

protein. Nonetheless, the data can be compared within each individual immunoassay, although not necessarily across assays.

As far as we are aware, this is the first report to look at α -syn levels in repeat blood samples taken from individual patients with PD (collected here over an initial 3-mo period, from the first 32 patients enrolled into an ongoing longitudinal study) and also the first to detect phosphorylated forms of α -syn in blood plasma. One of the most important and novel results arising from this study is that although it is clear that the concentrations of α -syn vary greatly between individuals (for reasons that are, as yet, unknown), they remain remarkably consistent (at least over 3 mo) within the vast majority of individuals. This was a general finding for all four of the immunoassays. This lack of fluctuation of α -syn levels within individuals is a prerequisite for establishment of any viable biomarker. It has been reported that only a very small proportion of the α -syn in whole blood is present in peripheral blood mononuclear cells, platelets, and plasma, with the majority being present in red blood cells (46). Considering the abundance and fragility of red blood cells, α -syn levels in plasma, or other bodily fluids, such as CSF, could be artificially elevated in some samples by contamination with intact or lysed red blood cells (32). However, this type of contamination cannot be a confounding factor here, given the very high degree of consistency of α -syn concentrations within the plasma samples prepared from blood taken on 4 different occasions from the same individual. Moreover, it is unlikely that conditions, such as anemia would confound the results because all recruits with PD had undergone regularly blood screening for hemoglobin levels, and if anemia had been detected, it would have been treated.

The results of the 4 different immunoassays reveal that there was, statistically, no difference between the levels of total α -syn, oligo- α -syn, or oligo-pS- α -syn when comparing the 32 patients with PD with the healthy (nondiseased) control group of 30 individuals. This is not consistent with our previous findings for oligo- α -syn (37), which was elevated in PD. However, in our previous study, the control samples were obtained from individuals with serious medical conditions, such as stroke, heart disease, and cancer, and they were also taken at a different institution from the PD samples. In our current study, the controls were from healthy people, and variables such as the collection, separation and storage of the blood samples were more stringently controlled for. It should be noted that the oligo- α -syn immunoassay is the same as that reported in our previous publication (37), except for the detection system, which was changed from alkaline phosphatase (absorbance-based assay) to streptavidin-europium (time-resolved fluorescence). This has improved assay sensitivity and has allowed us to dilute the plasma samples to 1:25, whereas previously (37), the samples were not diluted. It is possible that this has also contributed to the different findings reported here. We now find that only the levels of pS- α -syn were higher in

the PD group than in the control group ($P=0.053$), suggesting that the total phosphorylated protein may be the more useful diagnostic marker in plasma, and this is reflected in the ROC analysis for pS- α -syn (AUC=0.68). It should be noted that we did not make any formal adjustment for multiple testing (Bonferroni correction), because this is only a small-scale study, and this question will be addressed more fully in later work, when we have acquired and analyzed data from many more subjects.

The immunoblot results confirm that immunoreactive protein bands, with an intensity compatible with the immunoassay results, are detected when the protein from plasma is immunocaptured with N-19 and detected on immunoblots with pS129 (*i.e.*, the same capture and detection antibodies as those used for the pS- α -syn immunoassay). Some of these bands seem to represent ubiquitinated forms of the protein, since they also reacted with an antiubiquitin antibody. Whether these phosphorylated and ubiquitinated forms of α -syn originate from a cellular component in the blood itself or whether they originate from a peripheral tissue source elsewhere in the body, or from the brain (*via* CSF) is currently unknown.

Phosphorylation and ubiquitination are important secondary modifications of α -syn, with the protein deposited in LBs being predominantly phosphorylated at serine 129 (3, 47). Ubiquitination is a means of targeting a protein for destruction *via* the proteasome, and a defect in the ubiquitin-proteasome system is likely to be fundamental to the molecular pathogenesis of PD (48), although, recently, it has been suggested that α -syn phosphorylated at Ser-129 is targeted to the proteasome in a ubiquitin-independent manner (49). Phosphorylation of α -syn has been found to promote fibril formation, suggesting that hyperphosphorylation of α -syn might be a contributing factor in the pathogenesis of PD (47, 50). It has also been suggested that α -syn phosphorylated at serine 129 and its aggregation are involved in pathway responsible for α -syn toxicity in oligodendrocytes (51, 52). Given this pathological role for phosphorylated and ubiquitinated forms of α -syn, the levels of these modified proteins in body fluids, including blood plasma, are more likely to reflect the fundamental neuropathology of PD than the normal protein (33). This inference is borne out by the results of the present study. Observations that 10–37% of aged, neurologically healthy controls display some α -syn pathology in their brains (53, 54), with about half of such subjects showing abundant α -syn pathology (53), could explain why the levels of pS- α -syn did not better discriminate between patients with PD and healthy controls.

