control	70	10/60		85.71%
MSA	100	70/30	70.00%	
PD	51	36/15	70.59%	
Synucleinopathies	151	106/45	70.20%	
SUPPLEMENTARY FIGURES AND FIGURE LEGENDS				

ROC curve analysis confirmed the strong discriminatory potential of both kinetic parameters (Fig. 2d–f). When distinguishing pooled synucleinopathy patients from controls, lag time demonstrated a slightly superior performance (AUC = 0.88, 95% CI: 0.84–0.93) compared with ThT fluorescence (AUC = 0.86, 95% CI: 0.82–0.91; Fig. 2d). For the MSA versus control comparison, the AUCs were 0.84 (95% CI: 0.78–0.90) for ThT fluorescence and 0.86 (95% CI: 0.81–0.92) for lag time (Fig. 2e), whereas for PD versus controls, both ThT fluorescence and lag time achieved an AUC of 0.92 (95% CI: 0.87–0.97 and 0.87–0.98, respectively; Fig. 2f). Overall, the discriminatory performance of erythrocyte SAA was more pronounced for PD than for MSA, suggesting potential assay-dependent differences in seeding kinetics between these synucleinopathy subtypes.

Combined plasma and erythrocyte αSyn SAA synergistically enhance diagnostic accuracy for MSA

To evaluate whether the combination of erythrocyte and plasma αSyn RT-QuIC could improve diagnostic performance for MSA, we performed both assays on a subset of 80 MSA patients and 27 healthy controls (Table 3). Of the 27 controls, 20 tested negative for erythrocyte SAA and 25 for plasma SAA; of the 80 MSA patients, 59 were positive for erythrocyte SAA and 44 were positive for plasma SAA, reflecting the higher sensitivity of the erythrocyte assay in this cohort (Table 3).

We next assessed the diagnostic performance of each assay individually and in combination (Supplementary Fig. S2a, b; Supplementary Table 1). When used singly, erythrocyte SAA yielded AUCs of 0.81 (95% CI: 0.74–0.89) for ThT fluorescence and 0.85 (95% CI: 0.78–0.92) for lag time. Plasma SAA alone yielded AUCs of 0.89 (95% CI: 0.82–0.95) for ThT fluorescence and 0.74 (95% CI: 0.64–0.84) for lag time. Notably, combining the two assays resulted in markedly improved performance across both kinetic parameters: for ThT fluorescence, sensitivity of 84%, specificity of 89%, and AUC of 0.93 (95% CI: 0.89–0.98); for lag time, sensitivity of 83%, specificity of 93%, and AUC of 0.92 (95% CI: 0.86–0.97). Collectively, these findings demonstrated that combining erythrocyte and plasma α Syn SAAs significantly enhances diagnostic efficacy for MSA, outperforming either assay in isolation.

Plasma but not erythrocyte α Syn-seeding activity correlates with clinical severity and neuropsychological features in MSA

We next examined whether α Syn-seeding activity in plasma and erythrocytes was associated with clinical features in patients with MSA. In plasma SAA, ThT fluorescence showed significant positive correlations with SCOPA-AUT ($r = 0.25, p = 0.013 < 0.05$), PHQ-9 ($r = 0.23, p = 0.027 < 0.05$), and GAD-7 ($r = 0.21, p = 0.042 < 0.05$). Conversely, lag time exhibited significant negative correlations with UMSARS-I ($r = -0.28, p = 0.005 < 0.01$), UMSARS-II ($r = -0.26, p = 0.008 < 0.01$), UMSARS-IV ($r = -0.24, p = 0.023 < 0.05$), and SCOPA-AUT ($r = -0.30, p = 0.003 < 0.01$) (Fig. 3; Supplementary STable 2). No significant correlations were observed with age, age at onset, disease duration, H&Y stage, or other clinical measures.

When stratifying MSA patients by plasma SAA status, those with positive results had older age ($p = 0.038 < 0.05$) and older age at onset ($p = 0.038 < 0.05$) compared with negative cases, and also exhibited significantly higher PHQ-9 ($p = 0.026 < 0.05$) and GAD-7 ($p = 0.026 < 0.05$) scores. However, no significant differences were found between the two groups for disease duration, H&Y stage, UMSARS, SCOPA-AUT, Wexner, HRS, RBDSQ, or MMSE scores (Supplementary STable 3).

In contrast to the plasma findings, erythrocyte SAA kinetic parameters showed no significant correlations with any clinical scales in either MSA or PD patients (Supplementary STables 4, 5). Similarly, erythrocyte SAA status was not associated with any clinical scales in MSA patients (Supplementary STable 6). In PD patients, only sex ratio differed significantly between erythrocyte SAA-positive and -negative groups ($p = 0.031 < 0.05$) (Supplementary STable 7). Together, these results suggested that plasma α Syn-seeding activity, but not erythrocyte activity, was associated with clinical severity and neuropsychological burden in MSA.

Biochemical and biophysical characterization of α Syn aggregates amplified by RT-QuIC from plasma and erythrocytes

To confirm that the positive ThT fluorescence observed in our RT-QulC assays reflects the bona fide generation of α Syn aggregates formed through the prion-like recruitment and misfolding of the recombinant substrate, we conducted a multifaceted physicochemical characterization of the reaction end-products derived from both plasma and erythrocyte samples. This evaluation employed a panel of sophisticated techniques, as previously described[45, 47], including PK digestion to assess proteolytic resistance, FTA to quantify aggregate abundance, sucrose step-gradient ultracentrifugation to resolve species by density and size, and TEM for direct morphological visualization.

