BRAIN
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Alpha-synuclein seed amplification assay longitudinal outcomes in Lewy body disease spectrum

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Evidence from neuropathological cohorts indicates that a CSF α-synuclein (α-syn) seed amplification assay (SAA) might provide quantitative kinetic parameters correlating with α-syn pathology burden in patients with Lewy body disease (LBD). Studies are needed to assess their longitudinal trend during the presymptomatic and clinical disease phases and their correlation with measures of disease progression.

We aimed to assess the baseline α-syn CSF SAA kinetic parameters, their longitudinal variations and associations with clinical outcomes in a longitudinal cohort of repeatedly sampled LBD patients, including clinically unimpaired (asymptomatic LBD) and neurologically impaired individuals. Participants from the prospective BioFINDER-1 study with longitudinal CSF collections ($n = 718$) were screened by α-syn SAA. CSF samples were tested in four replicates blinded to clinical diagnoses. The number of positive replicates (N_{rep}), the time needed by the fluorescence signal to reach the threshold (Lag) and the highest intensity of the fluorescent signal were analysed at baseline (time of first positive SAA) in all participants and longitudinally in those with at least two α-syn-positive CSF samples available.

One hundred and ninety-six individuals (whole cohort) showing α-syn seeding activity were included. Of those, 170 participants tested positive by SAA in all available samples, while 26 converted from a negative to a positive test result during follow-up (LBD-converters), suggesting an early LBD stage. At baseline, LBD-converters showed lower N_{rep} ($P = 0.001$) and a longer Lag ($P = 0.001$) than subjects displaying α-syn seeding activity from the first available sample. The N_{rep} increased longitudinally in the whole cohort [$\beta = 0.09$, 95% confidence interval (95% CI) 0.06–0.12, $P < 0.001$], in asymptomatic LBD ($\beta = 0.15$, 95% CI 0.09–0.21, $P < 0.001$) and in Parkinson's disease individuals without dementia ($\beta = 0.07$, 95% CI 0.02–0.12, $P = 0.01$). The Lag decreased longitudinally in asymptomatic LBD ($\beta = -0.24$, 95% CI −0.42 to −0.06, $P = 0.008$). Baseline N_{rep} predicted the subsequent appearance of dementia in the whole cohort [hazard ratio (HR) 1.57, 95% CI 1.19–2.07, $P = 0.001$] and the Parkinson's disease subgroup (HR 1.83, 95% CI 1.17–2.85, $P = 0.008$). The difference between the Lag at each sampling and that at baseline was negatively associated with the appearance of dementia in the whole cohort (HR 0.76, 95% CI 0.59–0.99, $P = 0.04$) and in the Parkinson's disease subgroup (HR 0.69, 95% CI 0.50–0.95, $P = 0.02$). The α-syn SAA parameters N_{rep} and Lag showed associations with the LBD stage and the development of dementia. Furthermore, their longitudinal variation is coherent with progression of pathology over time. These data support the use of SAA kinetic parameters to monitor disease progression and therapeutic response.

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Introduction

Lewy body disease (LBD) is the second most prevalent neurodegenerative disorder and is characterized by the intraneuronal accumulation of misfolded α-synuclein (α-syn) in the form of Lewy bodies.[1] It is primarily the pathological hallmark of Parkinson's disease (PD) and dementia with Lewy bodies, but it also constitutes a frequent co-pathology in patients with Alzheimer's disease (AD).[2-4] Recently, given the neuronal localization of Lewy bodies, a definition of neuronal α-synuclein disease has been proposed.[5]

Recently, the α-syn seed amplification assays (SAA), which exploit the prion-like properties of α-syn to amplify misfolded α-syn from accessible fluids and tissues, have provided the first pathology-specific biomarker of LBD, allowing the ante-mortem diagnosis of this disorder with an accuracy of >90%.[6-15]

One limitation of current α-syn SAA protocols is the binary positive/negative classification of samples, which does not quantify α-syn seeds. However, recent data have shown associations between SAA kinetic parameters and disease progression, measured by the appearance of dementia in patients with Parkinson's disease.[16,17] Moreover, a recent study in a neuropathological cohort showed a significant correlation between the brain Lewy body pathology burden and SAA kinetic parameters.[13] These results raise expectations about using the α-syn SAA to assess the disease stage and predict clinical progression besides confirming or excluding α-syn pathology.

In this context, studies on CSF samples collected longitudinally in clinically monitored study participants represent a unique chance to investigate thoroughly the reliability of the assay at different disease stages and the association between SAA kinetics, clinical status and disease progression. Given the progressive course of the disease, a longitudinal increase of CSF α-syn seeds is expected. Moreover, as disease-modifying therapies for LBD are being developed,[18] defining the longitudinal trajectories of kinetic parameters in subjects with different clinical diagnoses is crucial because they represent potential biomarkers for treatment response.

In this study, we applied the α-syn CSF SAA real-time quaking-induced conversion (RT-QuIC) to a large longitudinal prospective cohort and evaluated the variations of the binary positive/negative outcome of the assay and associated quantitative kinetic parameters over a median of 3.9 years (range 1.2–10 years). Furthermore, we tested the associations between clinical outcomes and baseline and longitudinal kinetic parameters.

Materials and methods

Study population and clinical assessment

The initial study cohort included 718 BioFINDER-1 (NCT01208675) participants with at least two longitudinal CSF samples available. According to the study protocol,[19,20] every patient underwent periodic visits and CSF collections ~2 years apart. At baseline (time of the first positive SAA), study participants were either cognitively unimpaired (CU) or had PD, subjective cognitive decline, mild cognitive impairment (MCI) or dementia. Clinical diagnoses were attributed according to established criteria.[21-27] Patients with a diagnosis of PD and fulfilling criteria for MCI at baseline were denominated as PD-MCI.[25]

When available, scores on the following clinical tests were collected at each visit: Mini-Mental State Examination (MMSE), immediate and delayed word recall tasks from the Alzheimer's Disease Assessment Scale-Cognitive Subscale (ADAS-Cog) and the Brief Smell Identification Test (BSIT).

AD co-pathology was assessed by the CSF $A\beta_{42}$:p-tau181 ratio according to a validated cut-off[28] through the automated Lumipulse platform.