It is also worth noting that the levels of the nonphosphorylated protein in plasma showed a weak but positive correlation with sampling age (of the patients with PD) in the present study, whereas the phosphorylated protein showed no such correlation. Age has already been noted as a confounding variable for total α -syn levels in CSF (32). This lack of correlation between

phosphorylated α -syn and age could be an additional advantage in its development as a potential molecular biomarker.

In summary, we have validated some novel assays for assessing α -syn levels in blood plasma; shown that these levels are highly consistent in repeat blood samples taken over 3 mo from patients with PD; presented evidence for the presence of pS- α -syn (phosphorylated at Ser-129) in blood plasma; and found that the mean level of pS- α -syn was marginally significantly higher ($P=0.053$) in the PD samples than in the controls. We accept that the latter result is preliminary and will need to be confirmed in larger-scale studies. Nevertheless, on the basis of the data presented here, further study of phosphorylated α -syn as a potential biomarker for PD and related α -synucleinopathies is clearly warranted. Moreover, whether any of the different forms of α -syn can be used to monitor the progression of PD cannot be determined from the present study with longitudinal sampling over 3 mo only and must await data from our ongoing longer-term longitudinal studies. **[F]**

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Phosphorylated α -synuclein can be detected in blood plasma and is potentially a useful biomarker for Parkinson's disease

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ABSTRACT Parkinson's disease (PD) is characterized by the presence of Lewy bodies containing phosphorylated and aggregated α -synuclein (α -syn). α -Syn is present in human body fluids, including blood plasma, and is a potential biomarker for PD. Immunoassays for total and oligomeric forms of both normal and phosphorylated (at Ser-129) α -syn have been used to assay plasma samples from a longitudinal cohort of 32 patients with PD (sampled at mo 0, 1, 2, 3), as well as single plasma samples from a group of 30 healthy control participants. The levels of α -syn in plasma varied greatly between individuals, but were remarkably consistent over time within the same individual with PD. The mean level of phospho- α -syn was found to be higher ($P=0.053$) in the PD samples than the controls, whereas this was not the case for total α -syn ($P=0.244$), oligo- α -syn ($P=0.221$), or oligo-phospho- α -syn ($P=0.181$). Immunoblots of plasma revealed bands (at 21, 24, and 50–60 kDa) corresponding to phosphorylated α -syn. Thus, phosphorylated α -syn can be detected in blood plasma and shows more promise as a diagnostic marker than the nonphosphorylated protein. Longitudinal studies undertaken over a more extended time period will be required to determine whether α -syn can act as a marker of disease progression.—Foulds, P. G., Mitchell, J. D., Parker, A., Turner, R., Green, G., Diggle, P., Hasegawa, M., Taylor, M., Mann, D., Allsop, D. Phosphorylated α -synuclein can be detected in blood plasma and is potentially a useful biomarker for Parkinson's disease. *FASEB J.* 25, 4127–4137 (2011). www.fasebj.org

Key Words: Lewy body • oligomer • immunoassay • immunoblot

PARKINSON'S DISEASE (PD) is the second most common neurodegenerative disorder after Alzheimer's disease (AD) and is characterized clinically by the 3 cardinal motor symptoms of resting tremor, rigidity, and brady-

kinesia. Patients often exhibit further symptoms, including postural imbalance, gait disturbance, and a mask-like facial expression. In the advanced stages of PD, nonmotor symptoms can also appear, including anxiety, depression, dementia, and psychosis. The defining neuropathological features of idiopathic PD are the loss of dopaminergic neurons from the substantia nigra (SN) and the presence of Lewy bodies (LBs) and Lewy neurites (LNs) in surviving neurons of this and other brain regions (1). Similar lesions are present within the cerebral cortex in the related disorder of dementia with Lewy bodies (DLB; ref. 2). LBs and LNs contain a misfolded, fibrillar, and phosphorylated form of the protein α -synuclein (α -syn; refs. 1, 3). Pathological changes involving α -syn, chiefly in glial cells, also occur in multiple system atrophy (MSA), and, therefore, PD, DLB, and MSA are often collectively referred to as " α -synucleinopathies" (2). Duplication (4, 5), triplication (6), and mutation (7–9) of the gene encoding α -syn (*SNCA*) are all causes of hereditary forms of either PD or DLB. α -Syn oligomers are believed to be toxic to cells, as are oligomers derived from the various proteins associated with several other protein-misfolding neurodegenerative disorders, such as AD or the prion disorders (10, 11). Overexpression of wild-type or mutant α -syn in animal models can produce a phenotype resembling PD, including SN degeneration, movement problems and responsiveness to L-dopa therapy (12–15). Together, these observations suggest that α -syn plays a pivotal role in the development of the α -synucleinopathies (16).

