scientific reports

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Alpha-synuclein is increased in erythrocytes in parkinson's disease cases

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Idiopathic Parkinson's disease (iPD) is the second most common neurodegenerative disease after Alzheimer's disease (AD). Mutations in the SCNA gene, which encodes the protein alpha synuclein (α-syn), are associated with familial forms of Parkinson's disease (PD). Additionally, Lewy bodies (LBs) rich in α-synuclein are a hallmark of idiopathic Parkinson's disease (iPD) pathology. Unlike AD, there are no effective blood-based diagnostic assays for iPD. Recent studies show that measures of misfolded α-syn in cerebrospinal fluid (CSF) and skin biopsies reflect the diagnosis of iPD. The presence of misfolded α-syn suggests that the altered cellular processes in the brain that lead to aggregated α-syn may also occur in the periphery. However, CSF and skin biopsies are intrusive, highlighting the need for a blood-based diagnostic assay. Erythrocytes are the richest source of α-syn in the body, and we hypothesized that peripheral α-syn changes could be detected in erythrocytes in iPD. To test this hypothesis, we used a targeted liquid chromatography-mass spectrometry (LC-MS) assay, that included ^{15}N-enriched recombinant α-syn as an internal standard. We compared the levels of α-syn in erythrocytes from iPD patients, AD patients, and healthy controls (CN). α-syn concentrations were significantly elevated in iPD (48.1 (29.7) μg mL^{-1} of erythrocytes, median (IQR)) compared to CN (36.1 (28.4) μg mL^{-1}) and no difference was observed in AD (33.5 (18.1) μg mL^{-1}). Although α-syn levels were significantly elevated in iPD, the receiver operating characteristic (ROC) analysis yielded an area under the curve (AUC) of 0.62, indicating that erythrocytic α-syn levels alone are not sufficient for diagnostic purposes.

Keywords Red blood cells, Alpha synuclein, Parkinson's disease, Erythrocytes, QQQ, Proteomics

Idiopathic Parkinson's disease (iPD) is a neurodegenerative movement disorder in which there is early and prominent loss of dopaminergic neurons in the substantial nigra and with clinical features that include bradykinesia, rigidity, and resting tremor[1]. Lewy bodies (LBs) which consist of aggregates of misfolded alpha synuclein (α-syn) are a pathological hallmark of iPD[2]. The first gene associated with familial PD was SNCA (PARK1) that encodes for α-synuclein[2,3]. The cause of idiopathic Parkinson's disease (iPD) remains unclear although the presence of misfolded α-syn in LB suggests that α-syn may play a part in the pathophysiology particularly in the preclinical stages[4]. A blood-based biomarker could aid in preclinical detection and in understanding the pathophysiology of iPD and in the discovery of disease modifying therapies. α-syn has been studied as a potential biomarker for iPD due to its genetic and pathological association with the disease. α-syn is expressed in a wide range of tissues and biofluids including the central nervous system, peripheral nervous systems, skin, plasma, lymphocytes and erythrocytes[5–7]. Endogenous α-syn can undergo extensive post translations modifications (PTMs) that can affect its aggregation and toxicity[8]. Phosphorylation of serine 129 is a major PTM due to its prevalence in LBs[9] and many studies have explored phosphorylated α-syn as a potential biomarker[8,10,11]. Misfolded α-syn, detected via seed amplification, has been explored as a biomarker in cerebrospinal fluid (CSF), skin, plasma, and saliva with promising results[12–16]. Collecting CSF and skin is intrusive making it an impractical choice for large scale or repeated screening. Collecting blood samples is significantly easier and less invasive than other methods, therefore the development of blood-based biomarkers would be a significant advance for detection of presymptomatic Parkinson's disease.

Researchers have measured α-synuclein concentrations in plasma using ELISA kits and laboratory-made reagents. However, a consistent signature for changes in α-syn has yet to emerge[5,13]. With reports of increased

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values[17], decreased[18], and no-change[19] and with a varying level of diagnostic accuracy. Also, the concentrations of α-syn reported span seven orders of magnitude (0.1-2,000,000 pg ml^{-1})[17,20]. The inconsistency in detection is likely due to the absence of standard reference materials, the lack of a uniform method for measuring α-syn levels, and contamination from biological sources like erythrocytes. More than 99% of α-syn in blood is contained in erythrocytes thus hemolysis would release α-syn into plasma resulting in major contamination[21,22]. In general, we recycle approximately 5 million erythrocytes a second which provides ample opportunity to artificially elevate α-syn levels in plasma even without considering any hemolysis occurring during collection[23]. The variability in results may be improved with the application of a more robust detection method. Liquid chromatography-mass spectrometry (LC-MS) could remove variability introduced by different antibody pairs. However, detection limits and hemolysis would still be significant barriers to LC-MS quantitation of α-syn in plasma.

