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Aggregated α-synuclein in erythrocytes as a potential biomarker for idiopathic Parkinson's Disease

Konstantina Dimoula [a], Nikolaos Papagiannakis [b,c], Matina Maniati [c], Leonidas Stefanis [b,c], Evangelia Emmanouilidou [a,*]

[a] Laboratory of Biochemistry, Department of Chemistry, National and Kapodistrian University of Athens, Athens, Greece
[b] First Department of Neurology, Eginition Hospital, Medical School, National and Kapodistrian University of Athens, Athens, Greece
[c] Center of Clinical Research, Experimental Surgery and Translational Research, Biomedical Research Foundation of the Academy of Athens, Athens, Greece

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ABSTRACT

Background: Mostly known for its implication in synucleinopathies, including Parkinson's disease (PD), α-synuclein is predominantly expressed in the nervous system. Most of the peripheral α-synuclein is found in erythrocytes, and several studies have examined a possible association between erythrocytic α-synuclein and PD.
Methods: We have used a recently developed ELISA that selectively detects fibrillar and oligomeric α-synuclein to measure aggregated α-synuclein in red blood cells (RBCs) collected from PD patients and age/sex-matched control individuals (n = 35). The PD group included patients without any common mutation (genetically undetermined group, GU-PD, n = 56) as well as mutation carriers in the α-synuclein gene (A53T-PD, n = 28) and glucocerebrosidase gene (GBA-PD, n = 24).
Results: We found that the concentration of aggregated α-synuclein in erythrocytes was significantly increased in GU-PD patients compared to controls. A53T-PD and GBA-PD patients exhibited similar levels of erythrocytic aggregated α-synuclein as the control group. The levels of fibrillar/oligomeric α-synuclein in RBCs were reduced in respect to the age of control individuals suggesting that the observed increase in the GU-PD cohort was not due to normal aging. Parallel assessment of monomeric α-synuclein revealed that aggregated, but not total, α-synuclein could discriminate PD patients from control individuals.
Conclusions: The elevation of aggregated α-synuclein in GU-PD erythrocytes, which is not related to aging, suggests that these forms may be indicative of PD pathology and possibly accumulate upon disease establishment. As such, aggregated α-synuclein in RBCs could be a potential biomarker for PD diagnosis.

1. Introduction

Parkinson's Disease (PD) is one of the most prevalent neurodegenerative conditions causing motor and non-motor symptoms. PD pathology is associated with the presence of dense protein inclusions that gradually accumulate in dopaminergic neurons in the nigrostriatal axis, as well as the brainstem and, at later stages, the cortex [1]. These filamentous inclusions, called Lewy Bodies (LBs) and Lewy Neurites (LNs), primarily consist of aggregated α-synuclein, a small presynaptic protein that is abundant throughout the central nervous system (CNS) under normal conditions [2]. α-Synuclein is also genetically linked to PD pathology since point mutations as well as multiplications of the locus encoding for α-synuclein leads to early onset and familial PD [3]. Therefore, aggregated α-synuclein could potentially serve as a selective biomarker for the development and progression of PD pathology.

Most studies report that levels of oligomeric α-synuclein seem to be elevated in the cerebrospinal fluid (CSF) of PD patients [4–7] and the ratio of oligomeric to total α-synuclein seems to better support this trend [6,8]. However, CSF aggregated α-synuclein is not detectable by simple immunosorbent assays, unless there is amplification as performed in seeding amplification assays [9–11], rendering the CSF a challenging biomarker source.

Research efforts are focusing on peripheral biomarkers moving from CSF to blood mostly due to its convenient collection. Almost all erythroid precursors, megakaryocytes, platelets, lymphocytes, natural killer cells, monocytes and macrophages express α-synuclein. Erythrocytes, known as red blood cells (RBCs), contain >99 % of blood α-synuclein, almost ~1000-fold higher than the quantity found in the CSF

* Corresponding author.
E-mail address: eemman@chem.uoa.gr (E. Emmanouilidou).