PD is one of several neurological movement disor-

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ders that can produce similar symptoms, and a correct diagnosis is critically dependent on clinical examination to rule out disorders that can mimic PD. A diagnosis of PD is considered if the person exhibits more than one of the 3 cardinal motor symptoms mentioned above (17). The presence of resting tremor supports the diagnosis of PD more than the other two symptoms, but ~20% of patients with autopsy-confirmed PD fail to develop any resting tremor (18). Moreover, only 69–70% of people with autopsy-confirmed PD have at least two of the cardinal signs of the disease and 20–25% of people with two of these symptoms have a pathological diagnosis other than PD (19, 20). Perhaps even more surprising is the finding that 13–19% of people who demonstrate all three of the cardinal features have a pathological diagnosis other than PD (19, 20). Because the progression of neurological movement disorders and their treatment varies greatly, proper clinical diagnosis is essential for correct patient management. Furthermore, by the time PD is diagnosed, >60% of dopaminergic neurons in the SN can already be lost (21), making accurate early diagnosis, ideally before clinical symptoms appear, essential for any effective neuroprotective intervention strategy. Also, the clinical diagnosis of early PD may be difficult because although the patient might complain of symptoms suggesting PD, the neurological examination may be normal (22). These problems with clinical diagnosis have led to an increased interest in the development of diagnostic markers for PD, including advanced brain imaging methodologies (23) and molecular biomarkers (24). Genetic testing for mutations in genes linked to familial PD (including *SNCA*) is available (25), but it is only relevant when there is a strong family history, or when symptoms present at an unusually young age.

We have reported that α -syn is released from cells and is present in human body fluids, including cerebrospinal fluid (CSF) and blood plasma (26). This extracellular form of α -syn seems to be secreted from neuronal cells by exocytosis (27, 28) and could play an important role in cell-to-cell transfer of α -syn pathology in the brain (29). There is now an emerging consensus that the levels of α -syn are, on average, lower in samples of CSF taken from a group of patients with PD compared with a group of normal or neurological controls (30, 31), especially when the confounding variables of age and blood contamination are taken into account (32, 33). However, obtaining CSF is an invasive procedure, and analysis of α -syn levels in CSF is not generally amenable to longitudinal study. There are also some studies of α -syn as a potential biomarker in the much more accessible peripheral blood, with an initial report suggesting increased levels of this protein in plasma samples from patients with PD compared with those from healthy controls (34). However, subsequent studies have reported decreased levels of α -syn in PD plasma (35) or no significant change (36). We have shown that the levels of oligomeric α -syn appear to be significantly elevated in plasma samples from a group of patients with PD compared with a group of diseased

controls (37). To develop this line of enquiry further, we have now set up a longitudinal study in newly diagnosed patients with PD to examine the levels of various different forms of α -syn, including phosphorylated and/or oligomeric forms, in blood plasma. Because α -syn accumulates in a phosphorylated and aggregated form in LBs (3), it is possible that these modified, pathological forms of the protein will more accurately reflect the fundamental neuropathology of PD than straightforward measures of “total” α -syn (33, 38). Our ultimate aim is to develop a relatively simple test for the early diagnosis of PD, or a surrogate marker for monitoring the progression of PD. Here, we report the results obtained during the initial phase of this longitudinal study.

MATERIALS AND METHODS

Patient population and clinical method

Participants for this study were recruited (with ethical approval, using appropriate consenting procedures) from the neurological service based at the Royal Preston Hospital along with other similar departments in the northwest of England. The diagnosis of PD was based on the UK Parkinson's Disease Society diagnostic criteria for PD (39). Severity of disease was defined in terms of patients satisfying the criteria for stages 1 or 2 on the Hoehn and Yahr scale.

The overall target for the study was to follow a cohort of 200 patients meeting these criteria over a period of 2–3 yr, reviewing them at 4- to 6-mo intervals. This study is ongoing, but the plan was also to follow the first 32 patients more intensively over the initial phases of the study, and this group was seen at monthly intervals for the first 3 mo. The results from these 32 patients over the first 3 mo are presented here. Blood samples were obtained from the participating sites (Preston and Arroe Park). Around 3 ml of blood was collected in tubes containing EDTA, and the plasma was separated within 3 h by centrifuging the blood at 3000 *g* for 10 min. The plasma was immediately stored at -80°C . Appropriate care was taken to avoid contamination of the plasma samples with cells or components of the pellet obtained from the centrifugation. The samples were thawed at room temperature directly before analysis. Repeated freeze/thaw cycles were avoided.

Control subjects were healthy individuals with no apparent neurological or known psychiatric symptoms who were the spouses of patients attending the Cerebral Function Unit clinics at Hope Hospital (Salford Royal Hospital, National Health Service Foundation Trust) for investigation and diagnosis of dementia. These control subjects were recruited as part of an ongoing investigation into the genetics and molecular biology of dementia approved by the Oldham Local Research Ethics Committee. Blood plasma was prepared and stored as described above.