Characterization of plasma-derived RT-QulC end-products

We first assessed the proteolytic resistance of plasma-derived α Syn aggregates. Upon PK digestion, end-products generated from RT-QulC of MSA samples remained resistant to degradation at concentrations up to 10 μ g/mL, whereas those derived from control samples were completely digested under identical conditions (Fig. 4a). Two-way ANOVA revealed significant main effects for PK concentration ($F = 134.9$, $p < 0.001$) and diagnosis ($F = 28.02$, $p < 0.001$), alongside a significant interaction between these factors ($F = 11.39$, $p < 0.001$); protein band intensity densitometry confirmed that α Syn levels in MSA end-products were significantly higher than in controls at both 5 μ g/mL ($p < 0.001$) and 10 μ g/mL PK ($p = 0.013 < 0.05$) (Fig. 4b).

Next, we further characterized these aggregates, we employed sucrose step-gradient ultracentrifugation to profile their solubility and density. Serial fractions collected from the top (low density) to the bottom (high density) revealed that control end-products primarily sedimented in the top fractions 1–5, consistent with monomeric or small oligomeric species (Fig. 4c). In contrast, MSA-derived aggregates were detectable across all fractions, extending into the high-density bottom fractions 9–12, indicative of large, highly sedimentable aggregate species (Fig. 4d). Two-way ANOVA confirmed significant intergroup differences specifically in fractions 1–3 and 10–12 ($p < 0.05$) (Fig. 4e). Complementary FTA demonstrated a significantly greater immunoreactive α Syn signal in the MSA end-products compared to controls, indicating a higher abundance of aggregated material in the former (Fig. 4f, g).

Finally, fluorescence microscopy and TEM were employed to visualize the aggregate morphology; intense ThT fluorescence was readily observed in MSA reaction products but was absent in controls (Fig. 4h, i), and TEM imaging confirmed the presence of abundant, mature α Syn fibrils exclusively in the MSA samples (Fig. 4j, k).

Characterization of erythrocyte-derived RT-QulC end-products

We then evaluated the PK resistance of erythrocyte-derived α Syn aggregates. Notably, while the full-length 17 kDa α Syn band progressively diminished with increasing PK concentrations (1–6 μ g/mL), a smaller, PK-resistant fragment of approximately 15 kDa became clearly detectable, alongside the emergence of prominent higher-molecular-weight smears in the MSA and PD end-products (Fig. 5a). Two-way ANOVA demonstrated significant main effects of PK concentration ($F = 11.68$, $p < 0.001$) and

diagnosis ($F = 43.46$, $p < 0.001$); however, the interaction between these factors was not significant ($F = 1.315$, $p = 0.233$). Quantitative analysis of band intensities by densitometry confirmed significant differences between the combined disease groups (MSA and PD) and controls across all PK concentrations (1–6 $\mu\text{g/mL}$; $p < 0.05$), though no significant differences were detected between MSA and PD (Fig. 5b).

Corroborating these findings, FTA revealed significantly higher αSyn band intensities in both MSA and PD end-products relative to normal controls, again with no statistical distinction between the two disease groups (Fig. 5c, d). Consistent with the biochemical data, intense ThT fluorescence was visually apparent in both MSA and PD RT-QuIC end-products, but not in controls (Fig. 5e–g). Final ultrastructural examination via TEM confirmed the presence of mature, fibrillar αSyn assemblies in MSA and PD samples, whereas control reactions contained mainly non-fibrillar material (Fig. 5h–j).

DISCUSSION

In this study, we established and validated two novel blood-based αSyn RT-QuIC assays, one targeting plasma and the other erythrocytes, for the diagnosis of MSA and other synucleinopathies. Our findings demonstrate that both assays can effectively discriminate patients with MSA from healthy controls, with the erythrocyte assay showing superior sensitivity and the plasma assay providing exceptional specificity. Critically, we show that combining these two assays yields a synergistic enhancement in diagnostic accuracy, achieving an AUC of 0.93, which is comparable to the performance of CSF-based αSyn -SAA in PD and DLB [27–30]. This work offers a transformative, minimally invasive approach that overcomes the logistical and clinical barriers associated with CSF collection, potentially enabling widespread screening and surveillance.

The development of a high-performing erythrocyte-based SAA is a key innovation of this study. Erythrocytes are the primary peripheral reservoir of αSyn , yet their potential as a seed source for amplification assays has remained largely unexplored[6–8]. By incorporating an IP enrichment step into our RT-QuIC protocol, we successfully amplified seeding activity from erythrocyte lysates, achieving a sensitivity of 70% for both MSA and PD. This sensitivity, combined with a specificity of 85.7%, positions the erythrocyte assay as an effective diagnostic tool. Interestingly, while the erythrocyte assay exhibited slightly better overall sensitivity than the plasma assay for MSA (70.0% vs. 61.4%), the plasma assay's specificity was markedly higher (91.3% vs. 85.7%). This reciprocal performance profile suggests that the two biological matrices may harbor distinct conformational strains or states of αSyn aggregates, a concept consistent with the "strain hypothesis" of synucleinopathies [48, 49]. For instance, erythrocyte aggregates might be more abundant, making them easier to amplify (higher sensitivity), but they may also be more prone to non-specific amplification in some controls. Conversely, the stringent seeding requirements for plasma amplification may reflect a more pathologically specific, advanced conformational state that is less prevalent in healthy individuals. This hypothesis is supported by our physicochemical characterization, where plasma-derived MSA aggregates demonstrated increased PK resistance at high concentrations (10 $\mu\text{g/mL}$), a hallmark of highly stable, pathological conformers.