In this study we aim to determine if the levels of α-syn in erythrocytes are altered in iPD. We have previously developed a quantitative liquid chromatography triple quadrupole mass spectrometer (LC-QQQ) assay for α-syn in erythrocytes[24]. An advantage of this method is the use of full-length uniformly ^{15}N labelled α-syn as an internal standard. These standards are physiochemically identical to the endogenous molecules but can be distinguished by the mass spectrometer. In this study, we apply this assay to a well characterized samples from the Australian Parkinson's Disease Registry (APDR) to investigate whether α-syn levels in erythrocytes could aid in iPD diagnosis.

Materials and methods

Data acquisition and analysis followed approval from the University of Melbourne human ethics committee application ID1136882 and the study abided by the Helsinki Declaration of 1975. All secondary data used in the present study has been de-identified. Written informed consent was obtained from all participants following institutional ethics approval.

Diagnosis of subjects

Erythrocytes were obtained from the Australian Parkinson's Disease Registry (APDR) in Melbourne, Australia, and isolated as previously described[25]. This registry is a collaborative initiative involving researchers from Victoria, Western Australia, and New South Wales, and is managed by the Cooperative Research Centre (CRC) for Mental Health. Participants with Parkinson's disease have been characterized using clinical scales, including the Unified Parkinson's Disease Rating Scale (UPDRS). For healthy controls, only a brief family history of Parkinson's disease is available. Alzheimer's disease cases were from the Australian Biomarker Lifestyle Study of Ageing (AIBL). This cohort contains a higher male percentage (66%) in iPD patients while a lower male percentage (41%) in healthy controls. The full cohort characteristics are shown in Table 1.

Erythrocyte collection

Blood samples were collected into prostaglandin E_1 (PGE_1) EDTA tubes which were then inverted 8–10 times and placed on a rocker for 30 min. After this, the samples were centrifuged at 200 × *g* for 10 min. Next, the plasma was removed, and the remainder spun for 15 min at 1500 × *g*. The remaining plasma, buffy coat, and top layer of erythrocytes (supernatant) were removed. The packed erythrocytes were subsequently washed with 0.9% saline (v/v), centrifuged for 10 min at 650 × *g*, and the supernatant was removed. This step was repeated twice. After this, the packed erythrocytes underwent a final wash in saline and were subsequently centrifuged for 10 min at 1500 × *g*. The erythrocytes (without the supernatant) were stored as 500-µL aliquots in polypropylene tubes (Nalgene Nunc™) in a liquid nitrogen (− 178 °C) Dewar until further use.

Erythrocyte lysis

Packed erythrocytes (20 µL) were lysed by mixing with 250 µL of MilliQ water (18 MΩ, Merck-Millipore, USA) and frozen at -20 °C until needed. The cells were thawed, and 15µL of 40 ng*µL^{-1} of ^{15}N-labelled α-syn was added to achieve a final concentration of 2.105 ng*µL^{-1}. The mixture was vortexed and centrifuged at 17,000 × *g* for 20 min at 4 °C to separate cell debris from the supernatant. The protein concentration of each sample was estimated using absorbance 280 nm. Samples had an average protein concentration of 15 µg*µL after lysis.

	Controls	iPD	AD
N	144	141	28
Sex, %male	44*	66*	46^{ns}
Age, years (avg.(range))	67 (20–88)	64 (28–88)	69 (50–89)
HY, total score (mean(SD))	N.A.	1.79 (0.98)	N.A.
UPDRS3, total score (mean(SD)	N.A.	20.9 (15.1)	N.A.
Erythrocyte α-syn (µg mL^{-1}) (Median (IQR)	36.1 (28.4)	48.1 (29.7)**	33.5 (18.1)

Table 1. Demographics table for the Victorian disease PD registry. Significance of sex differences in the cohort was determined using the Chi-Squared (*p-value < 0.05). HY- Hoehn and Yahr scale, UPDRS3- unified parkinson's disease rating scale part 3, ** $p < 0.01$ by One-way ANOVA and dunnett's post-hoc analysis. N.A.-not applicable.