Study details for the BioFINDER-1 cohort have been reported elsewhere[19,20] and are briefly summarized here. For subjects with cognitive symptoms, the inclusion criteria were as follows: (i) referred to a participating secondary memory clinic owing to cognitive symptoms recognized by the patient, caregiver and/or the referring physician; (ii) age 40–100 years; and (iii) speaks and understands Swedish to the extent that an interpreter is not necessary for the patient to understand the study information and cognitive tests fully. The exclusion criteria were as follows: (i) significant unstable systemic disease or organ failure; and (ii) current significant alcohol or substance misuse. All patients were enrolled and underwent the first examination between 2007 and 2017. MCI was classified as not fulfilling the criteria for dementia according to the Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5)[29] and performing worse than −1.5 standard deviations in at least one of the cognitive domains of memory, attention/executive, verbal or visuospatial function. Dementia was classified according to the DSM-5 criteria for major neurocognitive disorders.[29] For cognitively unimpaired subjects, the inclusion criteria were as follows: (i) age 40–100 years; (ii) performs ≥24 points at the MMSE; and (iii) speaks and understands Swedish to the extent that an interpreter is not required for the patient to understand the study information and cognitive tests fully. None of the included participants fulfilled the clinical criteria for dementia or MCI at first examination. Participants with subjective cognitive decline had subtle cognitive symptoms (perceived by the participant or informant) but did not meet the MCI criteria. A neuropsychological assessment was performed by a senior neuropsychologist after a thorough battery, as described in detail elsewhere.[30]

Patients with parkinsonian disorders were included at the Neurology Clinic, Skåne University Hospital, Sweden, between 2008 and 2017. Participants were younger than 85 years old. The exclusion criteria were as follows: (i) significant unstable systemic

illness, such as terminal cancer or organ failure that made it difficult to participate in the study; (ii) current significant alcohol or substance misuse; (iii) significant neurological or psychiatric illness, including clinically diagnosed AD dementia, vascular dementia or frontotemporal lobe dementia; and (iv) participation in a clinical drug trial during the last 30 days. The cognitive status of patients with PD was assessed through a structured interview and cognitive tests [including MMSE, a quick test of cognitive speed (AQT), an animal fluency test, immediate and delayed word recall tasks from the ADAS-Cog and clock-drawing test].

SAA analyses

The α-syn SAA analyses were performed, blinded to the clinical diagnoses, according to an established RT-QuIC protocol.[11]

All samples were run initially in quadruplicates and considered positive when at least three of four replicates reached a threshold arbitrarily set at 30% of the median of the maximum fluorescence signal (Imax) values of the positive replicates of each plate's positive controls. Following recent data suggesting increased reliability of the final binary positive/negative classification of samples with repeated SAA runs,[11,31] all samples initially showing one or two positive replicates were run two additional times. They were then considered positive when at least four replicates out of 12 gave a positive result.

Assessment of SAA kinetic parameters

The number of positive replicates (N_{rep}) per sample corresponded to the number of replicates reaching the threshold. For samples analysed more than once, we considered the median of N_{rep}.

The time to the threshold (Lag) was calculated as the time (expressed in hours) needed to reach the fluorescence threshold in each run. As recently suggested,[31] we calculated two different Lag times for each sample: Lag_{med1} (the median of the Lag of each positive replicate) and Lag_{med2} (the median of the first two replicates reaching the threshold in each positive test).

The Imax was calculated as the highest intensity of the fluorescent signal (in relative fluorescent units). We calculated both $Imax_{med1}$ and $Imax_{med2}$, as indicated for the Lag.

Kinetic parameters were analysed at baseline in all patients and longitudinally in participants with at least two positive CSF samples ($n = 184$). Analyses were conducted in the whole cohort and subgroups according to the different diagnostic categories. These included:

(i) Cognitively unimpaired participants and individuals with subjective cognitive decline at baseline (asymptomatic LBD).
(ii) PD patients cognitively unimpaired at baseline not developing dementia during follow-up (PD-CU).
(iii) PD patients cognitively unimpaired at baseline developing dementia during follow-up (PD-PDD converters).
(iv) Cognitively impaired (CI) participants at baseline at stage of either mild cognitive impairment or dementia fulfilling clinical criteria for dementia with Lewy bodies, prodromal mild cognitive impairment stage of dementia with Lewy bodies (MCI-LB), PD-MCI or PD dementia (CI-LBD). Cognitively impaired participants at baseline not fulfilling these clinical criteria but not showing CSF biomarkers of AD pathophysiology (i.e. an abnormal $Aβ_{42}$:p-tau181 ratio; $n = 7$) were also included in this subgroup.
(v) Cognitively impaired participants at baseline responding to clinical criteria for dementia or mild cognitive impairment attributable to AD and showing CSF biomarkers of AD pathophysiology (CI-AD). A single cognitively impaired participant not fulfilling these clinical criteria, but showing a pathological $Aβ_{42}$:p-tau181 ratio, was also included in this subgroup.

Notably, we verified that the inclusion of PD participants in either the PD-CU or the PD-PDD converters subgroup was not affected by a different follow-up duration. The median time from baseline to last clinical assessment in PD-CU participants [6.4 years, interquartile range (IQR) 5.5–9.5] was indeed longer than the median time from baseline to the appearance of dementia in PD-PDD converters (4.2 years, IQR 3.6–5.9).

Statistical analyses

Analyses were performed using Stata SE v.14.2 (StataCorp, College Station, TX, USA) and GraphPad Prism v.7 (GraphPad, La Jolla, CA, USA). Depending on the number of groups, the Mann–Whitney U-test or the Kruskal–Wallis test (followed by the Dunn–Bonferroni test) was used for group comparisons. Fisher's test was applied to categorical variables. Spearman's ρ coefficients assessed the correlation between baseline kinetic parameters and between these and age.

A linear mixed-effect modelling analysis with random slope and intercept was applied to evaluate the longitudinal change of α-syn kinetic parameters. The dependent variables were kinetic parameters (one for each model), and the independent variable was time (in years). The same longitudinal models were implemented to evaluate the association between kinetic parameters (dependent variables) and scores at clinical scales (independent variables) over time. The results are expressed as β-coefficients and 95% confidence intervals (95% CI). Longitudinal change of the Lag was also evaluated qualitatively by comparing its value at baseline and at the time of the last available sample.