Our multifaceted biophysical characterization unequivocally confirms that the positive ThT fluorescence in our assays corresponds to the generation of bona fide α Syn aggregates. The PK resistance, high-density sedimentation profiles, and the presence of mature fibrils visualized by TEM in MSA and PD end-products are all classical features of prion-like, misfolded α Syn [29, 37, 45, 47]. These data collectively rule out non-specific fluorescence artifacts and provide compelling evidence that the RT-QuIC reaction faithfully propagates the pathological seed present in the patient's blood.

A particularly novel and clinically significant finding is the differential correlation of seeding activity with clinical features. Plasma-derived seeding kinetics (ThT fluorescence and lag time) showed significant correlations with core clinical outcome measures, including UMSARS motor scores and SCOPA-AUT for autonomic dysfunction, as well as with anxiety (GAD-7) and depression (PHQ-9) scales. In contrast, erythrocyte-derived seeding activity showed no correlation with any clinical parameters. This divergence has profound implications. It suggests that plasma α Syn seeds, perhaps originating from the brain or from systemic neurodegeneration, are more closely tied to the active, progressive phase of the disease, reflecting ongoing neuronal and glial dysfunction [50–53]. Conversely, erythrocytes might act as a persistent "sink" or repository for α Syn aggregates, accumulating them over time in a manner that does not directly mirror the current disease state or severity. The lack of correlation with disease duration supports this idea, as erythrocyte seeding activity might plateau early or be a cumulative measure of exposure to pathological α Syn. This distinction enhances the clinical utility of our dual-assay strategy: the erythrocyte assay could serve as a robust diagnostic test [8, 12], while the plasma assay could function as a valuable prognostic or staging biomarker to monitor disease progression and therapeutic response.

The strengths of this study include its well-characterized cohort, the rigorous use of orthogonal biochemical methods to validate assay specificity, and the direct comparison of two blood-based matrices within the same patients. However, several limitations must be acknowledged. First, the control group for the erythrocyte cohort was significantly younger than the patient groups, which, although a common challenge in clinical studies, could introduce a bias. Nevertheless, the robust ROC analysis and the fact that SAA positivity did not correlate with age within controls suggest this may not have a major impact. Second, the study was cross-sectional in design, limiting our ability to assess the assay's prognostic value in predicting disease conversion from prodromal stages or its sensitivity to change over time. Future longitudinal studies are essential to address these questions. Third, while our performance is promising, we did not directly compare our blood-based assays head-to-head with CSF α Syn-SAA in the same cohort, which would be a crucial next step to determine the relative and additive clinical value. Finally, the precise biological origin of the plasma seeds remains speculative. Whether they are directly released from the brain via the glymphatic system or originate from other peripheral sources, such as the skin or the erythrocytes themselves, requires further investigation [8, 33, 45, 54].

In conclusion, this work reveals erythrocytes as a viable and potent matrix for seed amplification, and the combined assay of erythrocytes and the plasma offers a practical, minimal-invasive alternative to lumbar puncture that could be rapidly integrated into clinical workflows. The divergent clinical correlations of

plasma versus erythrocyte seeding activity provide a new framework for understanding the systemic spread and heterogeneity of α Syn pathology. This dual-assay approach holds substantial promise for improving the early diagnosis, differential diagnosis, and clinical monitoring of patients with MSA and other synucleinopathies, and merits further validation in larger, multicenter, longitudinal cohorts.

Abbreviations

α Syn α -synuclein

AUC area under the curve

ANOVA analysis of variance

CSF cerebrospinal fluid

DLB Dementia with Lewy bodies

FTA filter trap assay

GCI glial cytoplasmic inclusions

GAD-7 Generalized Anxiety Disorder-7

HRS Hyposmia rating scale

H&Y stage Hoehn and Yahr stage

IP Immunoprecipitation

IQR interquartile range

iRBD idiopathic REM sleep behavior disorder

MMSE Mini-mental State Examination

MSA Multiple system atrophy

MSA-C MSA with predominant cerebellar ataxia

MSA-P MSA with predominant Parkinsonism

PD Parkinson's disease

PDD Parkinson's disease dementia

PHQ-9 Patient Health Questionnaire-9

PK proteinase K

p-αSyn phosphorylated αSyn

RBDSQ REM Sleep Behavior Disorder Screening Questionnaire

RT-QuIC real-time quaking-induced conversion

ROC receiver operating characteristic

REM rapid eye movement

SAA seed amplification assay

SD standard deviation

SCOPA-AUT The Scales for Outcomes in PD-Autonomic

TEM transmission electron microscopy

ThT Thioflavin T

UMSARS-I Unified Multiple System Atrophy Rating Scale Part I

UMSARS-II Unified Multiple System Atrophy Rating Scale Part II

UMSARS-III Unified Multiple System Atrophy Rating Scale Part III

UPDRS-III The Movement Disorder Society-Sponsored Revision of the Unified Parkinson's Disease Rating Scale Part III