Deoxycholate digestion protocol

An eight-point standard curve was created using a 1:2 dilution series of heavy α-syn from 10 ng*μl^{-1} – 0.039 ng*μl^{-1}, into a matrix of erythrocytes and was processed as followed along with the samples. Approximately, 150 μg total lysed erythrocyte protein (10 μL) spiked with full length heavy ^{15}N labeled α-syn, were transferred to a 96 well plate (Greiner bio-one U bottom plate #650201). The samples and standard curve were denatured, reduced, and alkylated in a total volume of 100 μL to a final concentration of 1% sodium deoxycholate, 10mM TCEP, 40mM chloroacetamide, and 100 mM Tris pH 8.5. Samples were heated at 95 °C for 10 min and allowed to cool to room temp and quickly centrifuges to collect condensation. Twenty microliters of the above material were transferred to a new plate for digestion of the sample. Samples were diluted up to 200μL with 100mM Tris-HCl containing a final protein to enzyme ratio of 1:25 Promega Tryp/LysC and incubated overnight at 37 °C. Following digestion, samples were adjusted to pH 2 through the addition of neat Formic Acid (10μL) and allowed to incubate for 20 min before centrifugation at 4347xg for 20 min. Digested material was desalted with the Oasis® PRiME HLB μElution Plate (Waters, Waxford, Ireland), 200 μL 0.1% FA and 180uL sample loaded to the plate together, washed once with 400 μL 0.1% FA and again with 200 μL of 5%MeOH, 0.1% FA and eluted twice with 25 μL of 70% ACN, 0.1% FA. Samples were concentrated in a centrifuge vacuum (Labcon, Kansas City, Missouri, USA) and stored at – 20 °C until further use.

QQQ method

Prior to analysis via the 6495 QQQ LC/MS (Agilent Technologies, Santa Clara, California, USA), the samples were resuspended to an approximate final peptide concentration of 1 μg*μL^{-1} in 25.7 μL of 0.1% FA and allowed to reconstitute on an oscillator for 30 min at room temperature. The protein content was estimated using absorbance at 280 nm to be at 1 μg*μL^{-1}. Approximately 4 μg of protein were injected on to the column with a 4 μL injection for each sample. The amount of heavy ^{15}N α-syn was calculated to be 0.140 ng* μL $^{-1}$ in the samples and 0.561 ng was injected in 4 μL. Peptide extracts were separated on an Agilent 1290 Infinity II LC system (Agilent Technologies) using an Agilent Advance Bio Peptide 2.7 μm analytical column (2.1 × 150 mm, with a guard column of the same media 5 × 50 mm) and developed at a flow rate of 0.4 mL*min^{-1}. Mobile phase A was 99.9% water with 0.1% FA and mobile phase B was 99.9% ACN with 0.1% FA. The following 8-minute gradient was used to separate the peptides in the samples: 0 min 7% B, 0–6.1 min 24% B, 6.1–6.3 min 80% B, 6.3–6.8 min 80% B, 6.8–7 min 2% B with 1 min of post time. The assay targeted three peptides specific to α-synuclein (EGVLYGSK, EQVTNVGGAVVTGVTAVAQK, EGVVAAAEK) (SNCA, UniProt P37840). The EGVV… peptide had a low signal-to-noise and was not used in analysis. Eluted peptides were detected using a 6495 QQQ MS system with AJS-ESI (Agilent Jet Stream-electrospray ionization with nitrogen gas) with a scan type of MRM with 20 ms dwell time for each transition. Data acquisition was performed in positive ionization mode. The gas temperature was set to 225 °C and the gas flow to 13 L*min^{-1}. The nebulizer was set to 35 psi. The sheath gas flow was set to 11 L*min^{-1}, with a temperature of 250 °C. The capillary voltage was set at 3500 V with a nozzle voltage of 1500 V. The voltages of the high-pressure RF and low-pressure RF were 150/60 V, respectively.