Preparation of recombinant α -syn

Recombinant α -syn (without any purification tag) was prepared at Lancaster University from *Escherichia coli* using the following protocol. pJEK2 was used to transform FB850, a *recA* derivative of BL21 (DES) pLysS. FB850 carrying this plasmid was grown in an 800-ml batch culture, and protein expression was induced through the addition of isopropyl- β -

D-thiogalactopyranoside (IPTG). A protein with a molecular weight of ~17 kDa started to accumulate in the cells 30 min after induction and reached maximum levels after 150 min. Immunoblot analysis identified this protein as α -syn using an anti- α -syn mouse monoclonal antibody (MAb 211; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After a 3-h induction, the suspension was centrifuged, and the cells were resuspended in buffer. The cells were lysed by sonication, and then cell debris and insoluble material were removed by centrifugation at 4°C for 1 h at 30,000 rpm. α -Syn was extracted from the supernatant by ammonium sulfate precipitation, then purified using two chromatography columns; mono Q and Superdex 200 (Amersham Biosciences, Piscataway, NJ, USA). After purification, 5 μ g of protein ran as a single band when observed on a Coomassie blue-stained SDS gel, corresponding to monomeric α -syn.

Preparation of phosphorylated recombinant α -syn

Phosphorylated α -syn was prepared from recombinant α -syn, as described previously (40). Briefly, α -syn (630 μ M) was incubated with casein kinase II (CK2; New England Biolabs, Ipswich, MA, USA) in 1 ml of buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl₂, and 1 mM ATP at 30°C for 24 h. For effective phosphorylation, CK2 was added to the reaction mixture at 2-h intervals for the first 10 h (7500 U \times 5). The reaction was stopped by boiling for 5 min, cleared by centrifugation at 113,000 g for 20 min at 4°C, and then loaded onto a Resource Q anion-exchange column (Amersham Biosciences) equilibrated with 20 mM Tris-HCl (pH 8.0) and 0.2 M NaCl, and then eluted with a linear gradient of NaCl from 0.20 to 0.35 M for 15 min at a flow rate of 1 ml/min. Fractionated samples were analyzed by immunoblotting with a phosphorylation-dependent anti- α -syn antibody, PSer129 (Epitomics, Burlingame, CA, USA), and mass spectrometry. The PSer129-positive phosphorylated α -syn recovered in the fractions with ~0.3 M NaCl was concentrated by ammonium sulfate precipitation.

Preparation of oligomeric forms of recombinant α -syn

To prepare a standard for the oligomeric α -syn immunoassay, the recombinant protein was oligomerized by incubation at 45 μ M in PBS in an orbital shaker at 37°C for 5 d, and the monomer and oligomer were separated by size-exclusion chromatography. A sample (0.5 ml) of preaggregated α -syn was loaded onto a Superdex 200 column (44 \times 1 cm) connected to a fast protein liquid chromatography (FPLC) system (Atka Purifier; GE Healthcare, New York, NY, USA) and eluted with running buffer (PBS) at a flow rate of 0.5 ml/min. Absorbance of the eluate was monitored at 280 nm; fractions of 1 ml were collected, and protein concentration was determined.

To prepare a standard for the oligo-phospho- α -syn immunoassay, the phosphorylated protein was oligomerized by incubation at 50 μ M in PBS in an orbital shaker at 37°C for 5 d. Aggregation of the protein was confirmed by thioflavin T assay. In this case, the amount of sample available was too small to fractionate by size-exclusion chromatography.

Immunoassay methods

We have already established immunoassay methods for the measurement of total and soluble oligomeric forms of α -syn in human biological fluids, including blood plasma (37, 41), and these methods have been further optimized.

Total α -syn

A 96-well microtiter plate (Iwaki, Holliston, MA, USA) was coated with 100 μ l/well of anti- α -syn monoclonal antibody 211 diluted 1:1000 (0.2 μ g/ml; Santa Cruz Biotechnology) in 50 mM NaHCO₃ (pH 9.6) and incubated at 4°C overnight. The wells were then washed 4 times with PBS containing 0.05% Tween-20 (PBS-T) and were incubated for 2 h at 37°C with 200 μ l/well of freshly prepared blocking buffer (2.5% gelatin in PBS-T). The plate was washed again 4 times with PBS-T, and 100 μ l of the assay standard or plasma samples was added to each well (each plasma sample was diluted 1:40 with PBS), and the assays were performed in triplicate. Following this, the plate was incubated at 37°C for 2 h. After a repeat washing with PBS-T, 100 μ l/well of the detection antibody, anti- α / β / γ -synuclein FL-140 (Santa Cruz Biotechnology), dilution 1:750 (0.27 μ g/ml) in blocking buffer was added, and the plate was incubated at 37°C for 2 h. After another wash with PBS-T, the plate was incubated with 100 μ l/well of secondary antibody [goat anti-rabbit horseradish peroxidase (HRP); Sigma, St. Louis, MO, USA], dilution 1:10,000 in blocking buffer at 37°C for 2 h. The plate was then washed again with PBS-T before adding 100 μ l/well of Sure Blue TMB microwell peroxidase substrate (KPL, Gaithersburg, MD, USA) and leaving the color to develop for 30 min at room temperature. Finally 100 μ l/well of stop solution (0.3 M H₂SO₄) was added, and absorbance at 450 nm was determined. Recombinant monomeric α -syn was used to create a standard curve.

