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Supplementary appendix

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Diagnostic and prognostic value of alpha-synuclein seed amplification assay kinetic measures in Parkinson's disease: a longitudinal cohort study

Supplementary material

Supplementary Methods

CSF sampling and a-syn SAA in the UK parkinsonism cohort

CSF samples from PROSPECT-UK PSP and control participants were centrifuged at 1750g at 4°C for 5 minutes within 15 minutes of lumbar puncture. The supernatant was then aliquoted into 500 microlitre aliquots before immediately storing at -80°C. In contrast, in Exenatide-PD3 PD participants the first 10 drops of obtained CSF were discarded and the remainder was collected and subsequently aliquoted into 1 millilitre aliquots before immediately storing at -80°C. Only aliquots with no previous freeze-thaw cycles were used for a-syn SAA testing in this study. Prior to testing, CSF samples were thawed and distributed into single-use ~65uL aliquots and re-frozen at -80°C. Single-use aliquots from each participant were then thawed for testing on the a-syn SAA assay.

The K23Q mutation of the αSyn sequence (Accession No. NM_000345.3) was engineered using Q5 Site-Directed Mutagenesis (NEB) using the primers CCACACCCTGTTGGGTTTTCTCAG and CAGAAGCAGCAGGAAAGAC, as previously described **(1)**, using a pET28 vector with an N-terminal His-tag (EMD Biosciences). The plasmid was transformed into BL21(DE3) Escherichia coli (EMD Biosciences). K23Q recombinant αSyn was purified as previously described with some modification. Briefly, following overnight auto-induction expression, a 1L Luria broth BL21(DE3) cell suspension was split into 4x250mL conical vessels and spun at 3273xg for 12min. Cell pellets were frozen at -80 °C for 20min and resuspended in 30ml of cold PBS per vessel. Next, each suspension was transferred into a 50ml falcon tube and probe sonicated in an ice bath (4 x 45sec with 15sec rests) at a power setting of 45% (Ultrasonic) to lyse the cells. Each tube was boiled for 20min and centrifuged at 9000xg for 60min. Then supernatants were combined and 50ml of Buffer A (20mM Tris pH7.4) were added. Next, the supernatant was filtered (0.22 μm vacuum filter) and loaded onto a 5mL Ni-NTA column (Cytiva 17525501). The middle section of the elution peak was collected, 20mL of Buffer A were added before moving onto a 5ml Q-HP (Cytiva 17115401) column using pre-chilled Buffers A and B2 (B2: 20mM Tris, 500mM Imidazole pH7.4). Following the collection of the middle peak fractions, the eluted material was filtered with 0.22 μm syringe filter and dialyze against pre-chilled PBS (3.5L overnight at 4 °C and another 3.5L PBS for 4 hrs the next day) using 3.5 kDa MWCO dialysis membrane (Thermo Scientific 68035). After another 0.22 μm syringe filtration, protein concentration was determined using a UV–VIS spectrophotometer and a theoretical extinction coefficient at 280 nm of 0.36 $(mg/mL)^{-1}$ cm^{-1}. Protein was dispensed in single use aliquots and stored at −80 °C.

UK parkinsonism cohort CSF samples were applied to an established a-syn SAA at the NIH Rocky Mountain Laboratories (RML) in a blinded fashion so that the experimenter did not known the diagnostic status of the samples being tested.[1] Each well of a black 96-well plate with a clear bottom (Nalgene Nunc International) was preloaded with six glass beads (0.8 mm in diameter, OPS Diagnostics). Assays were set up in quadruplicate reactions per patient and seeded with 15 μL of CSF. Prior to addition of CSF, each SAA reaction mix was 85 μL of solution adjusted to give final reaction concentrations of 40 mM sodium phosphate buffer, 170 mM NaCl, 0.1 mg/mL K23Q recombinant αSyn (filtered through a 100 kD MWCO Pall filter, immediately prior to use), 10 μM Thioflavin T (ThT) and 0.0015% sodium dodecyl sulfate (SDS). The plates were sealed (Nalgene Nunc International sealer) and incubated at 42°C in a BMG FLUOstar Omega plate reader. Plates were subjected to cycles of 1 min shaking (400 rpm double orbital) and 1 min rest for a minimum of 40 hours. ThT fluorescence measurements were taken every 45 min (450 +/− 10 nm excitation and 480 +/− 10 nm emission; bottom read). Each plate included a pool of non-diseased human CSF as a negative control and the same pool spiked with a 10^{-5} confirmed PD brain tissue dilution as a positive control (4 reactions each).