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[12,13]. The notion that peripheral α-synuclein can reflect pathological changes in the CNS is mostly based on its expected diffusion through the damaged blood brain barrier (BBB). Independently from BBB impairment that is characteristic of most neurodegenerative conditions, α-synuclein has been shown to navigate bidirectionally from the blood to the BBB in a free form and via extracellular vesicles [14,15]. Even though present in erythrocytes, the exact forms of α-synuclein aggregates in RBCs are not fully characterized. RBCs contain SDS-stable dimers in their membranes that can be observed by western blotting [16,17] but probably lack tetrameric α-synuclein as revealed using a small-angle X-ray solution scattering method in RBC samples [18].

To date, PD diagnosis is well established through the clinical assessment of a series of motor and non-motor symptoms. However, by the time motor symptomatology becomes evident, 60 % of neurons in substantia nigra pars compacta (SNc) are thought to be lost indicating that clinical diagnosis is not aligned with the onset of the disease [19]. This underlies the necessity of a sensitive disease-predictive biomarker. The potential of total, oligomeric or phosphorylated α-synuclein in RBCs to act as a diagnostic biomarker for synucleinopathies or dementia has been examined in several studies [20–26]. In particular, the measurement of oligomeric α-synuclein in RBCs, which reflects the presence of pathological conformers, was found to be elevated in the RBCs from PD patients compared to controls suggesting that peripheral aggregated α-synuclein could have diagnostic value for PD [20,23,26]. Here, we have used a recently developed conformation-specific ELISA to detect fibrillar and oligomeric α-synuclein in lysed RBCs from PD patients and control individuals. We found that the levels of high order synuclein multimers contained in erythrocytes, which tend to decrease during normal aging, could discriminate PD patients from controls. Our data support previous findings and suggest that aggregated, but not total, α-synuclein, in RBCs can be informative for PD diagnosis.

2. Materials and methods

2.1. Participants

Patients with PD (either genetic or non-genetic forms) and age-matched healthy subjects were enrolled from Attikon University Hospital and Eginitio University Hospital in Athens, Greece. Some additional participants harboring the G209A/A53T mutation in the SNCA gene (A53T-PD), were separately recruited in the MEFOPA study (MEndelian FOrms of PArkinsonism). PD patients negative for the A53T variant of the SNCA gene were screened for the presence of GBA variants, and were divided accordingly into two categories: patients without any known mutations in SNCA or GBA genes (Genetically-Undetermined PD patients, GU-PD) and patients harboring GBA mutations (GBA-PD). Enrolled healthy controls had no known family history of PD. They were not screened for any of the aforementioned mutations. All study procedures were approved by the scientific council and ethical committees of the respective hospitals and all the participants provided written informed consent. A total of 143 participants were recruited. Demographic information about all participants is summarized in Table 1.

2.2. Clinical assessment

All the key clinical features of the patients enrolled in the study are summarized in Table 2. The motor and cognitive performance of the patients was evaluated with the use of the Unified Parkinson's Disease Rating Scale (UPDRS Part-III) and the Mini-Mental State Examination (MMSE) test, respectively. Clinical assessment was conducted the same day with blood sample collection. All PD patients (with the exception of only one patient in the GU-PD group) were under dopaminergic replacement therapy as indicated by the calculated Levodopa Equivalent Daily Dose (LEDD) score computed based on the work of Jost et al. [27].

Table 1
Demographic information about enrolled subjects by group[a].

	Control	GU-PD	A53T-PD	GBA-PD
Number	35	56	28	24
Sex (M/F)	16/19	31/25	11/17	15/9
Age at study (years)	60.1 (11.8)	58.5 (12.3)	52.4 (13.8)	57.0 (11.1)

[a] Data represents mean (standard deviation).

Table 2
Clinical characteristics of enrolled patients by group[a].