Statistical analysis

The data was analyzed, and standard curve for heavy alpha synuclein (R^2 value 0.98) was plotted in Skyline (v22.2). This curve was generated by plotting the ratio of heavy to light peptides against the nanograms of known heavy labeled α-syn in each sample. Each sample was plotted on the line of best fit to calculate the concentration of endogenous α-syn. Average concentrations of α-syn were determined from the average of the two peptides detected (EGVL… and EQVT…). Comparison of the two peptides showed a strong linear correlation linear correlation in the peptides (supplemental Fig. 1, $r^2 = 0.75$). The concentration of α-synuclein in erythrocytes were compared using a one-way ANOVA followed by Dunnett's post-hoc test to assess the correlation between disease states and controls. Associations between the levels of α-syn and age, UPDRS, and Hoehn and Yahr (HY) were analyzed by linear regression (least squares regression). Analysis was conducted using GraphPad Prism 10.2.3 (GraphPad Software, La Jolla, California, USA). Raw data files are available through the following link (https://panoramaweb.org/renKJk.url).

Results

Soluble α-syn levels are elevated in erythrocytes from idiopathic Parkinson's disease

The calculated median (IQR) concentration of α-syn in erythrocytes were 36.1 μg*mL^{-1} (28.4) in healthy controls, 48.1 μg*mL^{-1} (29.7) in iPD, and 33.5 μg*mL^{-1} (18.1) in AD. Both peptide concentrations were not normally distributed) (Shapiro-Wilk test $p < 0.0001$), so log-transformed data was used for the statistical comparisons below. Figure 1 displays dot plots for the levels of α-syn in erythrocytes for healthy controls (CN), Parkinson's disease (iPD), and Alzheimer's disease (AD) (see Table 1 for cohort demographics). Using a one-way ANOVA and Dunnett's post hoc analysis there was a significant difference between iPD and controls ($p = 0.0020$). As a control for disease specificity, we also measured α-syn in Alzheimer's disease cases from the Australian Imagining and Lifestyle Study of ageing (AIBL) and found no significant difference with controls ($p = 0.76$). This is consistent with the increase in α-syn levels being specific to Parkinson's disease. To determine the discriminatory power of α-syn as a diagnostic biomarker the receiver operating characteristic (ROC) analysis was conducted. The resulting ROC curve showed weak diagnostic accuracy (AUC (95% CI) = 0.623 (0.559–0.687)). Because iPD occurs more commonly in males than females we did a sub-analysis to determine if there were any specific correlations related to sex or age. We found no significant differences between the male and female levels of erythrocytic α-syn in the healthy control ($p = 0.881$) or iPD groups ($p = 0.658$) using Mann-Whitney test. We did not find any significant correlations of erythrocyte α-syn levels with age (β (SE)=-0.200 (0.373) $p = 0.782$) using least squares regression (Supplemental Fig. 2). Next, we compared the levels of α-syn to clinical measures of disease. We did not observe any correlation to levodopa dose (LEDD) β (SE) = 0.003 (0.003) $p = 0.423$ (Fig. 2c).