The association of baseline kinetic parameters with the risk of dementia was assessed by Cox regression analyses with fixed covariates. To evaluate the relationship between the longitudinal trend of kinetic parameters and the risk of dementia, we calculated, for each follow-up sampling visit and kinetic parameter, a $\Delta_{parameter}/\Delta_{time}$ (kinetic parameter at the visit minus kinetic parameter at baseline divided by years between visit and baseline) expressing the annual change from baseline of that parameter. The association of $\Delta_{parameter}/\Delta_{time}$ and risk of dementia was evaluated with a time-dependent regression model with the covariates varying over time. The same time-dependent approach was used to evaluate the association between the time-varying kinetic parameters (i.e. time-varying N_{rep}, Lag and Imax) and the risk of dementia. The results for all Cox regressions are presented as hazard ratios (HR) and 95% CI. For these analyses, participants belonging to PD-CU and PD-PDD converter subgroups were merged into a single 'PD' subgroup. Individuals with dementia or last clinical assessment at baseline (or at second sampling for $\Delta_{parameter}/\Delta_{time}$ analyses) were excluded (Supplementary material). These analyses were also corrected for AD co-pathology. Differences were considered statistically significant at $P < 0.05$.

Results

Dichotomic α-syn SAA results across BioFINDER-1 participants with longitudinal assessment

Among the 718 patients screened, 197 had a positive CSF α-syn SAA at least once (whole cohort); 170 of them tested positive in all available CSF samples (463 CSF samples in total), whereas 26 converted to a positive α-syn status after one or more initial negative samples (LBD-converters). Only one participant with two samples available

tested negative for a sample collected 2 years after the baseline positive one. Being a single outlier, this subject was excluded from further analyses. Among the 26 LBD-converters, 10 had two, three had three, and one had four consecutive positive samples after the initial negative result, whereas 12 tested positive only once (at the last visit). In total, the analysis of CSF samples belonging to the whole cohort required 162 different α-syn RT-QuIC SAA experiments (i.e. plate runs).

Baseline demographic and clinical variables in the SAA-positive cohort

Details on the demographic and clinical data of the participants are reported in Table 1 and Supplementary Table 1.

At baseline, among the 196 selected SAA-positive individuals (whole cohort), 94 (47.9%) had a clinical diagnosis of PD, 11 (5.6%) of MCI attributable to AD (MCI-AD), 10 (5.1%) of prodromal dementia with Lewy bodies (MCI-LB), nine (4.6%) of PD dementia (PDD) and four (2.0%) of PD with mild cognitive impairment (PD-MCI). Thirty-nine (19.9%) participants were cognitively unimpaired at baseline, while 16 (8.2%) met the criteria for subjective cognitive decline. Seventy-two (36.7%) participants in the whole cohort developed dementia during follow-up.

Baseline SAA kinetic parameters

At baseline, age was unrelated to any kinetic parameter (Supplementary Table 2). Baseline N_{rep} was negatively associated with both Lag_{med1} and Lag_{med2} in the whole cohort (Lag_{med1}: $\rho = -0.25$, $P < 0.001$; Lag_{med2}: $\rho = -0.45$, $P < 0.001$) and in the asymptomatic LBD subgroup (Lag_{med1}: $\rho = -0.34$, $P = 0.01$; Lag_{med2}: $\rho = -0.61$, $P < 0.001$). In the other subgroups, N_{rep} was associated only with Lag_{med2} (PD-CU, Lag_{med2}: $\rho = -0.31$, $P = 0.01$; PD-PDD converters, Lag_{med2}: $\rho = -0.38$, $P = 0.03$; CI-LBD, Lag_{med2}: $\rho = -0.41$, $P = 0.02$; CI-AD, Lag_{med2}: $\rho = -0.73$, $P = 0.002$). No associations between baseline N_{rep} and Imax values were found in the whole cohort or any subgroup (Supplementary Table 3).

At the time of first positive assay (baseline), LBD-converters had significantly lower median N_{rep} ($P = 0.001$) and longer Lag (Lag_{med1}, $P = 0.001$; Lag_{med2}, $P = 0.003$) than participants already showing α-syn seeding activity in the first available sample (N_{rep}, LBD-converters: median 2.0, IQR 2.0–3.0; non-LBD-converters: median 3.0, IQR 2.0–4.0; Lag_{med1}, LBD-converters: median 19.6, IQR 17.7–21.0; non-LBD-converters: median 17.4, IQR 16.0–19.2; Lag_{med2}, LBD-converters: median 18.5, IQR 17.0–19.2; non-LBD-converters: median 16.6, IQR 14.9–18.6; Table 2, Fig. 1A and C and Supplementary Fig. 1A and B).

At baseline, median N_{rep} differed significantly among participant subgroups ($P < 0.001$), with PD-PDD converters showing higher values than PD-CU ($P = 0.01$) and asymptomatic LBD ($P < 0.001$; asymptomatic LBD: median 3.0, IQR 2.0–3.0. PD-CU: median 3.0, IQR 2.0–3.0; PD-PDD converters: median 4.0, IQR 3.0–4.0; CI-LBD: median 3.0, IQR 3.0–4.0; CI-AD: median 2.5, IQR 2.0–3.7). There were no significant differences in the baseline Imax and Lag across different subgroups (Table 2, Fig. 1D–F and Supplementary Fig. 1C and D).

Longitudinal variation of SAA kinetic parameters

Overall, the median N_{rep} increased over time. This was significant in the whole cohort ($\beta = 0.09$, 95% CI 0.06–0.12, $P < 0.001$) and in all subgroups, except for the PD-PDD converters, in which the parameter remained stable (Table 3).

There was a longitudinal decrease in the Lag only in the asymptomatic LBD (Lag_{med2}: $\beta = -0.24$, 95% CI −0.42 to −0.06, $P = 0.008$) and in the CI-AD subgroups (Lag_{med1}: $\beta = -0.57$, 95% CI −0.82 to −0.32, $P < 0.001$; Lag_{med2}: $\beta = -0.49$, 95% CI −0.71 to −0.27, $P < 0.001$). Lag duration was stable over time in the whole cohort and remaining subgroups (Table 3 and Fig. 2). When evaluated qualitatively, 67.3% (35/52) and 100% (10/10) of participants in the asymptomatic LBD and CI-AD subgroups showed a longer Lag_{med1} duration at baseline than at the last sample available. A similar behaviour was noted for Lag_{med2} (asymptomatic LBD: 36/52, 69.2%; CI-AD: 9/10, 90%; Supplementary Table 4).