Fluorescence threshold for a positive reaction was calculated as 10% of the maximum value any well reached on each plate, which meant that the threshold was calculated individually for each plate, thus accounting for differences across experiments and plate readers. For a CSF sample to be considered positive, the fluorescence signal needed to exceed the threshold in at least 75% of replicate wells (e.g., ≥3 of 4) by the 40-hour reaction cut-off time. Samples that had no positive wells were assigned a negative result. Samples that were positive in 1 or 2 replicate wells underwent a repeat experiment and were only assigned a positive result if the fluorescence threshold was exceeded in ≥2 of 4 replicate wells in the repeat experiment.

CSF Alzheimer's disease (AD) biomarker testing in the PPMI cohort and genetic stratification of all the studied cohorts

A subset of PPMI PD CSF samples underwent testing for biomarkers of Alzheimer's disease (AD) pathology using the high-precision Roche Elecsys electrochemiluminescence immunoassay. A validated method of using a total tau/Aβ42 ratio cut-off of 0.26 was used to define samples with AD pathology.[2]

In the UK parkinsonism cohort, LRRK2-G2019S status (rs34637584) was defined using the KASP genotyping platform at the Laboratory of the Government Chemist (LGC Ltd), UK.

In the PPMI cohorts, mutation screening was previously performed using genetic data from whole genome sequencing (WGS), whole exome sequencing (WES), and Sanger sequencing of select GBA variants. Variant data were extracted for the following genes using the same inclusion selection criteria as in the PD GENEration study (https://www.parkinson.org/understanding-parkinsons/causes/genetics/testing-counseling): LRRK2, GBA, SNCA, and PRKN. Variant data were reviewed to identify those that meet the current American College of Medical Genetics and Genomics criteria for pathogenicity, and data were compared across genetic platforms, to create a consensus variant resource for Parkinson's disease researchers. The downloaded genetic data was the most up-to-date version released on 2023-12-20.

Similarly, Tuebingen PD cohort participants were previously screened for pathogenic variants in LRRK2, GBA, DJ1, PINK1 and PRKN genes as previously described.[3]

Clinical data obtained in the UK parkinsonism, PPMI and Tuebingen PD cohorts

In the UK parkinsonism cohort, matched baseline clinical data, i.e. obtained on the same day as the lumbar puncture, from PD and PSP patients consisted of sex, age at symptom onset, age and disease duration at baseline assessment, MDS-UPDRS-III score in the 'Off' state and MoCA score. Additionally, in PSP patients we noted the dominant PSP clinical subtype and the PSP rating scale score. PD participants had serial clinical assessment data available at 24-months post-baseline assessment and this was not stratified by drug/placebo status due to the Exenatide-PD3 trial not meeting its primary endpoint.[4] Mortality and/or post-mortem data was not available on PD participants.

In the PPMI cohort, clinical data consisted of sex, age at symptom onset, age and disease duration at baseline assessment. Baseline and annual follow-up (up to 13 years post-baseline) scale scores for MDS-UPDRS-III in the 'Off' state, MoCA, SEADL and H&Y were recorded where available.

To replicate the main findings from the PPMI cohort analysis, the same clinical data was obtained from the Tuebingen PD cohort participants, with baseline and annual follow-up (up to 13 years post-baseline) MoCA scale scores were recorded where available. Of note, we used up-to-date clinical data which included more longitudinal follow-up and the exclusion of participants whose clinical diagnoses of PD had been revised since recent published studies of the cohort.[5]

Definition of dementia based on MDS Taskforce criteria for PD dementia

Our definition of dementia was based on the Movement Disorders Society Taskforce for Parkinson's disease dementia diagnostic criteria: 1) Montreal Cognitive Assessment (MoCA) scores ≤21; 2) At least two cognitive domains impaired in the MoCA scale (attention/serial sevens ≤2/3; language/verbal fluency 0/1; memory/delayed recall ≤4/5; visuospatial/executive ≤4/5); 3) Cognitive deficits severe enough to affect activities of daily living (Movement Disorders Society-Unified Parkinson's disease Rating Scale part I 1.1 score ≥2); 4) Absence of severe depression (Movement Disorders Society-Unified Parkinson's Disease Rating Scale part I 1.3 score <4).