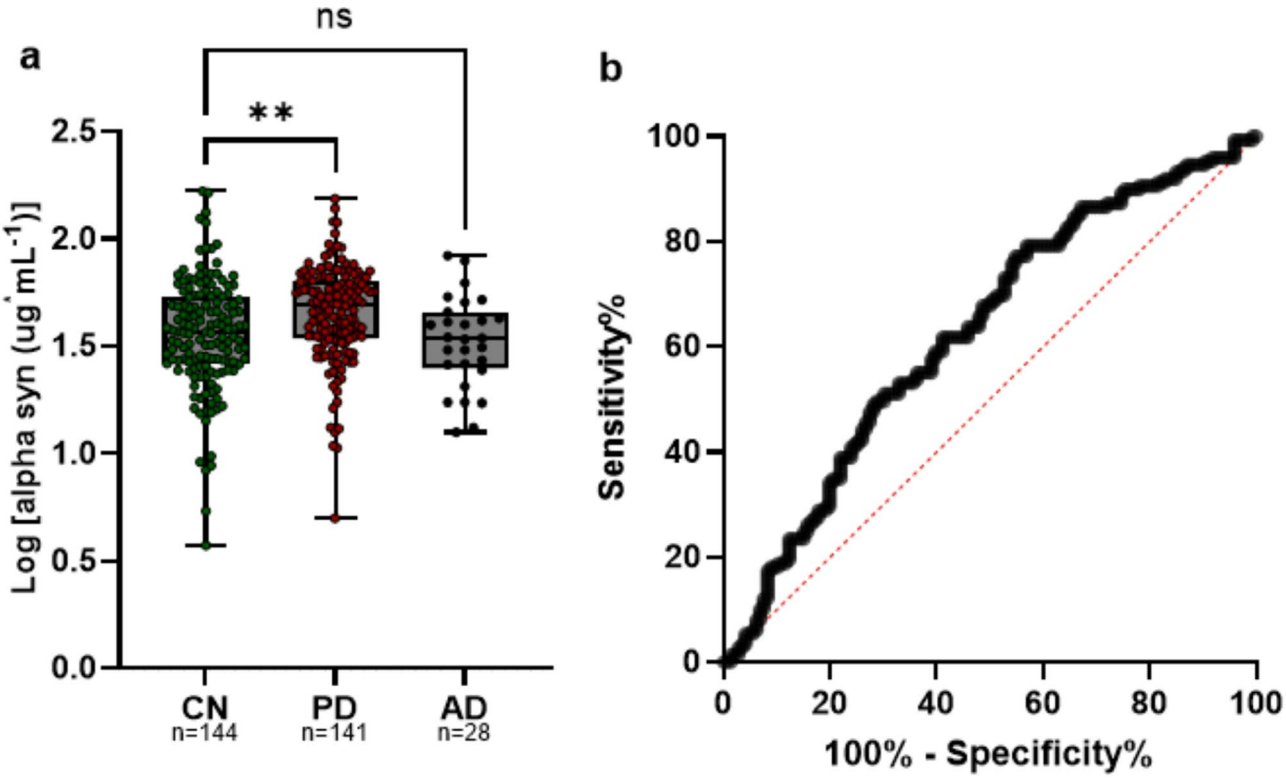

Fig. 1. Elevated α-synuclein in erythrocytes of iPD measured by quantitative LC-MS. Quantitation of α-syn in lysed erythrocytes was conducted using heavy ^{15}N full length labeled internal standard. (**a**) Log transformed dot plot of the average α-syn concentration (ug*mL^{-1}) from two peptides, EGVLYGSK and EQVTNVGGAVVTGVTAVAQK, calculated from the heavy to light ratio in each sample. (**b**) the receiver operating characteristic (ROC) analysis of CN vs. iPD levels of α-syn yielded an AUC of (AUC (95% CI) = 0.623 (0.559–0.687)). $^{**}p < 0.01$ one-way ANOVA and Dennett's post-hoc analysis.

We also did not observe a significant correlation between the Hoehn and Yahr scale (β (SE) = 0.440 (2.86) $p = 0.878$) or the UPDRS Scale 3 (UPDRS3, β (SE) = -0.204 (0.188) $p = 0.280$, Fig. 2). However, we did determine a significant correlation of Addenbrooke's cognitive exam (ACE-R) to α-syn concentration in the cohort β (SE) = -0.314 (0.131) $p = 0.018$ (Fig. 2d). To control for general changes in protein content between disease groups we used spectrin, a cytoskeletal protein, as a house keeping entity and did not observe any changes in spectrin between experimental groups indicating there wasn't a generalized change in protein abundance (Supplemental Fig. 3a). To compare raw α-syn concentration to generalized protein abundance we normalized the raw values to spectrin abundance, and the significant increase was conserved (Supplemental Fig. 3b).

Discussion

In this study we show that levels of α-syn in erythrocytes are significantly increased in iPD compared to controls. However, the diagnostic ability of α-syn as a blood-based biomarker was lacking (AUC (95% CI) = 0.623 (0.559–0.687)). While not of clinical significance, the change is indicative of a shift in the biology that controls α-syn due to iPD pathology.

Previous studies have reported conflicting data on the levels of α-syn in plasma. Some studies have reported increased levels compared to healthy controls while others have shown a decrease[17,20]. The variability in quantification of α-syn in plasma is likely attributed to inconsistent amounts of hemolysis occurring during the blood collection phase[21,22]. Because more than 99% of the α-syn in blood is contained in erythrocytes, hemolysis would result in high variability of plasma levels of α-syn. Here we aimed to directly measure α-syn levels in erythrocytes. The direct measurement of α-syn from erythrocytes presents a challenge due to the overwhelming abundance of hemoglobin and its potential interference with ELISA HRP based detection. To circumvent this, we applied an LC-MS assay for direct detection of α-syn from erythrocytes samples[24]. Using this assay, we show that levels of α-syn protein were increased in iPD patients compared with healthy control. The increase in α-syn was not observed in AD samples, however an effect size of 0.68 would be needed to detect a significant change in α-syn based on the 28 AD samples measured here. Thus, the power of the comparison between AD and controls is too low to detect a similar or smaller change to what was observed in the PD group. Application of this assay on different synucleinopathies may provide further insight on disease specificity but will likely require larger numbers (~100) to be sufficiently powered.