There were no significant longitudinal changes in the Imax in the whole cohort or any subgroup (Table 3).

Association of baseline SAA kinetic parameters with the appearance of dementia

Both in the whole cohort and in participants with PD, baseline N_{rep} was positively associated with subsequent dementia (whole cohort: HR 1.57, 95% CI 1.19–2.07, $P = 0.001$; PD: HR 1.83, 95% CI 1.17–2.85, $P = 0.008$; Table 4). Both analyses retained statistical significance after correcting for AD co-pathology (whole cohort: HR 1.69, 95% CI 1.26–2.27, $P < 0.001$; PD: HR 1.81, 95% CI 1.15–2.85, $P = 0.01$; Supplementary Table 5). There were no significant associations of baseline Lag and Imax with progression to dementia (Table 4 and Supplementary Tables 5 and 6).

Association of time-varying SAA kinetic parameters with clinical variables

The time-varying parameter $\Delta_{Lag}/\Delta_{time}$ was negatively associated with the risk of subsequent dementia in the whole cohort (Lag_{med1}: HR 0.76, 95% CI 0.59–0.99, $P = 0.04$) and participants with PD (Lag_{med1}: HR 0.69, 95% CI 0.50–0.95, $P = 0.02$; Table 4); however, the association was no longer significant after correcting for AD co-pathology (Supplementary Table 5).

Using the same time-varying approach, N_{rep} was positively associated and Lag negatively associated with progression to dementia both in the whole cohort (N_{rep}: HR 1.54, 95% CI 1.14–2.09, $P = 0.005$; Lag_{med1}: HR 0.91, 95% CI 0.83–0.99, $P = 0.04$; Lag_{med2}: HR 0.90, 95% CI 0.82–0.98, $P = 0.02$) and in the subgroup of PD participants (N_{rep}: HR 1.73, 95% CI 1.06–2.82, $P = 0.03$; Lag_{med1}: HR 0.87, 95% CI 0.75–0.99, $P = 0.05$; Table 4). The association between N_{rep} and dementia in the whole cohort remained significant after correction for AD co-pathology (HR 1.51, 95% CI 1.11–2.05, $P = 0.009$; Supplementary Table 5).

In the whole cohort, N_{rep} was negatively associated with the MMSE score over time ($\beta = -0.44$, 95% CI −0.68 to −0.19, $P < 0.001$), also after correction for AD co-pathology ($\beta = -0.45$, 95% CI −0.69 to −0.22, $P < 0.001$). In the whole cohort, worse immediate and delayed word recall scores were associated with higher N_{rep} over time (immediate: $\beta = 0.14$, 95% CI 0.02–0.27, $P = 0.02$; delayed: $\beta = 0.21$, 95% CI 0.02–0.40, $P = 0.03$) and lower Lag (immediate: Lag_{med1} $\beta = -0.08$, 95% CI −0.13 to −0.02, $P = 0.004$, and Lag_{med2} $\beta = -0.09$, 95% CI −0.15 to −0.03, $P = 0.002$; delayed: Lag_{med1} $\beta = -0.08$, 95% CI −0.15 to −0.01, $P = 0.04$, and Lag_{med2} $\beta = -0.09$, 95% CI −0.17 to −0.01, $P = 0.04$). All analyses, except for the association between Lag and delayed word recall scores, were also significant after AD co-pathology correction (Supplementary Tables 7 and 8).

In the asymptomatic LBD subgroup, N_{rep} was negatively associated with smell function measured using the BSIT score over

Table 1 Demographic and basic clinical variables in the whole cohort and subgroups

Variable	Whole cohort (*N* = 196)	Asymptomatic LBD (*n* = 55)	PD-CU (*n* = 60)	PD-PDD converters (*n* = 34)	CI-LBD (*n* = 31)	CI-AD (*n* = 16)	*P*-values[a]
Age at baseline, years[b]	69.8 (65.0–75.0)	74.0 (69.9–77.4)	64.0 (58.0–68.7)	69.0 (67.0–72.2)	70.0 (66.3–75.2)	75.4 (70.8–77.3)	**<0.001**[c]
Females, *n* (%)	65 (33.2)	26 (47.3)	16 (26.7)	10 (29.4)	5 (16.1)	8 (50.0)	**0.01**[d]
Education, years[b]	12.0 (9.5–15.0)	11.0 (9.0–13.0)	13.0 (10.0–15.4)	13.0 (9.4–16.1)	12.0 (9.0–14.0)	10.5 (9.0–12.7)	0.15
Motor symptoms duration, years[b,e]	4.0 (2.0–7.0)	n/a	4.0 (2.0–6.7)	4.0 (2.0–6.0)	11.5 (6.5–16.5)	n/a	**<0.001**
Sampling time duration, years[b]	3.9 (2.2–4.8)	4.2 (3.7–5.9)	3.9 (2.1–4.1)	3.9 (2.1–4.3)	2.2 (2.0–4.1)	4.2 (2.8–4.5)	**0.002**
CSF samples per patient[b], *n*	3.0 (2.0–3.0)	3.0 (2.0–4.0)	3.0 (2.0–3.0)	3.0 (2.0–3.0)	2.0 (2.0–3.0)	2.5 (2.0–3.0)	**0.01**
SAA-positive CSF samples per patient[b], *n*	2.0 (2.0–3.0)	3.0 (2.0–3.0)	3.0 (2.0–3.0)	3.0 (2.0–3.0)	2.0 (2.0–3.0)	2.0 (1.0–2.0)	**<0.001**
SAA positivity ratio, *n*/*n* (%)[f]	508/547 (92.9)	148/169 (87.6)	165/168 (98.2)	95/95 (100)	71/73 (97.3)	29/42 (69.0)	**<0.001**
Conversion to dementia, *n* (%)	72 (36.7)	14 (25.4)	0 (0)	34 (100)	14 (45.2)	10 (62.5)	**<0.001**
Time to progression to dementia, years[b]	3.8 (2.1–5.5)	4.8 (2.1–7.5)	n/a	4.2 (3.6–5.9)	2.0 (1.9–2.6)	2.8 (1.8–3.1)	**<0.001**
LBD-converters, *n* (%)	26 (13.3)	15 (27.3)	2 (3.3)	0 (0)	2 (6.4)	7 (43.7)	**<0.001**
APOE ε4 positive, *n* (%)	73 (37.2)	22 (40.0)	9 (15.0)	18 (52.9)	10 (32.2)	14 (87.5)	**<0.001**
CSF $A\beta_{42}$:p-tau181 ratio[b]	81.5 (41.1–102.1)	65.7 (29.1–107.2)	96.1 (86.5–110.6)	66.4 (41.4–98.7)	74.3 (52.3–94.7)	19.9 (16.5–28.0)	**<0.001**
Pathological CSF $A\beta_{42}$:p-tau181 ratio, *n*/*n* (%)	52/193 (26.9)	23/55 (41.8)	0/60 (0)	8/31 (25.8)	5/31 (16.1)	16/16 (100)	**<0.001**
Diagnosis at baseline, *n* (%)							
SCD	16 (8.2)	16 (29.1)	–	–	–	–	–
CU	39 (19.9)	39 (70.9)	–	–	–	–	–
PD	94 (47.9)	–	60 (100)	34 (100)	–	–	–
PDD	9 (4.6)	–	–	–	9 (29.0)	–	–
DLB	1 (0.5)	–	–	–	1 (3.2)	–	–
MCI-LB	10 (5.1)	–	–	–	10 (32.2)	–	–
PD-MCI	4 (2.0)	–	–	–	4 (12.9)	–	–
MCI-AD	11 (5.6)	–	–	–	–	11 (68.7)	–
AD	4 (2.0)	–	–	–	–	4 (25.0)	–
Others	8 (4.1)	–	–	–	7 (22.6)[g]	1 (6.2)[h]	–

