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

Konstantina Dimoula[a], Nikos Papagiannakis[b], Dimitris Anagnostou[a], Matina Maniati[c], Leonidas Stefanis[b], Evangelia Emmanouilidou[a]

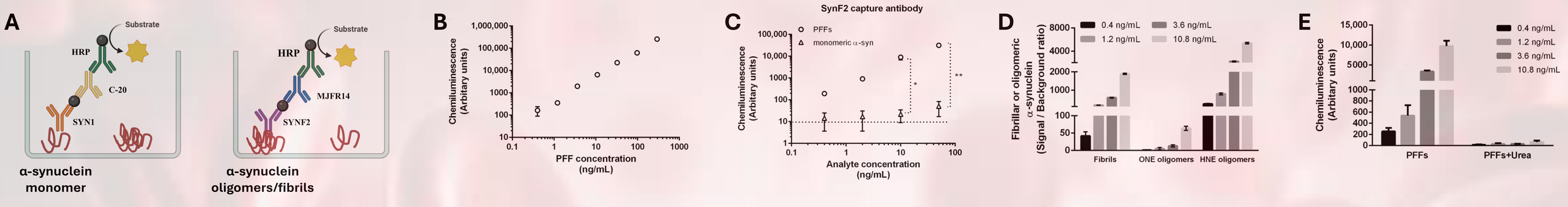
[A] Laboratory of Biochemistry, Department of Chemistry, National and Kapodistrian University of Athens, Panepistimiopolis Zografou, Athens, Greece
[b] First Department of Neurology, Eginition Hospital, Medical School, National and Kapodistrian University of Athens, Athens, Greece
[c] Center of Clinical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

Abstract

Objectives. Most of the peripheral α-synuclein is found in erythrocytes, and several studies have examined a possible association between erythrocytic α-synuclein and Parkinson's Disease (PD). 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, α-synuclein has been shown to navigate bidirectionally from the blood to the BBB in a free form and via extracellular vesicles. Even though α-synuclein aggregates are present in erythrocytes, their exact forms are not fully characterized, and their diagnostic value has not been clarified. **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, could discriminate PD patients from control individuals. **Conclusions**. The elevation of aggregated α-synuclein in 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.

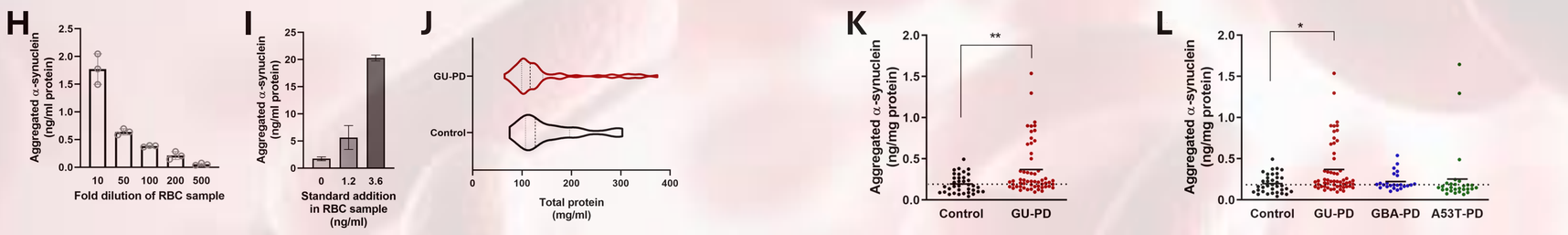
Results

Development and validation of a conformation-specific ELISA for fibrillar/oligomeric α-synuclein



A new conformation-specific ELISA was developed using the SynF2 mouse monoclonal antibody as the capture antibody (Vaikath et al, 2015) which was shown to preferentially bind to mature amyloid fibrils and high molecular weight oligomers. Anti-α-synuclein aggregate MJFR-14-6-4-2 antibody was used as the detection antibody (A). The detection limit of the assay was 0.1 ng/mL (with a signal-to-background ratio of 2) and the linear range 300 ng/mL (B). The new ELISA was selective for the aggregated forms of α-synuclein (C) as tested by measuring similar concentrations of monomeric α-synuclein (barely detectable only at 10 and 50 ng/mL), able to detect both fibrillar and oligomeric forms (D), and conformation-specific as assessed by measuring PFFs following urea treatment (E). The new assay was validated in human postmortem brain tissue (caudate nucleus and putamen) from PD patients and non-PD control individuals. Western blotting (F) using the C-20 polyclonal antibody indicated that SDS-stable aggregated α-synuclein conformers could be selectively detected in PD tissue homogenates whereas the levels of monomeric α-synuclein were similar between PD and control samples (N=8 samples per group per brain area). GAPDH was used as a loading control. The same homogenates were analyzed by the conformation-specific ELISA (G) which revealed that the levels of aggregated α-synuclein were highly elevated in the putamen compared with caudate nucleus. Importantly, low levels of α-synuclein aggregates were also present in control samples. Statistics: In (C) by multiple t-tests (* p = 0.0023 and ** p = 0.00036). In (G) by Student's t-test; *** p < 0.0001.