	GU-PD	A53T-PD	GBA-PD
Number	56	28	24
Age of onset (years)	52.2 (13.1)	47.8 (11.8)	50.6 (9.3)
PD duration (years)	6.2 (5.6)	4.0 (4.2)	6.4 (5.7)
UPDRS Part-III score	14.4 (8.0)	20.6 (26.2)	19.8 (8.7)
MMSE score	27.4 (4.6)	26.0 (6.2)	28.0 (2.2)
LEDD score	519 (263)	597 (391)	540 (255)

[a] Data represents mean (standard deviation).

2.3. Erythrocyte collection and isolation

RBCs were isolated from blood samples collected with EDTA-coated tubes (BD vacutainer) through spinning at 2200×*g* for 10 min at 4 °C followed by three washing steps with 1 vol of 0.9 % NaCl [16]. Following isolation, RBCs were mixed with an equal volume of 0.9 % NaCl and stored at −80 °C. Cell lysis was performed upon thawing the RBC samples twice at ambient temperature. Total cell RBC lysates containing both membranous and cytoplasmic proteins were used for all experimental procedures.

2.4. Measurement of protein concentration

Total protein concentration of lysed RBCs was measured by the Bradford assay using bovine serum albumin (BSA) as protein standard (Biorad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

2.5. ELISA for aggregated α-synuclein

The ELISA used to quantify α-synuclein aggregates has been described previously [28]. Shortly, 96-well plates (Corning Costar, Glendale, AZ, USA) were coated overnight at 25 °C with 1 μg/ml of mouse monoclonal antibody Syn-F2 (50 μl/well) in 100 mM $NaHCO_3$ (pH 9.5). The plates were washed three times with wash buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.1 % Tween-20). Recombinant α-synuclein pre-formed fibrils (PFFs) as standards and RBC samples were appropriately diluted in 10 mM Tris-Cl, pH 7.6, 100 mM NaCl, 0.1 % Tween-20 and 1 % BSA (TBS-T/BSA buffer), loaded on plates (50 μl/well) and incubated for 2.5 h at 37 °C on a shaker. The generation and characterization of PFFs have been described previously [29]. PFF storage and handling were performed as described (Anagnostou et al., 2023). After three washes, the rabbit monoclonal antibody MJFR-14-6-4-2 (Abcam, Cambridge, UK) was used at a concentration of 74 ng/mL in TBS-T/BSA buffer and plates were incubated shaking for 1 h at 25 °C. The plates were washed 3 times and the anti-rabbit IgG-HRP antibody (Dako Agilent, Santa Clara, CA, USA) was added at a concentration of 16.7 ng/mL in TBS-T/BSA buffer for 30 min at 4 °C. After three washes, 50 μl/well of HRP substrate (Luminata Crescendo ELISA HRP chemiluminescent substrate, Merck Millipore) was added and chemiluminescence was measured using a Biotek Synergy H1 multimode reader (Agilent, Santa Clara, CA, USA) after incubation for 5 min at 25 °C.

2.6. ELISA for total α-synuclein

The ELISA used to quantify total α-synuclein has been described previously and has been validated in human CSF and blood plasma and

serum [28,30,31].

2.7. Statistical analysis

Statistical analysis was performed using the GraphPad Prism 8.02 software. All measurements were analyzed with descriptive statistics and results were presented as mean ± Standard Error of the Mean (SEM). All data underwent a normality (Shapiro–Wilk) test. Normally distributed variables were analyzed by unpaired Student's *t*-test whereas non-parametric variables were compared using the Mann Whitney test. Kruskal-Wallis test followed by a Dunn's multiple comparisons test was used for skewed data to compare differences in more than two studied groups. Pearson's or Spearman's method was used to correlate aggregated α-synuclein concentration with participants' age and clinical characteristics. The p-value threshold was set at <0.05.