Wexner Wexner continence grading scale

Declarations

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Author's contributions

WQZ and HJ contributed to the conception and design of the work. XL performed all αSyn-related experiments. HY helped set up the initial αSyn RT-QuIC work. XL, LW, DC, RO, KD, XD, XW and LP selected and characterized patients for blood collection. XL, DC, RO, KD, XD, and XW processed plasma and

erythrocytes samples. ML and YG provided recombinant human α Syn for RT-QulC analyses. HY, YC, HW, and ZJ performed part of RT-QulC and Western blot analyses. ZZ and YW provided technical assistance during the study. XL, LW and HY performed statistical analysis. LW, ZC, CW, LP and RQ analyzed the data. All authors were responsible for the acquisition, analysis and interpretation of data. XL, HJ and WQZ wrote the first version of the manuscript. Critical revision of the manuscript for important intellectual content was performed by all authors. All authors agreed with the content of the article to be submitted. All authors reviewed and approved the final manuscript.

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Data availability

The raw data that supports the findings of this study are available from the corresponding authors upon request.

Ethics approval and consent to participate

The entire approval for this study was obtained from the ethic committees of the Xiangya Hospital of Central South University and the First Affiliated Hospital of Nanchang University (Approval No.202310206 and Approval No. IIT 2023 Clinical Ethics Review No. 145-2). Following the Declaration of Helsinki, written informed consent was obtained from all participants or their guardians.

Consent for publication

Not applicable.