P-values of statistically significant comparisons are reported in bold. Sampling time duration is the time between first and last sampling visits. AD = Alzheimer's disease dementia; CI = cognitively impaired; CU = cognitively unimpaired; DLB = dementia with Lewy bodies; LBD = Lewy body disease; MCI = mild cognitive impairment; MCI-AD = mild cognitive impairment attributable to Alzheimer's disease; MCI-LB = prodromal mild cognitive impairment stage of dementia with Lewy bodies; n/a = not applicable; PD = Parkinson's disease; PDD = Parkinson's disease dementia; PD-MCI = Parkinson's disease with mild cognitive impairment; SAA = seed amplification assay; SCD = subjective cognitive decline.

[a]Refer to the comparison among patients in the five different diagnostic categories.

[b]Expressed as the median (interquartile range).

[c]*Post hoc* analyses: PD-CU versus asymptomatic LBD, CI-LBD and CI-AD ($P < 0.001$); PD-PDD converters versus asymptomatic LBD and PD-CU ($P = 0.02$).

[d]Single significant analyses (Fisher's test): asymptomatic LBD versus PD-CU ($P = 0.03$); asymptomatic LBD versus CI-LBD ($P = 0.005$); CI-LBD versus CI-AD ($P = 0.02$).

[e]Data available for 105 patients.

[f]Ratio between SAA-positive and available CSF samples.

[g]Includes four participants with MCI attributable to vascular disease, two participants with MCI not otherwise specified and one participant with dementia not otherwise specified.

[h]Includes one participant with vascular dementia.

Table 2 Baseline kinetic parameters in the whole cohort and subgroups

Parameter	Whole cohort (N = 196)	Asymptomatic LBD (n = 55)	PD-CU (n = 60)	PD-PDD converters (n = 34)	CI-LBD (n = 31)	CI-AD (n = 16)	P-values[a]
N_{rep}	3.0 (2.0–4.0)	3.0 (2.0–3.0)	3.0 (2.0–3.0)	4.0 (3.0–4.0)	3.0 (3.0–4.0)	2.5 (2.0–3.7)	**<0.001**[b]
$Imax_{med1}$	127 672 (105 086–156 596)	126 785 (110 803–150 958)	132 289 (106 480–157 628)	113 463 (102 759–157 999)	119 584 (97 927–161 148)	145 722 (116 681–163 682)	0.57
$Imax_{med2}$	133 317 (111 596–160 711)	131 571 (114 503–150 958)	142 947 (112 057–161 246)	127 246 (111 950–158 635)	133 248 (101 360–168 367)	147 604 (118 443–163 415)	0.69
Lag_{med1}	17.6 (16.3–19.7)	18.1 (16.8–20.6)	17.7 (16.7–19.5)	17.6 (16.1–20.5)	17.2 (15.5–19.8)	16.7 (15.9–19.1)	0.40
Lag_{med2}	16.8 (15.2–18.7)	17.1 (15.5–18.8)	17.0 (16.0–18.9)	16.4 (14.4–18.8)	15.8 (14.5–18.0)	16.3 (14.7–18.8)	0.16

	LBD-converters (n = 26)	Non-LBD converters (n = 170)	P-values
N_{rep}	2.0 (2.0–3.0)	3.0 (2.0–4.0)	**0.001**
$Imax_{med1}$	133 623 (112 948–164 368)	126 748 (102 897–155 875)	0.38
$Imax_{med2}$	136 468 (118 468–174 687)	133 161 (111 258–158 706)	0.40
Lag_{med1}	19.6 (17.7–21.0)	17.4 (16.0–19.2)	**0.001**
Lag_{med2}	18.5 (17.0–19.2)	16.6 (14.9–18.6)	**0.003**

Kinetic parameters are expressed as the median (interquartile range). Imax values are measured in relative fluorescence units (RFU); Lag values are expressed in hours. *P*-values of statistically significant comparisons are reported in bold. AD = Alzheimer's disease; CI = cognitively impaired; CU = cognitively unimpaired; IQR = interquartile range; LBD = Lewy body disease; N_{rep} = number of positive replicates; PD = Parkinson's disease; PDD = Parkinson's disease dementia.

[a]Refer to the comparison among patients belonging to the five different subgroups.

[b]*Post hoc* analyses: asymptomatic LBD versus PD-PDD converters ($P < 0.001$); PD-CU versus PD-PDD converters ($P = 0.01$).

time (β = −0.37, 95% CI −0.71 to −0.03, $P = 0.03$; Supplementary Table 8).

Discussion

The development of accurate assays for misfolded α-syn has significantly improved the *in vivo* diagnosis of LBD, prompting proposals for new biologically based research diagnostic criteria.[2,5] However, biomarkers in neurodegenerative diseases are also needed to indicate the pathology stage and predict clinical progression. Taking advantage of a unique cohort of patients with LBD followed longitudinally, probably harbouring various burdens and stages of α-syn pathology, we provided several insights into this critical issue.