3. Results

To evaluate the use of aggregated α-synuclein present in erythrocytes as a potential biomarker for PD, we assessed the levels of fibrillar and oligomeric α-synuclein in homogenates from RBCs collected from a cohort of PD patients including 56 patients without any common mutation (GU-PD), 28 patients carrying the A53T SNCA mutation (A53T-PD), and 24 patients carrying one of D409H, H255Q, L444P and N370S GBA mutations (GBA-PD). The results from the PD groups were compared with a group of 35 age/sex-matched control individuals (control). The demographic and clinical characteristics of the groups enrolled in the study are presented in Table 1. Aggregated α-synuclein was measured in lysed RBCs using a conformation-specific ELISA recently developed in our laboratory that selectively detects fibrillar and oligomeric α-synuclein [28]. The specificity of the assay in erythrocytes was confirmed by measuring five serial dilutions (10, 50, 100, 200 and 500-fold) of an RBC sample from a control individual. Our measurements showed that the signal for aggregated α-synuclein was conversely proportional to dilution fold as expected (Fig. 1A). The 50-fold dilution was followed for the assessment of the RBC samples due to higher signal-to-noise ratio compared to the other dilutions. Since RBCs constitute a complicated biological sample, we also examined the background interference in a 50-fold diluted RBC sample. The concentration of aggregated α-synuclein in an RBC sample was assessed before and after the spiking of 1.2 and 3.6 ng/ml of fibrillar α-synuclein (Fig. 1B). The concentration of aggregated α-synuclein measured directly from the 50-fold diluted sample did not differ from the one estimated through the standard addition method indicating that the inherent background of 50-fold diluted erythrocytes did not affect the ELISA results. Finally, measurement of total protein using the Bradford assay showed similar distributions in GU-PD vs control RBC samples (mean values 154.8 ± 10.7 and 142.3 ± 9.5 mg/ml for the control and GU-PD groups, respectively) suggesting comparable conditions of storage and handling for samples in both cohorts (Fig. 1C).

We found a significant increase in RBC-associated aggregated α-synuclein in the GU-PD group vs the control group (0.197 ± 0.019 and 0.369 ± 0.043 ng aggregated α-synuclein/mg total protein, for control vs GU-PD, respectively) (Fig. 2A). Similar analysis showed that the levels of aggregated α-synuclein in the A53T-PD and GBA-PD groups were similar to the control group (0.222 ± 0.022 and 0.252 ± 0.067 ng/mg, for GBA-PD and A53T-PD, respectively) (Fig. 2B). We next assessed the potential ability of aggregated α-synuclein contained in RBCs to discriminate PD patients from controls compared with total α-synuclein. We measured fibrillar/oligomeric and total α-synuclein in 9 RBC samples from participants from the control and GU-PD groups using two different ELISA assays (Fig. 2C). Our data revealed that aggregated, but not total, α-synuclein could differentiate PD patients from controls. The use of aggregated:total ratio further reinforced the discrimination capacity in the same RBC samples (Fig. 2D). For the assessment of both aggregated and total α-synuclein, the concentrations were normalized to total erythrocytic protein since the erythrocyte content of blood samples is reported to have substantial variability by subject [23].

We found a negative correlation, albeit with a low rho value, between aggregated α-synuclein levels and the age of control participants suggesting that α-synuclein aggregates in RBCs tend to decrease during normal aging (Fig. 3A). There was no such correlation in the cohort of GU-PD patients, possibly due to the accumulation of these forms upon disease development and progression (Fig. 3B). We also found that the levels of aggregated α-synuclein in PD RBCs were not related to the age of disease onset or disease duration in the GU-PD group (Fig. 3C and D). To further reinforce this observation, we further examined potential correlations of aggregated α-synuclein in RBCs with clinical features reflecting the motor and cognitive performance of the patients in the GU-PD group. Our analysis suggested that aggregated α-synuclein is not linked with either UPDRSIII or MMSE scores (Fig. 3E and F). Extension of this analysis for the A53T-PD and GBA-PD groups yielded similar results (Supplementary Figs. 1 and 2).