The cellular localization of α-syn in erythrocytes could be a key factor for future studies based on the work by Tian et al.[26] which measured α-syn, using antibody-based assays, in erythrocytes cytosolic fraction and

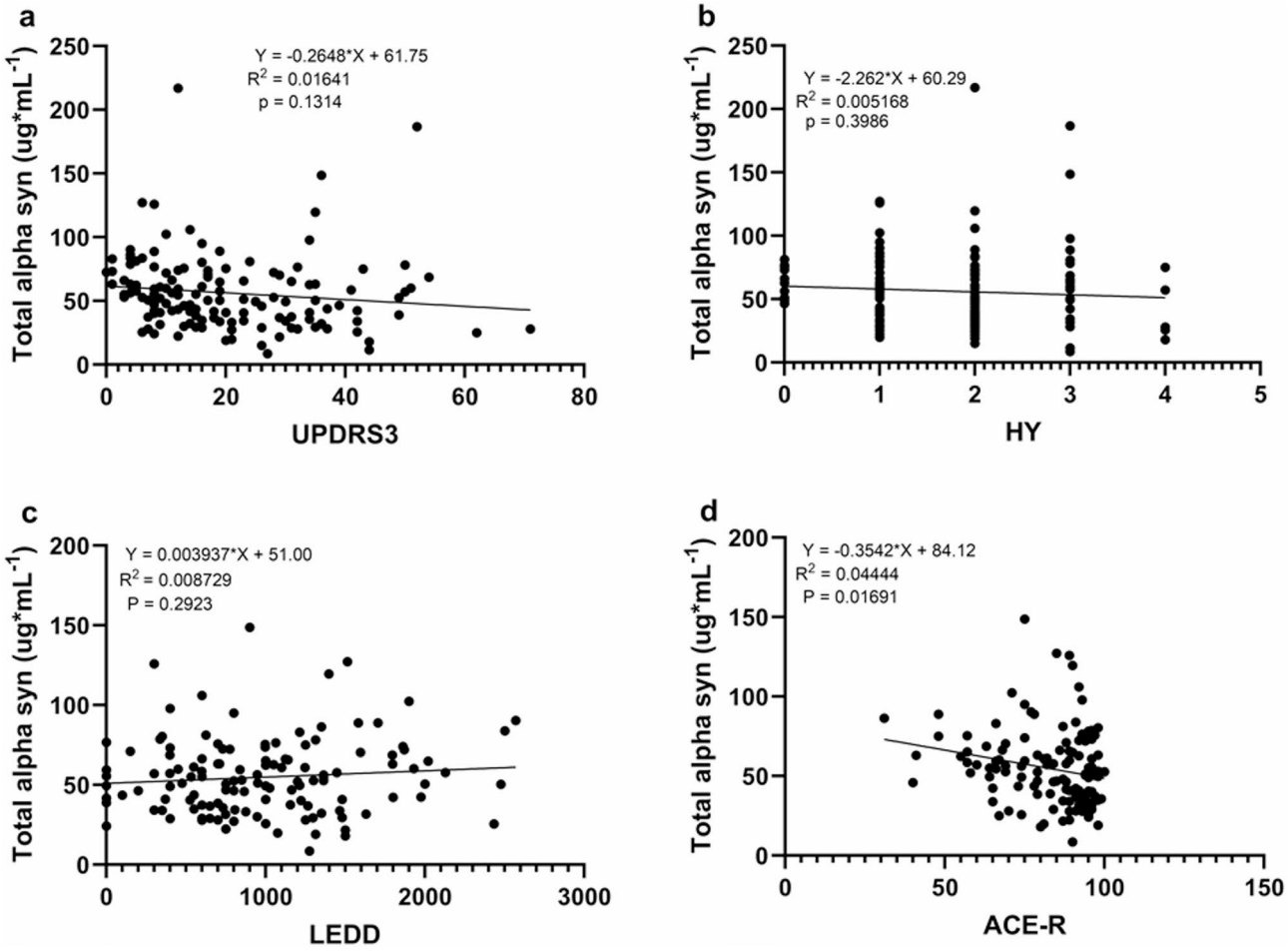

Fig. 2. Clinical data relation to analytical results indicates no significance in motor control but slightly significant in cognitive ability. At the time of sample collection several clinical tests were taken to assess motor and cognitive ability. (**a**) Unified Parkinson's Disease Rating Scale (UPDRS3) was measured to assess the motor capability, and we observed no significant correlation β (SE) = − 0.204 (0.188) $p = 0.280$. (**b**) Hoehn and Yahr (HY) were measured to assess the motor progression of disease, and we observed no significant correlation β (SE) = 0.440 (2.86) $p = 0.878$. (**c**) Levodopa dose (LEDD) was monitored, and we observed no significant correlation β (SE) = 0.003 (0.003) $p = 0.423$. (**d**) Addenbrooke's cognitive exam (ACE-R) was collected to assess the cognitive ability, and we observed a small significant correlation β (SE) = − 0.314 (0.131) $p = 0.018$.

membrane bound forms and only observed a significant elevation in the membrane bound form of α-syn. They also investigated misfolded α-syn and phosphorylation at S129 both which were significantly elevated in iPD. Remarkably the amount of total α-syn in erythrocytes reported by Tian et al.[26] was 34 $\mu g^{*}mL^{-1}$ very similar to what we measured in Klatt et al.[24], and by Elhadi et al.[8] and reported here. Overall, our results are consistent with Elhadi et al.[8] that show an increase in total α-syn in erythrocytes of PD. The increase in α-syn in erythrocytes provides further supporting evidence that changes in α-syn are not isolated to the brain in PD but also affects the periphery tissues. This is consistent with the detection of misfolded α-syn detected in skin[13,14].

One hypothesis to explain the increased levels is that treatment of iPD with levodopa causes a decrease in erythrocyte clearance resulting in elevated aged erythrocytes. Catechols, including dopamine, have been shown to increase the life cycle of erythrocytes[27]. The decreased clearance could cause a drug induced increase of α-syn in erythrocytes in iPD. However, we did not observe a relationship with levodopa dose and α-syn levels (Fig. 2c). This would need to be further studied in a cohort containing treatment free iPD patients or models of erythropoiesis. Another explanation is that as α-syn expression is altered during terminal differentiation of erythroid cells in an upstream signaling event that causes increased production of α-syn. The expression of α-syn increases