Competing interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Figures

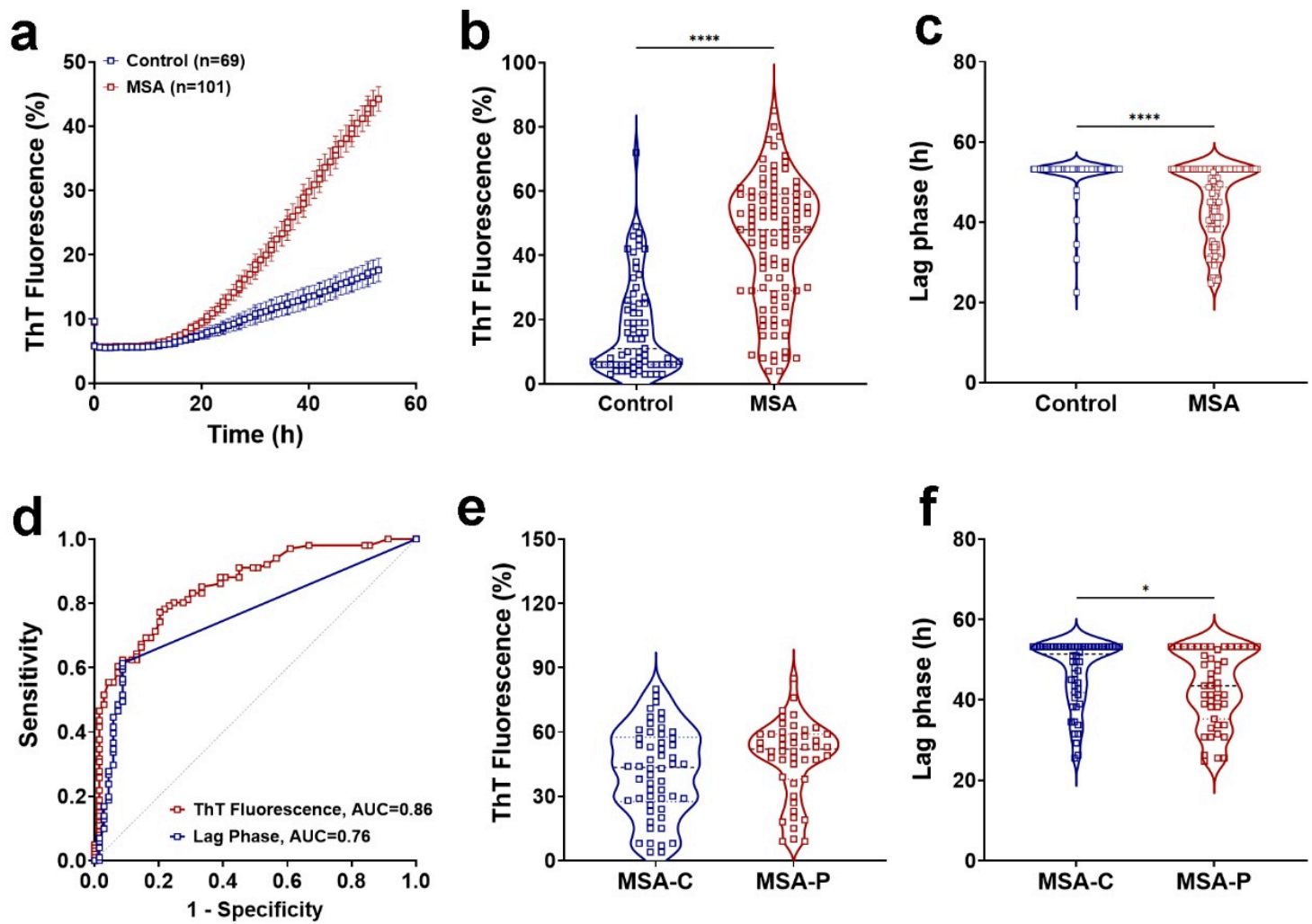


Figure 1

α Syn-seeding activity of plasma in different groups measured by RT-QulC. **a.** Kinetic curves showing the ThT fluorescence (mean $\pm$ standard error) over time for seed amplification in plasma samples from control (n = 69) and MSA (n = 101) groups. **b.** Comparison of end-point ThT fluorescence of plasma α Syn RT-QulC between control and MSA groups (Mann-Whitney U test). **c.** Comparison of the lag phase of plasma α Syn RT-QulC between control and MSA groups (Mann-Whitney U test). **d.** ROC curves for plasma α Syn RT-QulC to distinguish between control and MSA groups. **e.** Comparison of end-point ThT fluorescence of plasma α Syn RT-QulC between MSA-C and MSA-P groups (Mann-Whitney U test). **f.** Comparison of the lag phase of plasma α Syn RT-QulC between MSA-C and MSA-P groups (Mann-Whitney U test).

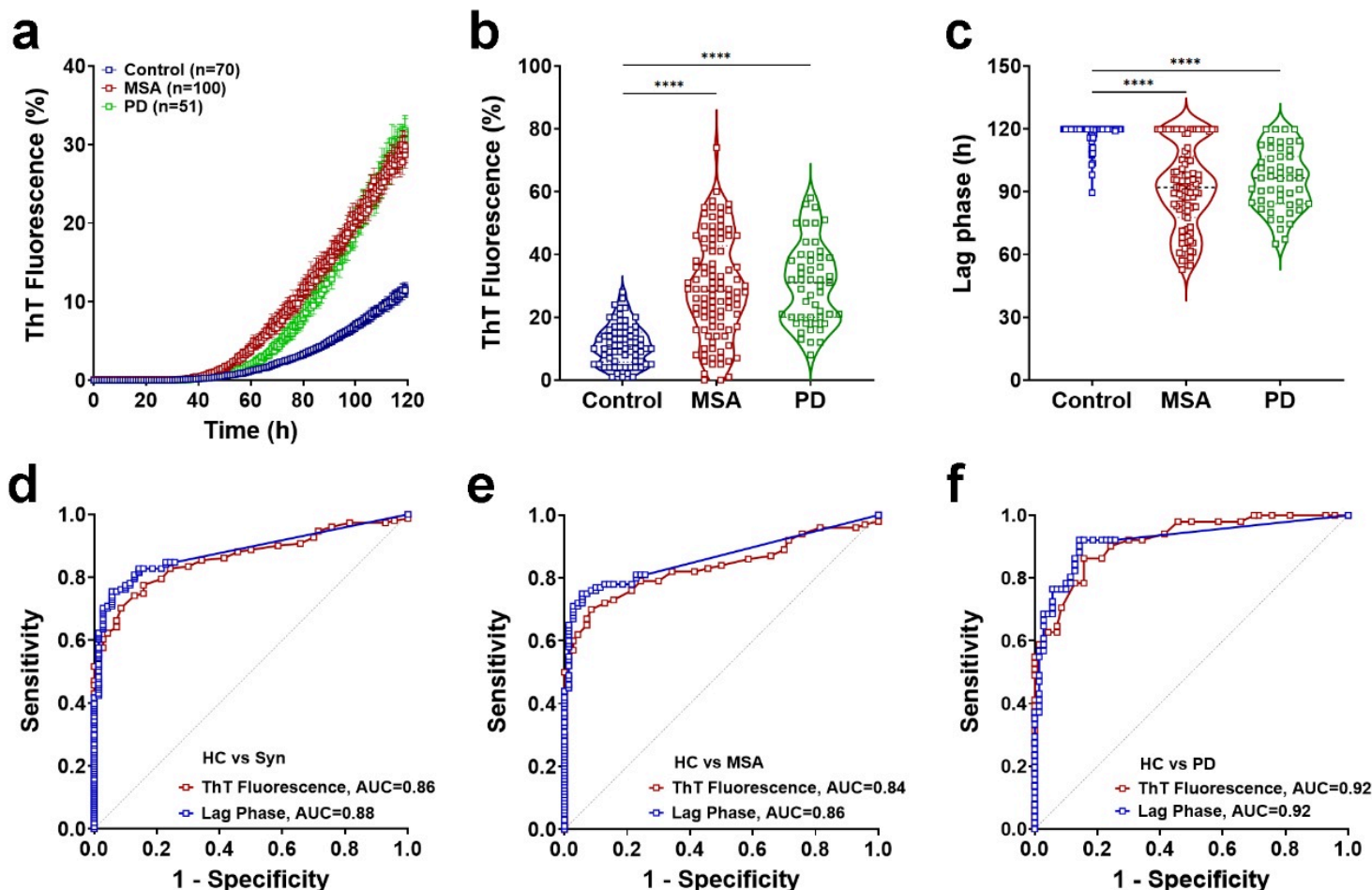


Figure 2

αSyn-seeding activity of erythrocytes in different groups measured by RT-QulC. **a.** Kinetic curves showing the ThT fluorescence (mean $\pm$ standard error) over time for seed amplification in erythrocyte samples from control (n = 70), MSA (n = 100), and PD (n = 51) groups. **b.** Comparison of end-point ThT fluorescence of erythrocyte αSyn SAA between control, MSA, and PD groups (one-way ANOVA). **c.** Comparison of the lag phase of erythrocyte αSyn SAA between control, MSA, and PD groups (Kruskal-Wallis test). **d.** ROC curves for erythrocyte αSyn SAA to distinguish between control and synucleinopathies groups. **e.** ROC curves for erythrocyte αSyn SAA to distinguish between control and MSA groups. **f.** ROC curves for erythrocyte αSyn SAA to distinguish between control and PD groups. **** $p < 0.0001$.