4. Discussion

The idea that pathological forms of peripheral α-synuclein could provide useful information about the CNS has been neglected for years. Even though the high levels of α-synuclein in RBCs are related to its erythroid origin, various forms of neuronal α-synuclein are thought to escape from the distorted BBB and can circulate at low levels in peripheral tissues including blood plasma [32,33]. These forms of α-synuclein, which can be informative for CNS pathology, can be actively transported into RBCs via receptor-dependent endocytic pathways as

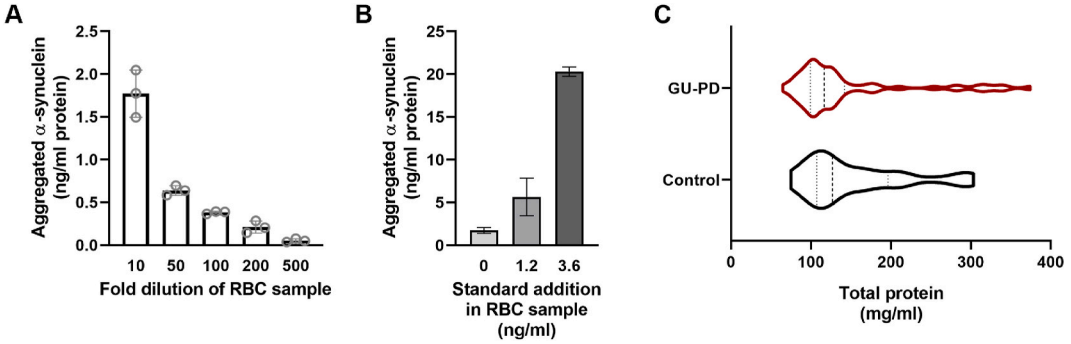

Fig. 1. Specificity of the measurements using the conformation-specific ELISA in lysed RBCs. (A) Assessment of serial dilutions of a control RBC sample. Dilutions were made in TBS-T/BSA buffer and measured in triplicate. (B) Assessment of a PD RBC sample before and after the addition of 1.2 or 3.6 ng/ml PFFs. Aggregated α-synuclein after the PFF addition was estimated using the standard addition method. Data in A and B are presented as mean ± SEM. (C) Measurement of total protein concentration in lysed RBCs from the control (n = 34) and GU-PD (n = 56) groups using the Bradford method. Statistics were performed by Student's t-test (*p=0.4024*).