throughout the process of terminal differentiation of healthy erythroid cells[28]. Therefore, an investigation of the levels of expression of α-syn during terminal differentiation in healthy erythroid cells compared with iPD erythroid cells could provide valuable insight into the mechanisms of iPD pathology. Lastly, the increased levels of α-syn in iPD erythrocytes may be due to an increase absorption of α-syn from external sources. It is shown that in mouse erythrocytes that added external α-syn is collected into the erythrocytes increasing the localized concentration of α-syn in the cell[29]. This would explain how the levels of α-syn were increased in iPD but it does not explain where the increased α-syn originates from. These areas of investigation

are important for understanding the role of α-syn iPD and what factors modulate α-syn expression, post-translational modification and aggregation. Overall, the increased levels of α-syn help to provide valuable insight on the pathobiology of iPD while not providing diagnostic ability.

Data availability

The dataset generated during this study are available through the following link (https://panoramaweb.org/renKJk). All data and analysis generated are also available from the corresponding author on reasonable request.

Received: 6 December 2024; Accepted: 14 July 2025
Published online: 29 August 2025

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Acknowledgements

We would like to acknowledge the research team of The Australian Imaging, Biomarker & Lifestyle Flagship Study of Ageing (AIBL; a complete list of researchers can be found at www.aibl.csiro.au), the Victorian Parkinson's Disease Research team, the volunteers and their families. We would also like to thank Agilent Technology and the Cooperative Research Centre (CRC) for Mental Health for research support. We thank Amber Thomas for producing the heavy labeled 15 N alpha synuclein. Funding was provided by Emory Start-up and the Michael J. Fox Foundation for Parkinson's Research (BR, MJFF-021151). The funder played no role in study design, data collection, analysis and interpretation of data, or the writing of this manuscript.

Author contributions

B.R., R.C. and A.R. designed the experiments. R.C., A.R. prepared the samples and acquired the data. R.C.

analyzed data wrote the main manuscript text. C.M, C.F and M.H oversaw the collection of clinical samples. All authors reviewed the manuscript and approved the completed version.

Declarations

Competing interests

Dr. Blaine Roberts receives research support from ThermoFisher, Agilent technologies, and Bruker.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-11979-8.

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