Oligomeric α -syn (oligo- α -syn)

The microtiter plate was coated and blocked using the same method as the assay for total α -syn. The wells were then washed 4 times with PBS-T, and 100 μ l of the plasma sample (diluted 1:25 with PBS) or assay standard (oligo- α -syn) was added to each well, in triplicate. Following this, the plate was incubated at 37°C for 2 h. After a repeat wash with PBS-T, 100 μ l/well of the detection antibody, biotinylated anti- α -synuclein 211 (diluted 1:1000 in blocking buffer) was added, and the plate was incubated at 37°C for 2 h. After another wash with PBS-T, the plate was incubated with 100 μ l/well of streptavidin-europium, diluted 1:500 in streptavidin-europium buffer (Perkin Elmer, Wellesley, MA, USA) and shaken for 10 min. After a further 50-min agitation on a rotating platform, the plate was washed again with PBS-T, before adding 100 μ l/well enhancer solution (Perkin Elmer). Finally, the plates were read on a Wallac Victor² 1420 multilabel plate reader (Perkin Elmer), using the time-resolved fluorescence setting for europium.

Total phosphorylated α -syn (pS- α -syn)

The antibody-sandwich ELISA for total α -syn was modified to detect only the protein phosphorylated at Ser-129 by replacing the 211 phospho-independent capture antibody with polyclonal anti- α -synuclein N-19 (Santa Cruz Biotechnology), diluted 1:3,000 (0.07 μ g/ml). The phospho-dependent rabbit monoclonal antibody, Phospho (pS129) antibody (Epitomics), used at a dilution of 1:3000, was the chosen detection antibody. This antibody detects only α -syn phosphorylated at Ser-129. The preferred secondary antibody was human serum absorbed goat anti-rabbit HRP, 1:3000 (KPL), rehydrated in 1 ml H₂O. Recombinant pS- α -syn was used as the assay standard.

Oligomeric phosphorylated α -syn (oligo-pS- α -syn)

The antibody-sandwich immunoassay for oligo- α -syn was modified to detect only phosphorylated, oligomeric forms of the

protein, by replacing the 211 phospho-independent capture antibody with the phospho-dependent rabbit monoclonal antibody, pS129 (Epitomics), used at a dilution of 1:3000. The detection antibody was biotinylated pS129 at a dilution of 1:400. Recombinant oligo-pS- α -syn was used to generate a standard curve.

Preparation of biotinylated antibodies

To prepare the biotinylated antibody, 200 μ g Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA) was reacted with the required antibody (1 ml, 200 μ g/ml) in PBS and then placed on ice for 2 h. The mixture was desalted on Bio-Spin-6 columns (Bio-Rad, Richmond, CA, USA) to remove excess uncoupled biotin and the biotinylated antibody was stored at -20°C until use.

Immunocapture of α -syn from plasma

Dynabeads covalently coupled with recombinant protein G were derivatized with goat polyclonal anti- α -syn synuclein N-19 antibody (Santa Cruz Biotechnology), as recommended by the manufacturer (DynaL Biotech, Wirral, UK). Plasma (500 μ l) was added to the beads and incubated overnight at 4°C . The plasma samples were chosen according to the immunoassay results, with one sample giving a high signal for the phosphorylated protein, the other a low signal, and a control containing PBS only. The beads were then washed 3 times with 0.1 M phosphate buffer (pH 8.2). Any captured protein was eluted from the beads by boiling for 10 min in NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA, USA)

and was examined by gel electrophoresis and immunoblotting.

Gel electrophoresis and immunoblotting

The protein eluted from the magnetic Dynabeads was separated on 12.5% acylamide gels. The separated proteins were transferred to nitrocellulose membranes (0.45 μ m, Invitrogen) at 30 V, 125 mA for 1 h. Membranes were blocked with 5% dried skimmed milk dissolved in PBS-Tween (PBST), for 1 h. The membranes were probed overnight at 4°C with either phospho-dependent rabbit anti- α -synuclein monoclonal antibody pS129 (Epitomics) at a dilution of 1:5000; rabbit polyclonal anti-synuclein antibody FL-140 (Santa Cruz Biotechnology) at a dilution of 1:1000 (0.2 μ g/ml); or rabbit anti-ubiquitin antibody FL-76 (Santa Cruz Biotechnology) at a dilution of 1:1000 (0.2 μ g/ml) in PBST. The membranes were washed 3 times in PBST, followed by incubation with human serum absorbed HRP-conjugated goat anti-rabbit (Sigma), 1:10,000 in PBST, for 1 h. The protein bands were visualized using ECL reagents (Pierce), as described by the manufacturer.