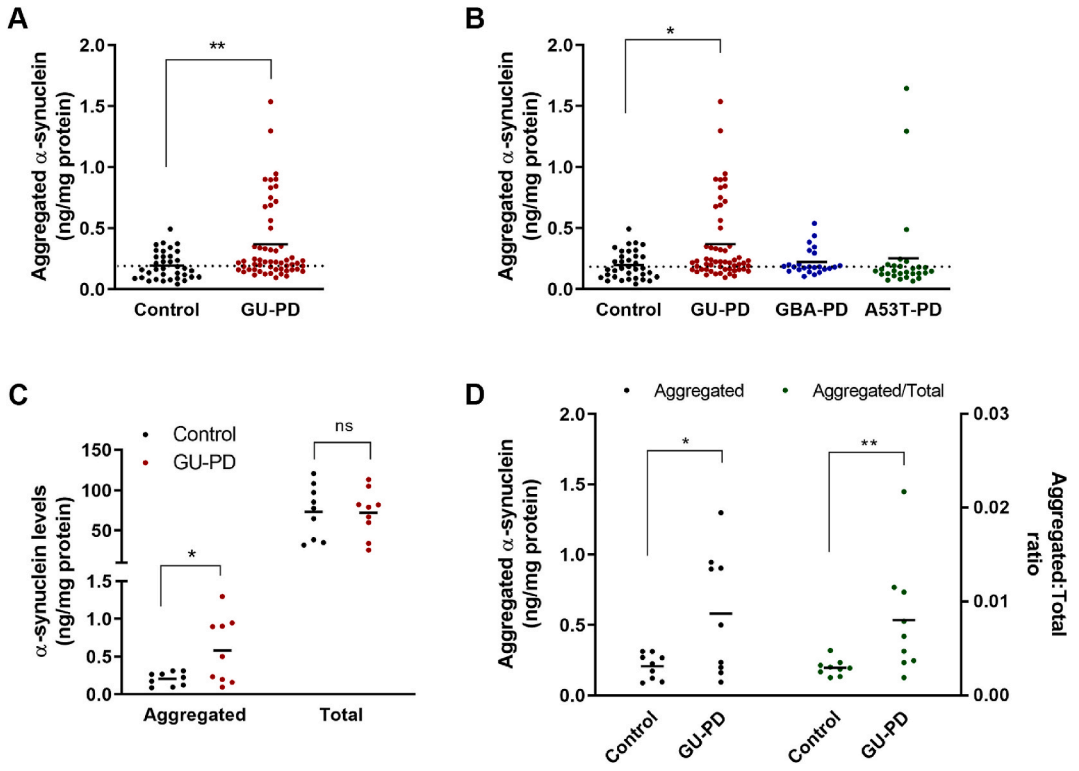

Fig. 2. Aggregated, but not total, α-synuclein is elevated in erythrocytes from PD patients compared to controls. (A) Aggregated α-synuclein was measured in RBCs from controls (n = 35) and GU-PD patients (n = 56). Statistics were performed by Mann Whitney test (***p=0.0067*). (B) The levels of aggregated α-synuclein found in GU-PD patients were compared to PD patients carrying GBA and A53T mutations. Statistics by Kruskal-Wallis test followed by Dunn's multiple comparisons test (*p=0.0102*, *0.9616*, and *>0.9999* for GU-PD, GBA-PD and A53T-PD vs controls, respectively). (C) The concentration of aggregated α-synuclein is compared to total α-synuclein in RBCs from 9 controls and 9 GU-PD patients. In each case, control vs GU-PD groups were compared by Student's t-test (**p=0.02319* for aggregated α-synuclein, *p=0.9327* for total α-synuclein). (D) The concentration of aggregated α-synuclein is compared to the aggregated:total ratio in RBCs from 9 controls and 9 GU-PD patients. Control vs GU-PD groups were compared by Mann-Whitney test (**p=0.0326* for aggregated α-synuclein, ***p=0.0078* for aggregated:total ratio).