Supplementary Table 1: Clinical profile of the a-syn SAA 'low and slow' PD samples in comparison with unequivocally a-syn SAA positive PD samples in the UK parkinsonism cohort

	Age at symptom onset, years	Disease duration at baseline, years	MDS-UPDRS-III at baseline, score	2-year change in MDS-UPDRS-III, score	MoCA at baseline, score	2-year change in MoCA, score
PD a-syn 'low and slow' 1	46	7.8	32	+4	30	0
PD a-syn 'low and slow' 2	51	4.5	17	+5	29	0
PD a-syn 'low and slow' 3	68	6.3	39	+28	25	-1
PD a-syn 'low and slow' 4	47	5.6	35	NA	26	NA
PD a-syn 'low and slow' 5	48	3.5	33	+2	30	0
PD a-syn 'low and slow' 6	35	4.0	34	+3	30	0

	Age at symptom onset, years	Disease duration at baseline, years	MDS-UPDRS-III at baseline, score	2-year change in MDS-UPDRS-III, score	MoCA at baseline, score	2-year change in MoCA, score
PD a-syn 'low and slow' 7	47	7.6	23	NA	29	NA
PD a-syn 'low and slow' 8	49	6.8	31	-3	30	-1
PD a-syn positive group (n=55)*	53.4 (9.0)	6.7 (3.9)	34.1 (9.2)	+0.4 (7.2)	28.3 (1.5)	-0.2 (1.4)

Movement Disorder Society-Unified Parkinson's Disease Rating Scale part III: MDS-UPDRS III, Montreal Cognitive Assessment: MoCA, * Values for the PD a-syn SAA positive group are expressed as mean (standard deviation).

Supplementary Table 2: Pathogenic variants detected in monogenic PD participants in the PPMI cohort

	Amprion 24h assay cohort	**Amprion 150h assay cohort**
LRRK2	G2019S, n=38 R1441G, n=16 R1441C, n=1 I2020T, n=1 N1437H, n=1	G2019S, n=109
GBA	N409S, n=15 L483P, n=4 IVS2+1G>A, n=3 L29Afs*18, n=2 R502C, n=1 R159W, n=1 F216Y, n=1	N409S, n=47 L483P, n=2
SNCA	A53T, n=1	A53T, n=11
PRKN	Q25X / G430D, n=1 Q34Rfs*5 / Q34Rfs*5, n=1 p.Pro113fs, n=1 p.Gln34fs, n=1 Exon deletion, n=3	R275W, n=4 Exon duplication, n=2 Exon deletion, n=2 P133_A134insP, n=1 P37L, n=1 R42P, n=1

Leucine-rich repeat kinase 2: LRRK2, Beta-glucocerebrosidase: GBA, Synuclein Alpha: SNCA, Parkin RBR E3 ubiquitin protein ligase: PRKN.

Supplementary Table 3: Baseline a-syn SAA kinetic measures predicting unfavourable outcome and individual components of unfavourable outcome (cognitive decline (MoCA ≤21), postural instability (H&Y ≥3), motor progression (≥5 point increase in MDS-UPDRS-III score relative to baseline score), dependency (SEADL <80) and death)) in the PPMI cohort

	MaxThT		TTT		AUC	
Event **Event rate x/y (%)**	**HR** **(95% CI)**	**p-value**	**HR** **(95% CI)**	**p-value**	**HR** **(95% CI)**	**p-value**
Unfavourable outcome **593/810 (73%)**	1.06 (0.88-1.28)	0.55	0.92 (0.76-1.10)	0.35	0.98 (0.81-1.18)	0.81
Cognitive decline **(MoCA ≤21)** **113/824 (14%)**	1.27 (0.84-1.91)	0.26	**2.36** **(1.60-3.46)**	**0.001**	1.67 (1.12-2.51)	0.01
Postural instability **(H&Y ≥3)** **144/814 (18%)**	1.11 (0.76-1.62)	0.59	1.14 (0.77-1.67)	0.52	1.14 (0.77-1.67)	0.52
Motor progression **(≥5 point increase in MDS-UPDRS-III score relative to baseline score)** **534/737 (72%)**	1.01 (0.83-1.23)	0.89	0.94 (0.77-1.14)	0.54	0.99 (0.82-1.20)	0.93
Dependency **(SEADL <80)** **231/814 (28%)**	1.00 (0.74-1.34)	0.99	1.10 (0.81-1.48)	0.55	1.19 (0.88-1.60)	0.25
Death **50/709 (7%)**	1.07 (0.47-2.44)	0.87	0.97 (0.50-1.88)	0.92	1.03 (0.53-2.00)	0.93

Hazard ratio: HR, 95% confidence interval: 95% CI, Movement Disorder Society-Unified Parkinson's Disease Rating Scale part III: MDS-UPDRS III, Montreal Cognitive Assessment: MoCA, Schwab and England Activities of Daily Living Scale: SEADL, Hoehn and Yahr stage: H&Y, Maximum Thioflavin T fluorescence value: MaxThT, Time to threshold: TTT, Area under the curve: AUC. Cox proportional hazards models that adjusted for sex, age and disease duration at baseline were used to assess whether baseline a-syn SAA kinetic measures predict various milestones of clinical decline. Bonferroni significance (red) $p<0.003$.