Initially, we demonstrated that LBD-converters, with an expected early neuropathological LBD stage, show a lower median N_{rep} and a longer median Lag than participants already showing seeding activity in the first available sample. This supports previous preliminary findings indicating that these kinetic parameters are the most promising in estimating the α-syn pathology burden.[13] We obtained similar results when comparing baseline parameters among participants with different clinical phenotypes, probably reflecting various LBD pathology burdens. Accordingly, asymptomatic LBD and PD-CU subgroups displayed a lower baseline median N_{rep} than PD-PDD converters. The lack of significant differences in the baseline Lag in this analysis might reflect a lower sensitivity of this parameter to pathology burden changes compared with N_{rep}. The expected partial overlap of neuropathological LBD stages across subjects with different clinical diagnoses should also be considered.

Regarding the longitudinal trend of α-syn seeding activity, we demonstrated, for the first time, a significant increase in the median N_{rep} in a cohort representative of the full LBD spectrum and confirmed the result in all subgroups except for PD-PDD converters. The latter result likely reflects a threshold effect of this parameter in patients with a high pathology load owing to the high baseline median N_{rep} in this subgroup (four out of four positive replicates). Notably, the presence of a significant increase of median N_{rep} in the CI-LBD subgroup, mainly including participants with mild cognitive impairment developing dementia at follow-up, fully supports the idea that, in patients with LBD, the progression of cognitive impairment implies a further spreading of LBD pathology.

Unlike for N_{rep}, we found no significant changes over time in median Imax and Lag in the whole cohort. However, there was a significant longitudinal Lag reduction in the asymptomatic LBD and the CI-AD subgroups. One possible explanation for these findings is that our current SAA protocol detects a significant longitudinal variation of the Lag, with a reduction implying an increase in LBD load,[13] only in the case of consistent progression in the pathology burden, as possibly occurring in the asymptomatic LBD and CI-AD subgroups given the higher β-coefficients of progression of median N_{rep}. Alternatively, the increase in CSF α-syn seeding activity might occur at different rates in subjects at different LBD stages. Notably, a recent study also reported a significant decrease in the Lag duration over time in participants converting from a negative to a positive α-syn SAA reflecting an early-stage LBD.[32]

Our results also have implications for the potential use of α-syn SAA as a surrogate marker of treatment response in therapeutic trials targeting α-syn. Notably, we showed a consistently positive outcome across longitudinal CSF samples once the positivity appeared and a significant stability (or changes indicating an increased LBD pathology burden) of kinetic parameters. Therefore, significant changes (i.e. decreased N_{rep} or increased Lag) in these parameters in the treatment group, or even the appearance of negative SAA results after initial positive tests, would support a positive effect of the drug through a reduction of α-syn seeds.

Of note, we evaluated the change of kinetic parameters over time as a 'group effect' in the whole cohort and the different subgroups according to baseline diagnostic categories. However, in the subgroups showing significant longitudinal changes for N_{rep} and/or Lag duration, not all patients showed a coherent variation over time of that parameter. This highlights the need

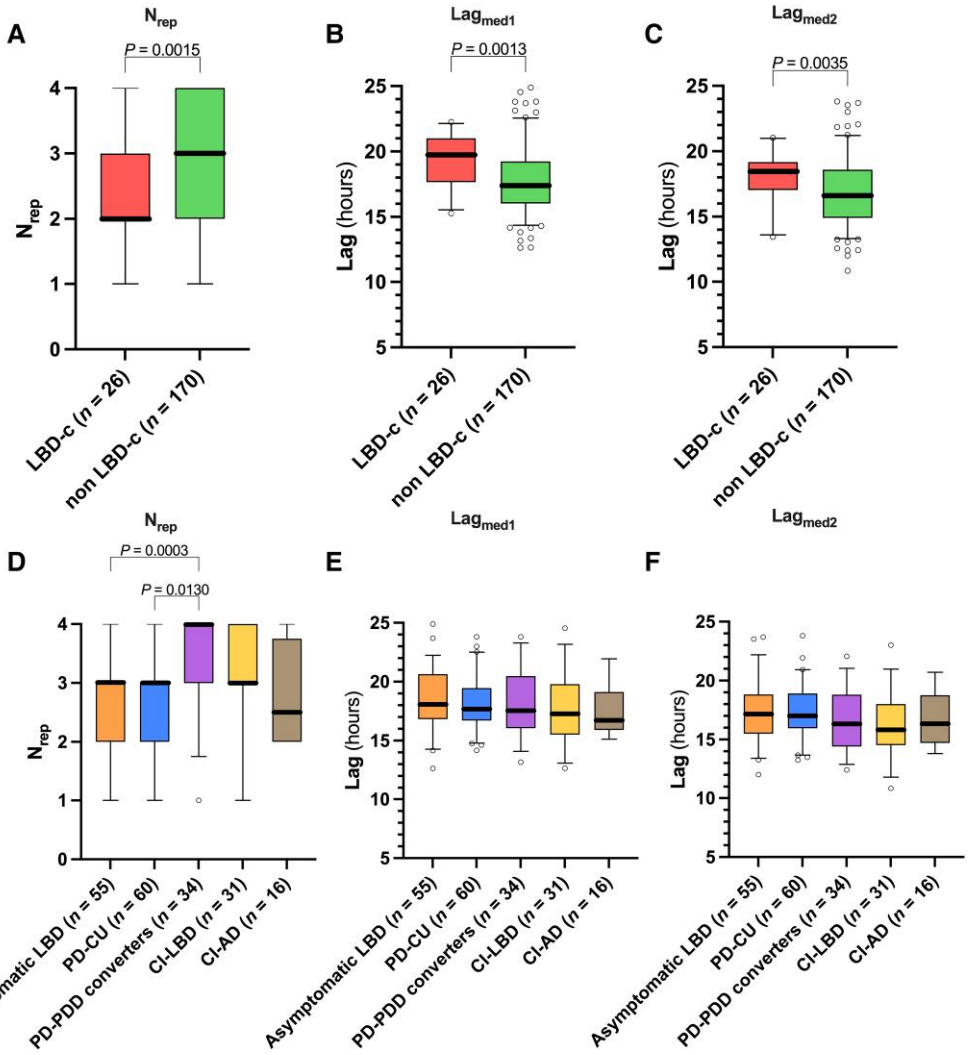

Figure 1 Comparison of baseline N_{rep} and Lag values between LBD-converters and non-LBD-converters and among different subgroups. (**A–C**) Values of N_{rep} (**A**), Lag_{med1} (**B**) and Lag_{med2} (**C**) in LBD-converters and non-LBD-converters. (**D–F**) Values of N_{rep} (**D**), Lag_{med1} (**E**) and Lag_{med2} (**F**) in participants belonging to different subgroups. Horizontal bold lines represent the median value, boxes represent the interquartile range, and whiskers represent the 5th and the 95th percentiles. Open circles represent the outliers. AD = Alzheimer's disease; CI = cognitively impaired; CU = cognitively unimpaired; N_{rep} = number of positive replicates; LBD-c = Lewy body disease converters; non-LBD-c = non-Lewy body disease converters; PD = Parkinson's disease; PDD = Parkinson's disease dementia.

for further improvement in the quantification of α-syn seeds by SAA.