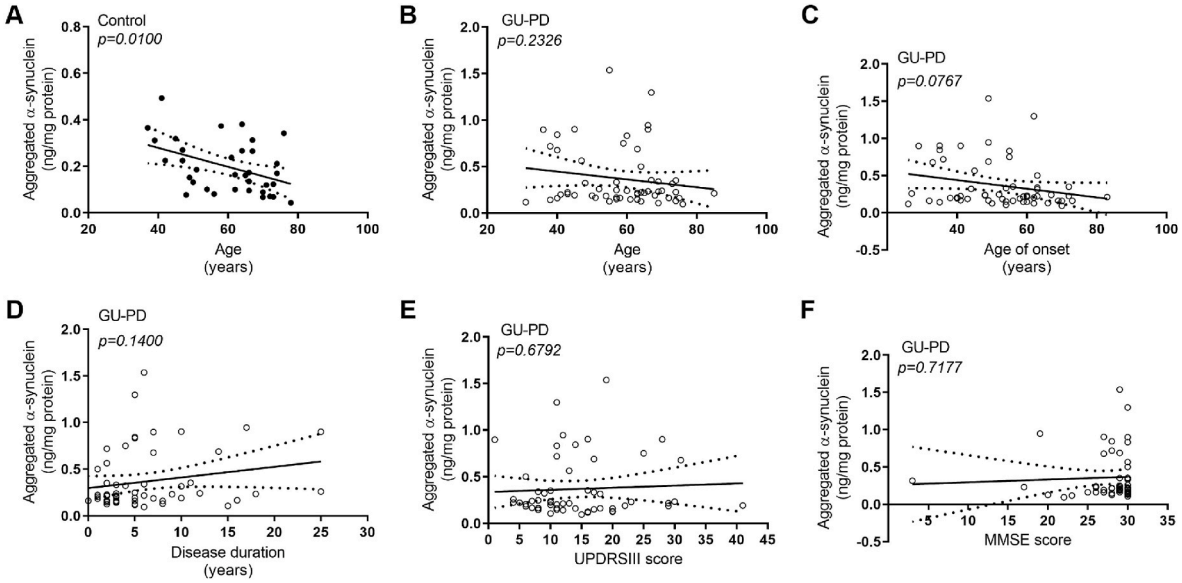

Fig. 3. The accumulation of α-synuclein aggregates in GU-PD erythrocytes is not due to aging. (A, B) Correlation analysis of aggregated α-synuclein concentration with the age of the control (A, $R^2 = 0.1845$) or the GU-PD (B, $R^2 = 0.0263$) groups. (C–F) Correlations between aggregated α-synuclein and clinical metrics of disease severity; age of onset (C, $R^2 = 0.0569$), disease duration (D, $R^2 = 0.0399$), UPDRS Part-III score (E, $R^2 = 0.0032$), and MMSE score (F, $R^2 = 0.0027$).

was recently shown for oligomeric α-synuclein [34].

In this study, we have used a recently developed ELISA that detects aggregated (fibrillar and oligomeric) α-synuclein to evaluate whether the levels of α-synuclein aggregates in erythrocytes could be helpful in PD diagnosis. The new assay was previously tested in tissue homogenates from post-mortem human brains, CSF and CSF-isolated exosomes from PD patients and controls [28]. We found that aggregated α-synuclein is significantly increased in GU-PD RBCs compared to RBCs from

control individuals. Our further analysis showed that the detected conformers in RBC lysates have the tendency to decline by age indicating that the reported increase in GU-PD RBCs is not due to normal aging. We also found that the levels of RBC-related aggregated α-synuclein do not depend on the age of onset, disease duration, or extent of motor/cognitive impairment, suggesting that the observed accumulation is associated with the presence of PD pathology per se and does not depend on disease progression or severity. This finding is in accordance with our previous results from post-mortem brain tissue samples from PD patients with established pathology in which the concentration of aggregated α-synuclein was not correlated with disease duration [28].

Considering the easy accessibility of blood compared to CSF, several studies have addressed the potential contribution of peripheral α-synuclein to PD diagnosis and severity assessment. Even though erythrocytes contain almost all the blood α-synuclein (~99 %) compared to plasma (~0.1 %), serum (~0.05 %) and platelets (~0.2 %), most studies measure peripheral α-synuclein in plasma or serum, yet with inconsistent results reporting either increased, decreased or equal levels of α-synuclein in PD vs control samples (reviewed in Ref. [35]). One possible explanation for this variability is the use of different immunoassays and the extent of hemolysis which results in high signal variability due to the addition of RBC-contained α-synuclein as contaminant. Assessment of α-synuclein in lysed RBCs, the major source of α-synuclein, eliminates such inconsistency due to hemolysis interference.

Erythrocytes contain various conformations of α-synuclein including the oligomeric and phosphorylated forms that are considered toxic in the context of PD pathology. Our results clearly show that measurement of total aggregated (i.e. oligomeric and fibrillar) α-synuclein in RBCs can discriminate idiopathic PD patients from controls which is in agreement with previous studies detecting oligomeric or aggregated α-synuclein [16,20,23,24]. Using the same conformation-specific assay, these conformers were undetectable in CSF from control and PD patients, as we have previously reported [28]. The present study confirms the low abundancy of these conformers also in erythrocytes since we found that they constitute on average only the 0.3 % and 0.8 % of total α-synuclein in control and PD participants, respectively. Conversely, the levels of total α-synuclein, which is abundant in RBCs, were similar between controls and PD patients suggesting that most of α-synuclein present in erythrocytes does not relate to pathology. This is in accordance with previous studies reporting that the majority of α-synuclein present in RBCs stems from erythropoietic lineage [12,36]. The use of aggregated-to-total α-synuclein ratio further improved the discrimination capacity as also observed by others [20,23].