Aggregated, but not total, α-synuclein is elevated in erythrocytes from PD patients compared to controls



The specificity of the measurements using the conformation-specific ELISA in lysed RBCs was verified using serial dilutions (H) or using the standard addition of 1.2 or 3.6 ng/ml PFFs (I) in a control RBC sample. Data presented as mean ± SEM. Measurement of total protein using the Bradford assay showed similar distributions in GU-PD (n=56) vs control (n=34) RBC samples suggesting comparable conditions of storage and handling for samples in both cohorts. Statistics by Student's t-test (*p=0.4024*).

Aggregated α-synuclein was measured in RBCs from controls (n=35) and GU-PD patients (n=56) (K) and compared to PD patients carrying GBA and A53T mutations (L). Parallel measurement of aggregated or total α-synuclein in RBCs from 9 controls and 9 GU-PD patients showed that total α-synuclein could not discriminate between control and PD groups (M). The use of aggregated:total ratio further reinforced the discrimination capacity in the same RBC samples (N). 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.

Statistics: In (K) by Mann Whitney test (***p=0.0067*). In (L) 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. In (M) by Student's t-test (**p=0.02319* for aggregated α-synuclein, *p=0.9327* for total α-synuclein). In (N) by Mann-Whitney test (**p=0.0326* for aggregated α-synuclein, ***p=0.0078* for aggregated:total ratio.

Table 1. Demographic information about enrolled subjects by group

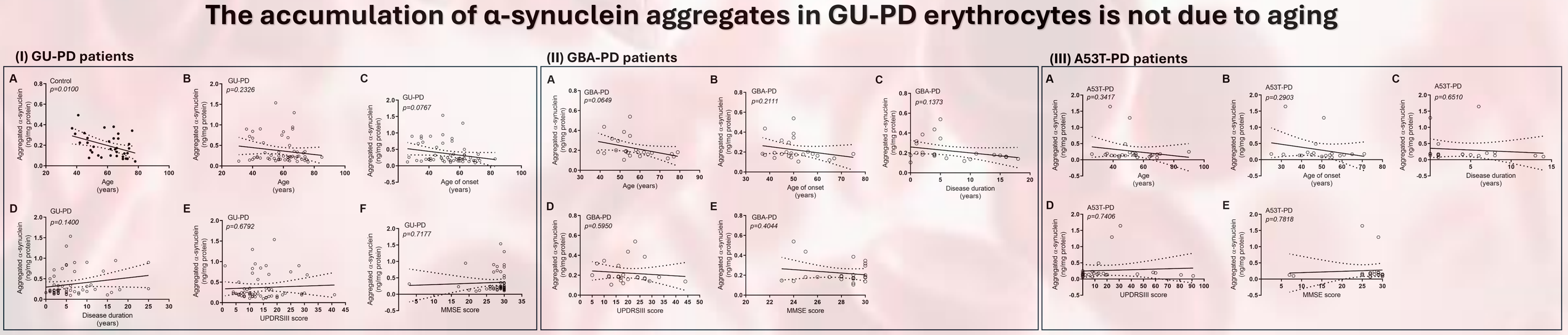
	Control	GU-PD	A53T-PD	GBA-PD
Number	35	56	28	24
Sex (M/F)	16/19	31/25	11/17	15/9
Age at study (years)	60.1 (11.8)	58.5 (12.3)	52.4 (13.8)	57.0 11.1)

Table 2. Clinical characteristics of enrolled patients by group

	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)

Patients with PD 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 were screened for the presence of GBA variants, and were divided 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 and were not screened for the aforementioned mutations. Data in tables represents mean (STDEV).

The accumulation of α-synuclein aggregates in GU-PD erythrocytes is not due to aging



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 (I, A). 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 (I, B). 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 (I, C 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 (I, E and F). Extension of this analysis for the GBA-PD and A53T-PD groups yielded similar results (panels II and III, respectively).

Conclusions

- **A new conformation-specific ELISA was developed for the selective detection of α-synuclein fibrils and high-order oligomers without cross-reacting with monomers.**
- **Using this ELISA, aggregated forms of α-synuclein in total RBC homogenates containing both membranous and cytoplasmic proteins were readily detected.**
- **Aggregated, but not total, α-synuclein is elevated in erythrocytes from idiopathic PD patients compared to controls suggesting that erythrocytic aggregated α-synuclein could be a potential biomarker for PD.**
- **The accumulation of α-synuclein aggregates in erythrocytes is not due to aging and does not depend on the severity of disease.**

Acknowledgements & Funding

We would like to acknowledge Omar El Agnaf and Nishant N Vaikath for producing and providing the Syn-F2 antibody and the ONE- and HNE-oligomers.

This work was funded by Hellenic Foundation for Research and Innovation (HFRI) grant (#581) and a RESEARCH–CREATE–INNOVATE grant (AlphaSyn, #T2EDK-02813) to E.E. Also by a PhD scholarship grant to D.A. (Scholarship No. 2022-050-0502-52576) from the Greek State Scholarships Foundation (I.K.Y.) through the action "Scholarships Programs for post-graduate studies". This project was also supported by the National Network for Research of Neurodegenerative Diseases on the basis of Medical Precision (Grant number 2018SE01300001), funded by the General Secretariat of Research and Innovation (GSRI), and Brain Precision (TAEDR-0535850), funded by GSRI, through funds provided by the European Union (Next Generation EU) to the National Recovery and Resilience Plan.