Regarding the relevant issue of the correlation between baseline kinetic parameters and clinical measures, we found a significant association between baseline median N_{rep} and the subsequent appearance of dementia both in the whole cohort and in the participants with PD, which was retained after correcting for AD pathology. Notably, in our PD cohort, those developing dementia at follow-up already showed the highest median N_{rep} at baseline. This suggests that some PD patients might show a high LBD pathology burden years before the appearance of dementia.

In contrast to a recent study,[17] but similar to other published data,[9] we did not find any association of baseline Lag or Imax with the risk of progression to dementia. This result is probably related to the multiple determinants of cognitive impairment in patients with LBD, including co-pathologies, which might vary in different PD cohorts. In this regard, it might be noteworthy that, in contrast to the previously studied cohort,[17] the present one was characterized by a relatively high percentage of PD participants displaying AD co-pathology. Nonetheless, the lack of association between baseline kinetic parameters and clinical measures underlies the need to refine SAA protocols to quantify the α-syn seeds better.

Regarding clinical associations, the longitudinal analysis of kinetic parameters provided more interesting insights than the baseline analysis. Using time-varying parameters, we found that a higher N_{rep} and shorter Lag predicted a higher risk of subsequent dementia in the whole cohort and the participants with PD. Likewise, at follow-up visits, a decrease in the Lag was associated with a risk of progression to dementia. Taken together, these data demonstrate that a longitudinal progression of pathology is a determinant of cognitive impairment in individuals with LBD. Supporting this view, we found significant associations between the MMSE score and N_{rep} and between immediate and delayed memory measures, N_{rep} and Lag. Interestingly, previous studies using the same cognitive tests already showed the effects of LBD pathology on the longitudinal impairment of memory function.[11,12]

Table 3 Longitudinal variation of SAA kinetic parameters in positive samples in the whole cohort and subgroups

Parameter	Whole cohort ($N = 184$)	Asymptomatic LBD ($n = 52$)	PD-CU ($n = 58$)	PD-PDD converters ($n = 34$)	CI-LBD ($n = 30$)	CI-AD ($n = 10$)
N_{rep}						
P-value	**<0.001**	**<0.001**	**0.01**	0.41	**0.03**	**0.004**
β-Coefficient (95% CI)	0.09 (0.06 to 0.12)	0.15 (0.09 to 0.21)	0.07 (0.02 to 0.12)	0.02 (−0.03 to 0.07)	0.10 (0.01 to 0.19)	0.12 (0.04 to 0.20)
$Imax_{med1}$						
P-value	0.47	0.68	0.051	0.15	0.87	0.52
β-Coefficient (95% CI)	−432.4 (−1614.1 to 749.2)	−488.6 (−2785.1 to 1808.0)	−1868.0 (−3742.7 to 6.78)	1803.5 (−685.1 to 4292.1)	−339.9 (−4285.4 to 3605.6)	1031.8 (−2104.0 to 4167.7)
$Imax_{med2}$						
P-value	0.89	0.83	0.11	0.22	0.96	0.37
β-Coefficient (95% CI)	−88.8 (−1293.8 to 1116.1)	266.4 (−2118.2 to 2651.1)	−1597.5 (−3547.8 to 352.8)	1537.5 (−928.2 to 4003.3)	−106.5 (−4059.9 to 3846.9)	1665.5 (−1997.1 to 5328.1)
Lag_{med1}						
P-value	0.61	0.18	0.07	0.58	0.80	**<0.001**
β-Coefficient (95% CI)	−0.03 (−0.12 to 0.07)	−0.13 (−0.33 to 0.06)	0.15 (−0.01 to 0.31)	−0.05 (−0.25 to 0.14)	−0.04 (−0.34 to 0.26)	−0.57 (−0.82 to −0.32)
Lag_{med2}						
P-value	0.21	**0.008**	0.21	0.97	0.91	**<0.001**
β-Coefficient (95% CI)	−0.06 (−0.15 to 0.03)	−0.24 (−0.42 to −0.06)	0.09 (−0.05 to 0.24)	0.002 (−0.17 to 0.17)	−0.01 (−0.29 to 0.26)	−0.49 (−0.71 to −0.27)

Only participants with at least two CSF samples available were included. β-Coefficient values are reported as the main value (95% confidence interval). *P*-values of statistically significant results are reported in bold. AD = Alzheimer's disease; CI = cognitively impaired; CU = cognitively unimpaired; LBD = Lewy body disease; N_{rep} = number of positive replicates; PD = Parkinson's disease; PDD = Parkinson's disease dementia; 95% CI = 95% confidence interval.

Figure 2 Longitudinal Lag variation in the asymptomatic LBD and the CI-AD subgroups. (**A**) Longitudinal trend of Lag_{med2} in the asymptomatic LBD subgroup. (**B**) Longitudinal trend of Lag_{med1} in the CI-AD subgroup. (**C**) Longitudinal trend of Lag_{med2} in the CI-AD subgroup. Grey dotted lines represent the longitudinal variation at a single-patient level; thick lines represent the overall kinetic parameter longitudinal trend. AD = Alzheimer's disease; CI = cognitively impaired; LBD = Lewy body disease.