In agreement with our previous results in brain tissue [28], aggregated α-synuclein was readily detected by the conformation-specific ELISA in RBCs from control participants suggesting that the presence of aggregates in low levels is physiological and likely stems from the inherent propensity of α-synuclein to multimerize. It is also noteworthy that even though we observed a clear increase in the levels of aggregated α-synuclein in RBCs from the GU-PD group, there was no difference in the genetically determined PD groups, in which PD development is linked with either the A53T mutation in the SNCA gene or point mutations in the GBA gene. This could partly be attributed to the lower number of patients in the A53T-PD (n = 28) and GBA-PD (n = 24) groups compared to the GU-PD group (n = 56). The difference in participant number stems from the rare occurrence of the genetic forms of PD compared with the idiopathic cases and remains a limitation of our study. It is also possible that, in the genetically defined groups, pathology-related α-synuclein forms have different biochemical characteristics that minimize their diffusion into the periphery and therefore could not be detected by our assay. Indeed, increasing evidence in cell and animal models but also in brain tissue from patients suggests that the presence of A53T or GBA mutations significantly alters the biochemical characteristics of α-synuclein aggregates. The A53T mutation enhances both the nucleation and the fibril elongation processes resulting in faster accumulation and insufficient clearance of toxic insoluble α-synuclein aggregates [37,38]. Mutations in human glucocerebrosidase encoded by the GBA gene have been linked with increased levels of detergent-insoluble Lewy body-like α-synuclein aggregates mostly due to alterations in membrane lipids that either stabilize monomeric α-synuclein or enhance fibril formation [39–41]. As such, the abnormal conformations increase in size and have low solubility that can slow down (but not prevent) the transportation of aggregated α-synuclein from the brain to the periphery when the A53T or GBA mutations are present. Finally, there is a possibility that part of the aggregated α-synuclein is produced in the periphery, favored by high local concentrations of the protein, whereas this would not be the case for the genetic forms, as argued in previous studies [16,31]. For instance, it has been reported that mRNA and protein levels of α-synuclein derived from the mutant allele are reduced in carriers of the p. A53T mutation, thereby leading to overall reduced levels of α-synuclein in the periphery [42,43]. In sum, further investigation is necessary to evaluate the levels of aggregated α-synuclein in genetically defined PD RBCs.

The ability of oligomeric and/or aggregated α-synuclein from RBCs to discriminate PD patients from controls has been reported in several studies. The membranes isolated from lysed RBCs are mostly used for the assessment of multimeric a-synuclein. Tian and colleagues reported a significant increase in oligomeric α-synuclein in RBC membranes from PD patients compared with controls using a specific electrochemiluminescence (ECL) immunoassay [23]. Using western blotting, our group has also reported that dimeric α-synuclein is significantly increased in the membranes of PD erythrocytes and the densitometric quantification of the dimer-to-monomer α-synuclein ratio could differentiate GU-PD and GBA-PD patients from controls [16,17,44]. In the current study, we have used a conformation-specific immunoassay for the determination of both aggregated and oligomeric α-synuclein in RBCs using a more simplified approach; cell membranes were not isolated and total RBC homogenates containing both membranous and cytoplasmic proteins were analyzed. Our results using such total RBC homogenates also agree that multimeric α-synuclein is increased in PD vs control erythrocytes.

In sum, our study supports the diagnostic potential of aggregated α-synuclein in erythrocytes to differentiate sporadic PD from controls. Further validation is required to understand the clinical applicability of this finding, as well as the source of presumably aberrant α-synuclein in this promising and easily accessible biomaterial.

CRediT authorship contribution statement

Konstantina Dimoula: Writing – original draft, Methodology, Investigation, Data curation. **Nikolaos Papagiannakis:** Writing – original draft, Resources, Methodology, Investigation, Data curation. **Matina Maniati:** Resources, Methodology. **Leonidas Stefanis:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Data curation. **Evangelia Emmanouilidou:** Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parkreldis.2025.107321.

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