Supplementary Table 4: Baseline clinical profile and CSF a-syn SAA status of the Tuebingen cohort.

	Sporadic PD (n=107)	LRRK2-PD (n=10)	GBA-PD (n=91)	DJ1-PD (n=2)	PINK1-PD (n=2)	PRKN-PD (n=17)
Sex (% male)	68.2%	30.0% †	68.1%	50.0%	0%	58.8%
Mean age at symptom onset, years (SD)*	56.9 (10.4)	52.6 (14.1) †	55.6 (10.0) †	50.0 (7.1)	39.0 (2.8) †	46.5 (13.5) †
Mean age at baseline, years (SD)*	64.0 (9.2)	65.0 (12.1)	63.4 (9.5)	60.3 (10.3)	67.0 (4.4)	59.6 (11.8) †
Mean disease duration at baseline, years (SD)*	7.1 (6.1)	12.4 (8.1) †	7.8 (5.4) †	10.3 (3.2) †	28.0 (1.6) †	13.1 (10.9) †
Mean MDS-UPDRS-III at baseline, score (SD)^	25.0 (11.0)	26.0 (8.9)	27.3 (11.8)	42.5 (10.6) †	22.0 (NA)	22.0 (12.1)
Mean MoCA at baseline, score (SD)^	25.0 (4.2)	24.0 (4.1)	24.5 (4.7)	20.5 (6.4)	28.0 (NA)	26.0 (3.9)
Median H&Y at baseline (IQR)	2 (2-2)	2 (2-2.8)	2 (2-2)	2.5 (2.3-2.8)	2.5 (2.3-2.8)	2 (2-2)
CSF a-syn SAA positive, n, (95% CI)	98/107 92% (85-96%)	7/10 70% (35-93%)	83/91 91% (83-96)	2/2 100%	0/2 0%	10/17 59% (33-82%)

Alpha-synuclein: a-syn, Movement Disorder Society-Unified Parkinson's Disease Rating Scale part III: MDS-UPDRS III, Montreal Cognitive Assessment: MoCA, Hoehn and Yahr stage: H&Y, Interquartile range: IQR, Not applicable: NA, Standard deviation: SD, 95% confidence interval = 95% CI. Fisher's exact test used for group comparisons of sex distributions. Group comparisons of continuous variables were done using linear regression that was either unadjusted (*) or adjusted (^) for sex, age and disease duration at baseline. CSF a-syn SAA positive 95% CI calculated using the binomial formula for all proportions >0% and <100%. † $p<0.05$ vs. sporadic PD group.

Supplementary Table 5: Pathogenic variants detected in monogenic PD participants in the Tuebingen PD cohort

Gene	Tuebingen PD cohort
LRRK2	G2019S, n=4 R1441C, n=2 I2020T, n=2 N1437S, n=2
GBA	E326K, n=35 L444P, n=15 T369M, n=13 N370S, n=12 c.115+1G>A, n=3 D140H, n=2 S271G, n=2 W184R, n=2 c.1265_1319del, n=1 D409H, n=1 E388K, n=1 G202R, n=1 R359X, n=1 R39C, n=1 T297S, n=1
DJ1	Exon 1+2+3+4+5 duplication, n=1 Exon 4+5 duplication, n=1
PINK1	p.Q126P homozygous, n=2

Gene	Tuebingen PD cohort
PRKN	p.R275W, n=4 p.P437L, n=3 ex3+4del, n=2 p.R234Q, n=1 c.101_102delAG, ex3+4del, n=1 Exon 8+9 deletion, n=1 Exon 2 duplication, n=1 Exon 2 deletion, n=1 Exon 2 deletion, Exon 7 duplication, n=1 Exon 3+4+5+6 deletion homozygous, n=1 Exon 4 deletion, Exon 8+9 deletion, n=1

Leucine-rich repeat kinase 2: LRRK2, Beta-glucocerebrosidase: GBA, Parkinsonism associated deglycase: DJ1, PTEN induced kinase 1: PINK1, Parkin RBR E3 ubiquitin protein ligase: PRKN.

Supplementary References

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