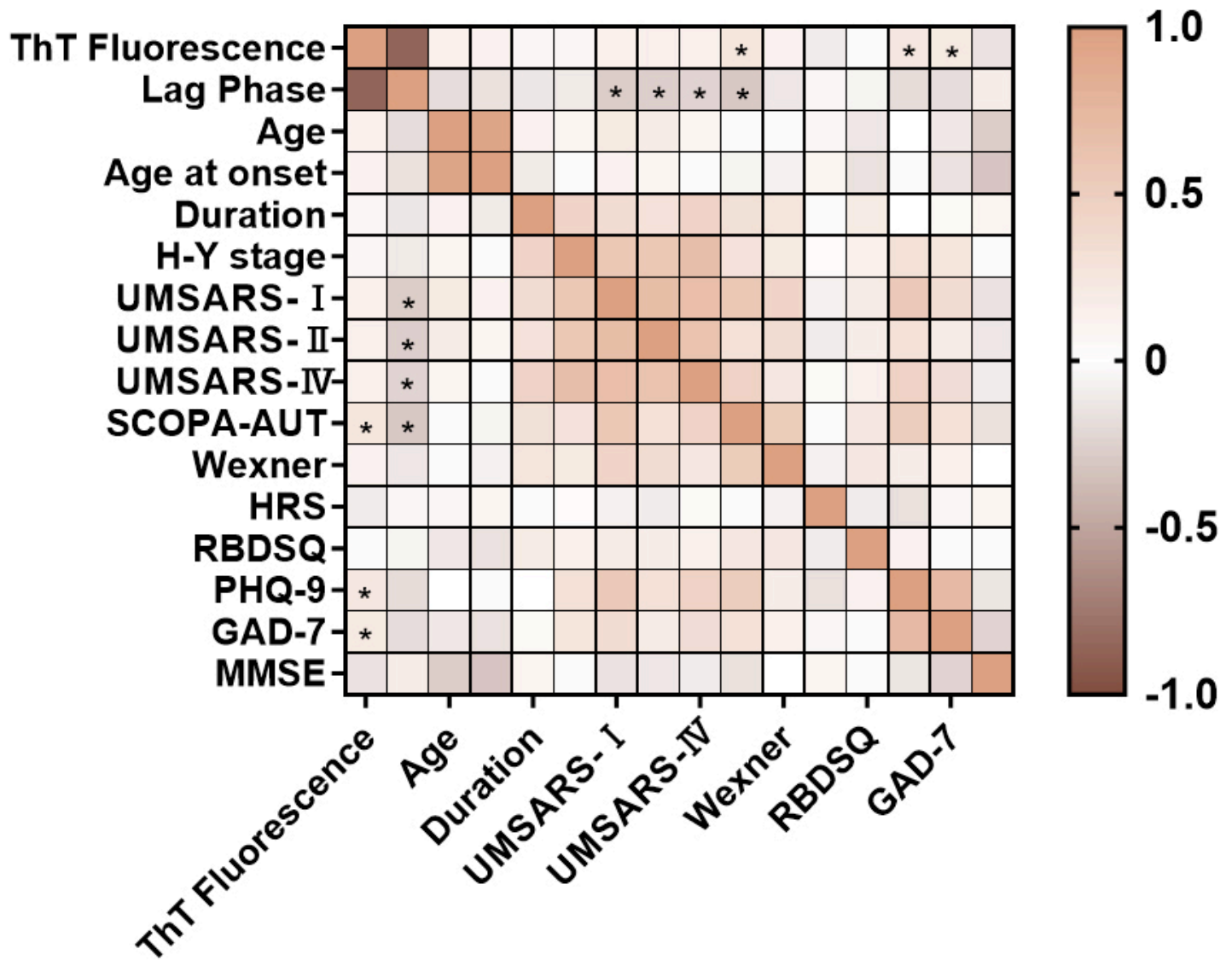


Figure 3

Correlation analysis of plasma αSyn-seeding activity with clinical characteristics in MSA.

Spearman's rank correlation was used to assess correlations between plasma αSyn-seeding activity and clinical characteristics in MSA. * $p < 0.05$; **** $p < 0.0001$. AUC, area under the curve; GAD-7, Generalized Anxiety Disorder-7; H&Y stage, Hoehn and Yahr stage; HRS, Hyposmia rating scale; MMSE, Mini-mental State Examination; MSA, Multiple system atrophy; PHQ-9, Patient Health Questionnaire-9; RBDSQ, REM Sleep Behavior Disorder Screening Questionnaire; SCOPA-AUT, The Scales for Outcomes in PD-Autonomic; UMSARS-I, Unified Multiple System Atrophy Rating Scale Part I; UMSARS-II, Unified Multiple System Atrophy Rating Scale Part II; UMSARS-III, Unified Multiple System Atrophy Rating Scale Part III; Wexner, Wexner continence grading scale.