Analysis of immunoassay data

A set of standards for one of the 4 different assays (*i.e.*, for total α -syn, oligo- α -syn, pS- α -syn, or oligo-pS- α -syn) was included on each microtiter plate, as appropriate for the type of protein being measured on that plate. Standard curves were fitted using nonlinear least squares (see Fig. 1 for representative examples of standard curves for each of the four different immunoassays). The samples of blood plasma from

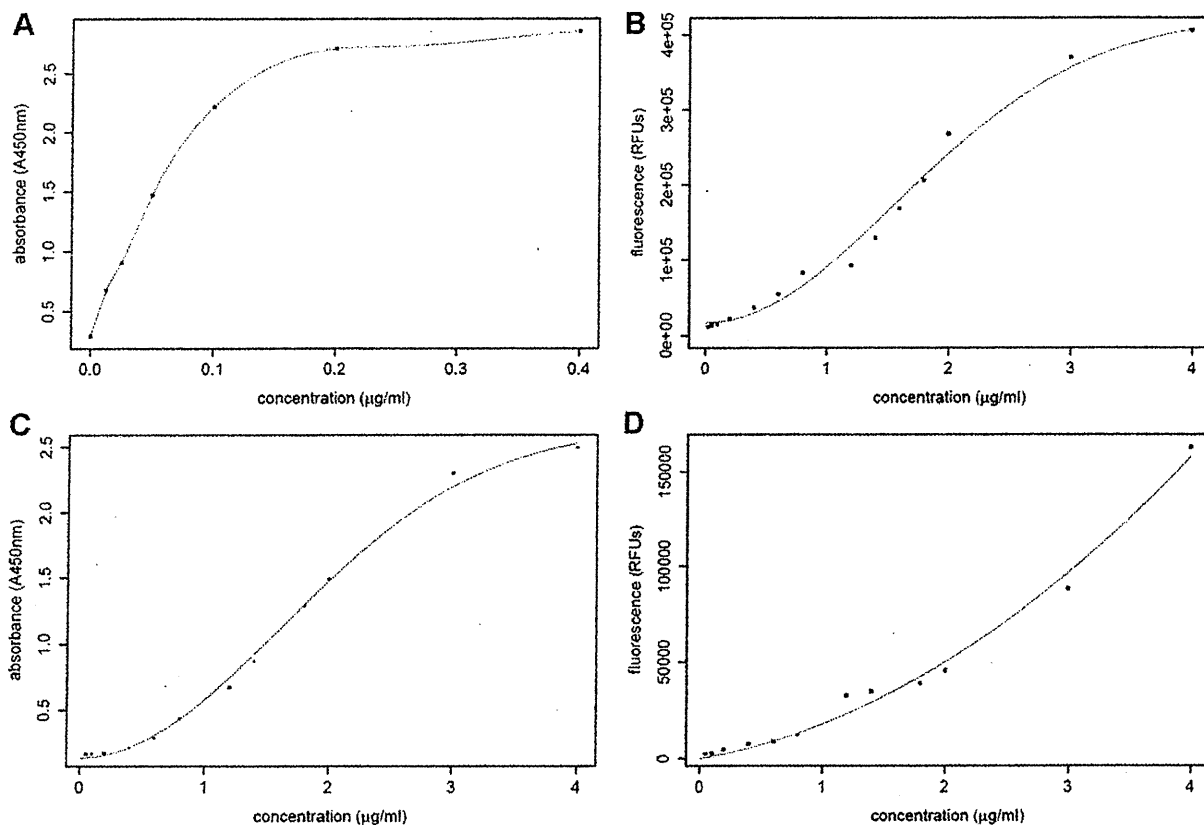


Figure 1. Examples of standard curves obtained for total α -syn (A), oligo- α -syn (B), pS- α -syn (C) and oligo-pS- α -syn (D). These are representative curves, each obtained from a single ELISA plate.

patients with PD and controls, diluted as indicated above, were measured in triplicate for each individual at each time point. The standard curves for each individual plate were used to transform the absorbance values (total α -syn, pS- α -syn) or relative fluorescence units (RFU; oligo- α -syn, oligo-pS- α -syn) for that particular plate into protein concentrations, and, in this way, any variation between plates was accounted for. The specificity of the oligo- α -syn immunoassay toward aggregated forms of α -syn has been reported previously (37, 41) but was confirmed here by analysis of fractions obtained by gel filtration of preaggregated, recombinant α -syn; only the peak containing α -syn oligomers, and not the monomer peak, was detected by the oligo- α -syn immunoassay. Also, as expected, the nonphosphorylated form of α -syn gave no signal in the pS- α -syn immunoassay, and the oligo-pS- α -syn immunoassay detected only pS- α -syn that had been preaggregated (data not shown). Further, when the blood plasma samples were immunodepleted with anti- α -syn antibodies C211 or FL-140, each coupled to magnetic Dynabeads, and then tested in the immunoassays, only trace signals could be detected above background compared to the nonimmunodepleted samples (data not shown).