The finding of a negative association between N_{rep} and measures of smell function in the asymptomatic LBD subgroup, indicating that participants with a more advanced LBD pathology show worse performance in smell tests, deserves a final comment. Misfolded α-syn deposition in the olfactory bulb is thought to occur in the initial LBD stages[33,34]; however, an increase in α-syn load and

Table 4 Association of baseline and time-varying N_{rep} and Lag with the appearance of dementia in the whole cohort and subgroups

Parameter	Whole cohort	Asymptomatic LBD	PD	CI-LBD	CI-AD
Baseline kinetic parameters, HR					
	***n* = 179**	***n* = 54**	***n* = 94**	***n* = 20**	***n* = 11**
N_{rep}	**1.57 (1.19–2.07)**[a]	1.47 (0.78–2.78)	**1.83 (1.17–2.85)**[b]	1.30 (0.72–2.32)	0.96 (0.43–2.12)
Lag_{med1}	0.97 (0.88–1.07)	1.03 (0.85–1.27)	1.02 (0.87–1.18)	1.08 (0.83–1.41)	0.91 (0.61–1.35)
Lag_{med2}	0.93 (0.84–1.02)	1.00 (0.81–1.23)	0.95 (0.81–1.11)	1.09 (0.82–1.45)	0.92 (0.69–1.25)
Time-varying kinetic parameters, HR					
	***n* = 179**	***n* = 54**	***n* = 94**	***n* = 20**	***n* = 11**
N_{rep}	**1.54 (1.14–2.09)**[b]	1.58 (0.76–3.26)	**1.73 (1.06–2.82)**[c]	1.33 (0.73–2.42)	0.84 (0.40–1.77)
Lag_{med1}	**0.91 (0.83–0.99)**[c]	1.00 (0.82–1.23)	**0.87 (0.75–0.99)**[c]	1.02 (0.79–1.34)	0.90 (0.60–1.34)
Lag_{med2}	**0.90 (0.82–0.98)**[c]	0.91 (0.73–1.13)	0.89 (0.77–1.03)	1.06 (0.81–1.39)	0.96 (0.72–1.29)
	***n* = 142**	***n* = 44**	***n* = 87**	***n* = 7**	***n* = 4**
$\Delta N_{rep}/\Delta time$	0.90 (0.43–1.88)	1.20 (0.12–12.29)	0.81 (0.33–1.98)	2.22 (0.08–62.90)	0.25 (0.002–30.40)
$\Delta Lag_{med1}/\Delta time$	**0.76 (0.59–0.99)**[c]	0.83 (0.43–1.59)	**0.69 (0.50–0.95)**[c]	0.004 (0.0002–545)	0.75 (0.08–7.54)
$\Delta Lag_{med2}/\Delta time$	0.82 (0.62–1.08)	0.52 (0.23–1.15)	0.82 (0.57–1.16)	0.70 (0.12–4.00)	20.8 (0.01–50200.6)

HR values are reported as the main value (95% confidence interval). HR values of statistically significant associations are reported in bold. AD = Alzheimer's disease; CI = cognitively impaired; CU = cognitively unimpaired; HR = hazard ratio; LBD = Lewy body disease; N_{rep} = number of positive replicates; PD = Parkinson's disease; PDD = Parkinson's disease dementia.

[a]$P \leq 0.001$.

[b]$P \leq 0.01$.

[c]$P \leq 0.05$.

degeneration in this area are expected as pathology progresses.[34] Future studies should test the hypothesis that a progressive impairment of olfactory function in asymptomatic LBD patients might be used as a surrogate of pathology progression.

Of significance, the overall results of our work seem to suggest that N_{rep} might be a stronger indicator of α-syn load than Lag. This might rely on the fact that Lag duration might be affected by other factors in addition to seed concentration, such as sample manipulations and matrix composition.[31] Moreover, the lack of associations between Imax and measures of clinical progression probably depends on the high inter-plate variability of this parameter, suggesting a stronger dependence on pure analytical factors than on initial seed concentration.[31]

The major strengths of our work are the large cohort of deeply phenotyped LBD participants, the longitudinal CSF samples and the robustness of our SAA assay, as shown here and in several studies.[6,7,11-13,35] Additionally, including a significant proportion of LBD-converters allowed us to strengthen the correlation between kinetic parameters and α-syn pathology burden.

The study also has limitations. Firstly, we acknowledge that the current 'quantitative' α-syn SAA still has significant imperfections in predicting pathology burden. In a recent study,[31] we significantly increased the repeatability of the N_{rep} estimate by testing each CSF sample repeatedly. Moreover, we found that N_{rep} is positively associated with the likelihood of positive SAA results at increasing sample dilutions. Thus, examining each sample at different dilutions using a higher replicate number (eight instead of four) for each dilution is expected to refine the α-syn seed quantification by SAA. Future studies should apply this extended protocol to baseline and longitudinal samples. Secondly, the high proportion of our PD patients with AD co-pathology is likely to represent a limit in the definition of the contribution of LBD pathology progression, estimated by SAA kinetic parameters, to the development of dementia. Finally, although preliminary data indicate that α-syn SAA parameters are not significantly affected by pre-analytical factors except for blood contamination,[31] further studies are required in this direction, especially on the effect of CSF storage time.

In conclusion, our analysis of longitudinally sampled LBD patients shows that α-syn SAA might provide quantitative parameters coherent with the longitudinal pathology progression. Asymptomatic LBD and AD subjects with concomitant LBD might show a more consistent longitudinal increase in α-syn burden than clinical LBD. Given their stability over time, N_{rep} and Lag might be promising treatment response biomarker candidates in clinical LBD. Finally, in longitudinal assessment, a lower Lag and higher N_{rep} are associated with a higher risk of progression to dementia.

Data availability

Raw α-syn SAA data were generated at the Laboratory of Neuropathology, Istituto delle Scienze Neurologiche di Bologna, Italy. Derived data supporting the findings of this study are available from the corresponding author on request.

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Competing interests

O.H. has acquired research support (for the institution) from AVID Radiopharmaceuticals, Biogen, C2N Diagnostics, Eli Lilly, Eisai, Fujirebio, GE Healthcare and Roche. In the past 2 years, he has received consultancy/speaker fees from AC Immune, Alzpath, BioArctic, Biogen, Bristol Meyer Squibb, Cerveau, Eisai, Eli Lilly, Fujirebio, Merck, Novartis, Novo Nordisk, Roche, Sanofi and Siemens. S.P. has acquired research support (for the institution) from ki elements/ADDF and Avid. In the past 2 years, he has received consultancy/speaker fees from Bioartic, Biogen, Esai, Lilly and Roche. None of the other authors has any disclosures.

Supplementary material

Supplementary material is available at *Brain* online.

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