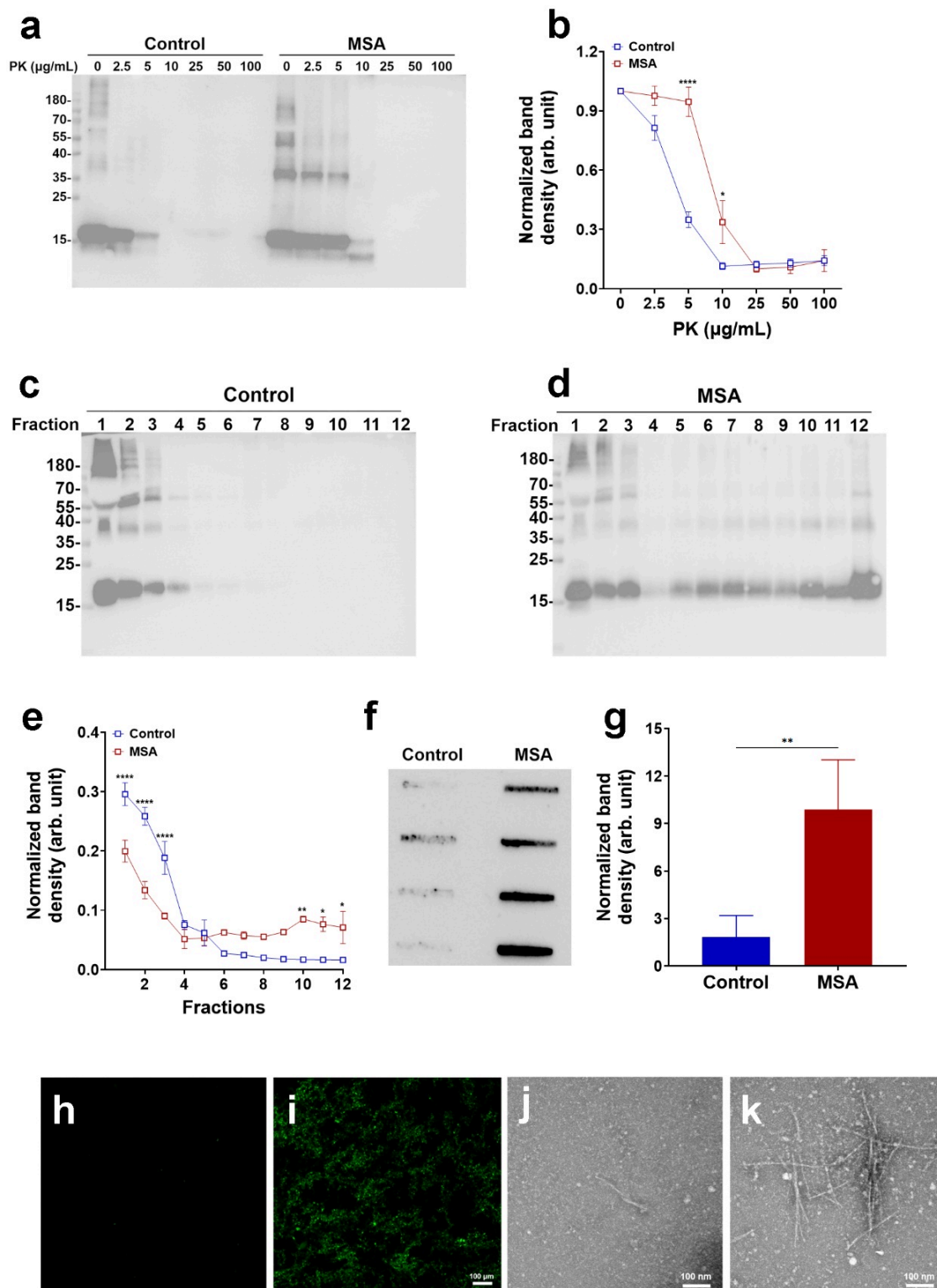


Figure 4

Biochemical and structural analysis of plasma αSyn RT-QuIC end-products.

a. Representative Western blots of plasma αSyn SAA end products from MSA (n = 3) and control (n = 3) samples following treatment with different concentrations of PK at 0, 2.5, 5, 10, 25, 50, and 100 μg/mL, probed with anti-αSyn antibody MJFR1. **b.** Quantification of αSyn band intensity from Western blots of

PK-treated plasma α Syn SAA end-products of MSA (n = 3) and control (n = 3) samples (two-way ANOVA). **c & d.** Representative Western blots of sucrose gradient sedimentation fractions of plasma α Syn SAA end-products from MSA (n = 3) and control (n = 3) samples, probed with anti- α Syn antibody MJFR1. **e.** Quantitative Western blot analysis of sucrose gradient sedimentation fractions of plasma α Syn SAA end-products from MSA (n = 3) and control (n = 3) samples (two-way ANOVA). **f & g.** FTA and quantification of α Syn aggregates in plasma α Syn SAA end-products from MSA (n = 4) and control (n = 4) samples, probed with the conformation-specific antibody MJFR14 (one-way ANOVA). **h.** Fluorescence image of plasma α Syn SAA end-products from control. **i.** Fluorescence image of plasma α Syn SAA end-products from MSA. **j.** TEM image of plasma α Syn SAA end-products from control. **k.** TEM image of plasma α Syn SAA end-products from MSA. Scale bar in panels **h & i**: 100 μ m and panels **j & k**: 100 nm. * p < 0.05; ** p < 0.01; **** p < 0.0001.