To investigate whether the protein levels changed over time (*i.e.*, during the first 3 mo) a linear mixed model was fitted to the longitudinal data from each assay (details in Supplemental Data).

A classic 2-sample *t* test was used to determine whether there was any significant difference between the mean levels of each of the different forms of α -syn when comparing the plasma samples from the patients with PD with those from the healthy controls. To better satisfy the assumptions underlying this test, the empirical distributions were constructed on the logarithmic scale to obtain a more symmetric distribution than was obtained on the original scale.

RESULTS

Patient population and demographics

Demographic details of the cohort of 32 patients with PD that was followed at monthly intervals for 3 mo are summarized in **Table 1**. The mean age of this cohort on ascertainment and initial sampling was 68.2 yr (youngest 56 yr, oldest 85 yr). Among the 30 recruited healthy controls, there were 13 males and 17 females, with a median age of 63 yr and mean age of 61.5 yr (youngest 42 yr, oldest 75 yr). The PD case and control subjects were recruited in parallel, at the same clinical centers, and the blood samples were taken and processed by the same personnel at each site. Moreover, the plasma

TABLE 1. Demographic details of the cohort of 32 patients with PD

Parameter	Value
Gender (male/female)	23/9
Hoehn and Yahr 1.0	5
Hoehn and Yahr 1.5	3
Hoehn and Yahr 2.0	24
Median PD onset age (yr)	61.9 (55.5–69.7)
Age at study recruitment (yr)	68.4 (62.3–73.8)
Disease duration at study recruitment (yr)	4.9 (3.1–9.3)

Values in parentheses indicate interquartile range.

samples were effectively randomized for analysis, with both control and PD samples being assayed together on the same microtiter plates.

Longitudinal data from patients with PD

Figure 2 presents a bar plot of the total α -syn plasma concentrations for each individual with PD over time (*i.e.*, for mo 0, 1, 2, 3) where, within each time point, we have averaged over triplicate measurements. It can be seen that the levels of total α -syn varied greatly between individuals, within an overall range of 0.01–6 μ g/ml. Although a few individuals did show small, stepwise increases or decreases of total α -syn levels over this (very short) sampling period (see, for example, patient 32 in Fig. 2B), one of the most striking findings from this study was that, overall, the immunoassay results from the repeat PD plasma samples were remarkably consistent within each individual over time. This was a general finding for the results from all four of the different α -syn immunoassays, but it is illustrated here (Fig. 2) for the total- α -syn data only. Data for the other 3 assays (Supplemental File S2), together with a linear mixed model-based analysis (Supplemental File S1) for all 4 α -syn assays, are available in Supplemental Data. From the latter analysis, it is clear that the variation in α -syn levels across time within an individual is negligible relative to the variation across individuals. The model specifies a time trend, in addition to accounting for inherent differences in protein levels between individuals and differences across time within an individual. In all cases, the confidence interval for the estimated temporal effect covered 0. Thus, we conclude that there was no significant change over time for the levels of α -syn being measured by any of the immunoassays.

Comparison of patients with PD and controls

Empirical distributions of the α -syn concentrations for each assay were highly skewed on the original scale. **Figure 3** presents box plots pertaining to each assay, stratified according to patients with PD and controls. Note that the whiskers of the box plots extend by no more than the range of the data (largest minus smallest value) multiplied by the interquartile range. Extending the whiskers to the largest and smallest values would yield a rather compressed box. An apparent feature of the box plots is that the median concentration of α -syn for the patients exceeds that of the controls for both of the assays for phosphorylated α -syn (*i.e.*, pS- α -syn in Fig. 3B, and oligo-pS- α -syn in Fig. 3D). The reverse is true regarding the nonphosphorylated assays (total- α -syn in Fig. 3A and oligo- α -syn in Fig. 3C). Further, the interquartile range (*i.e.*, box height) reports that the concentrations are far less dispersed for controls compared to patients for both of the phosphorylated α -syn assays. For the nonphosphorylated assays, the controls display a larger spread of concentrations.