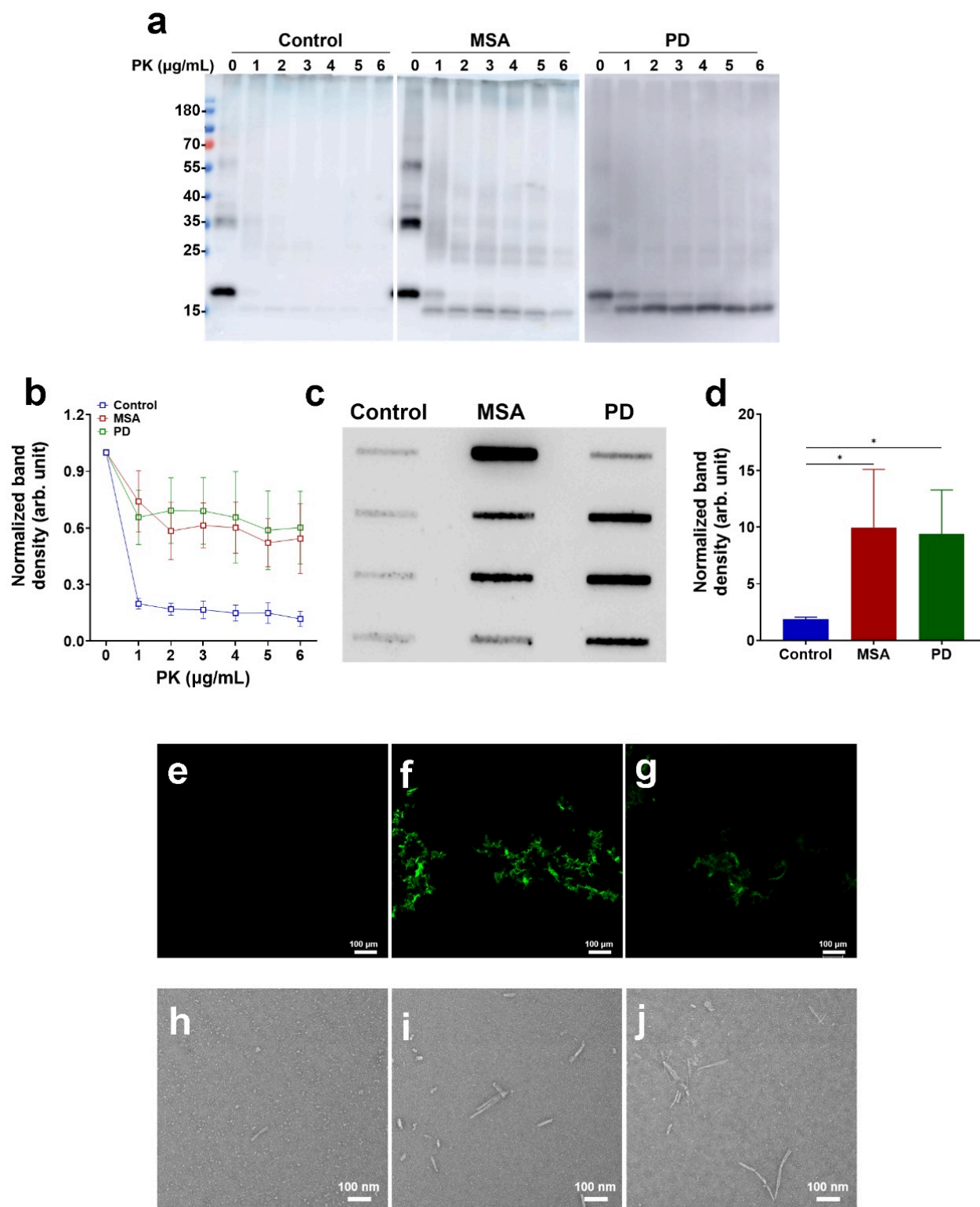


Figure 5

Biochemical and structural analysis of erythrocyte αSyn RT-QulC end products. **a.** Representative Western blots of erythrocyte αSyn SAA end products from MSA (n = 3), PD (n = 3), and control (n = 6) samples following treatment with different concentrations of proteinase K, ranging from 0, 1, 2, 3, 4, 5, and 6 μg/mL, probed with anti-αSyn antibody MJFR1. **b.** Quantification of αSyn band intensity from Western blots of PK-treated erythrocyte αSyn SAA end products of MSA (n = 3), PD (n = 3), and control (n

= 6) samples (two-way ANOVA). **c.** and **d.** FTA and quantification of α Syn aggregates in erythrocyte α Syn SAA end products from MSA (n = 4), PD (n = 4), and control (n = 4) samples, probed with the conformation-specific antibody MJFR-14-6-4-2 (one-way ANOVA). **e.** Fluorescence image of erythrocyte α Syn SAA end products from control. **f.** Fluorescence image of erythrocyte α Syn SAA end products from MSA. **g.** Fluorescence image of erythrocyte α Syn SAA end products from PD. **h.** TEM image of erythrocyte α Syn SAA end products from control. **i.** TEM image of erythrocyte α Syn SAA end products from MSA. **j.** TEM image of erythrocyte α Syn SAA end products from PD. Scale bar in panels **e-g**: 100 μ m and panels **h-j**: 100 nm. * $p < 0.05$.

Supplementary Files

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