To investigate the potential of α -syn as a means of

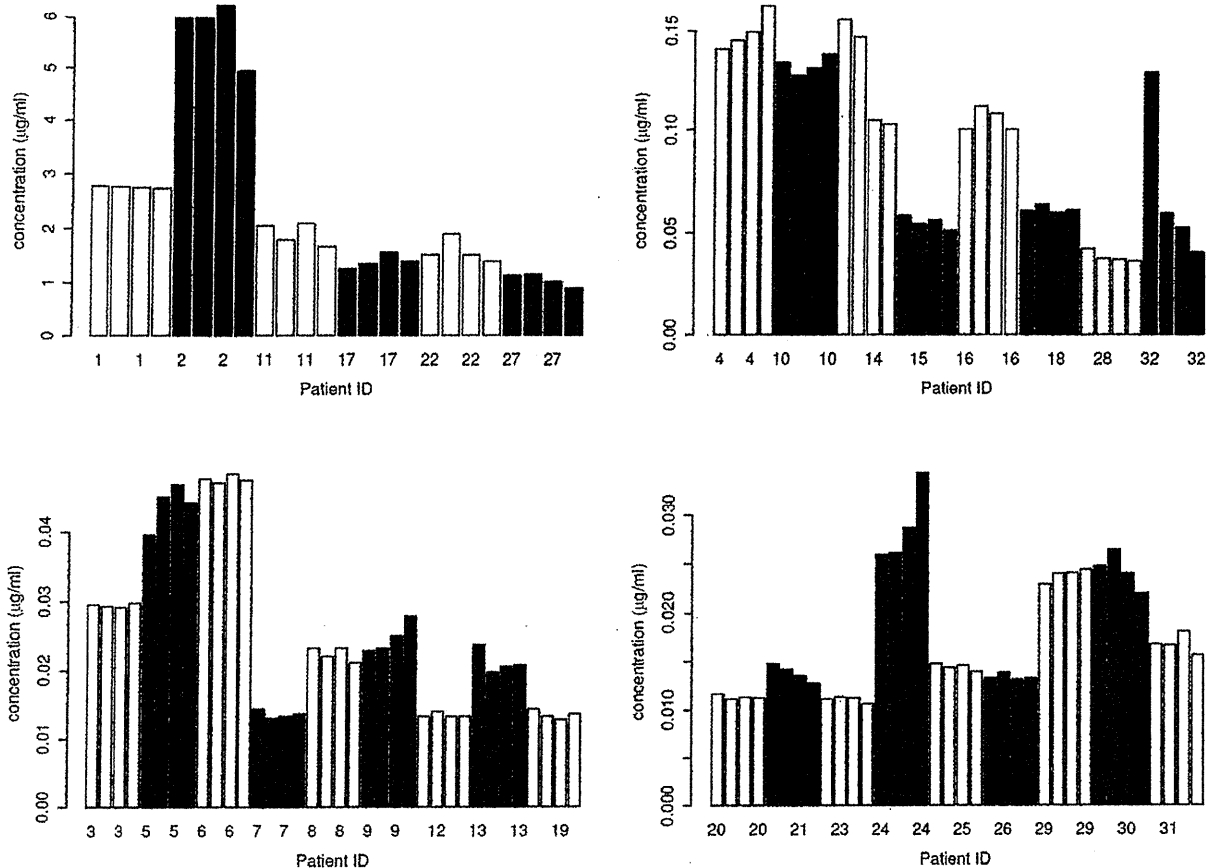


Figure 2. Longitudinal data for the levels of total α -syn in plasma samples from all of the 32 patients with PD. Consecutive bars for each patient represent the level of total α -syn in blood plasma samples taken at 0, 1, 2 and 3 mo. The participants are assigned to one of the 4 sections depending on their overall levels of protein.

discriminating between patients with PD and controls, we determined whether there was any significant difference between the average level of α -syn (on the logarithmic scale) across patients and controls, within all 4 α -syn assays. Because there was no consistent change in α -syn levels over time, nor across replicates within time, in the plasma samples from patients with PD, the concentrations for mo 0, 1, 2 and 3 were averaged over time and replicates in order to obtain a single mean value for each individual patient. Under a classical two-sample *t* test, the mean level of pS- α -syn was found to be marginally significantly higher for the patients than for the healthy controls ($P=0.053$). On the other hand, there was no difference across the average levels of patients and controls with regard to total α -syn ($P=0.244$), oligo- α -syn ($P=0.221$), or oligo-pS- α -syn ($P=0.181$).

Association with gender and age

The levels of α -syn showed no association with gender. For the total and oligo- α -syn assays, sampling age was a marginally significant -0.049 ($-0.099, -0.001$; $P=0.052$) and significant -0.009 ($-0.019, -0.001$; $P=0.045$) predictor, respectively, of α -syn levels in the patients with PD. On the

other hand, the *P* values corresponding to the effect of sampling age on α -syn levels in the patients, under the pS- α -syn and oligo-pS- α -syn assays, were 0.412 and 0.274, respectively. The levels of α -syn showed no correlation with age in the control group. We did also analyze the data adjusting for age, but found no significant effects, and the adjustments did not materially change the (lack of) significance of the relevant assay results. Therefore, we chose to report the unadjusted results for simplicity.

Receiver operating curve (ROC) analysis

Figure 4 displays an ROC curve constructed to evaluate the utility of plasma pS- α -syn levels in discriminating patients with PD from healthy controls. The area under the curve (AUC) of 0.68 suggests that pS- α -syn has some potential value as a discriminant between patients and controls. AUC curves for 2 of the other 3 assays gave AUC values of less than 0.5 (0.28 for total α -syn and 0.22 for oligo- α -syn), which would also indicate a potentially informative result, with plasma levels of these being lower in patients than in controls. An AUC of 0.62 for oligo-pS- α -syn, however, suggests that in this

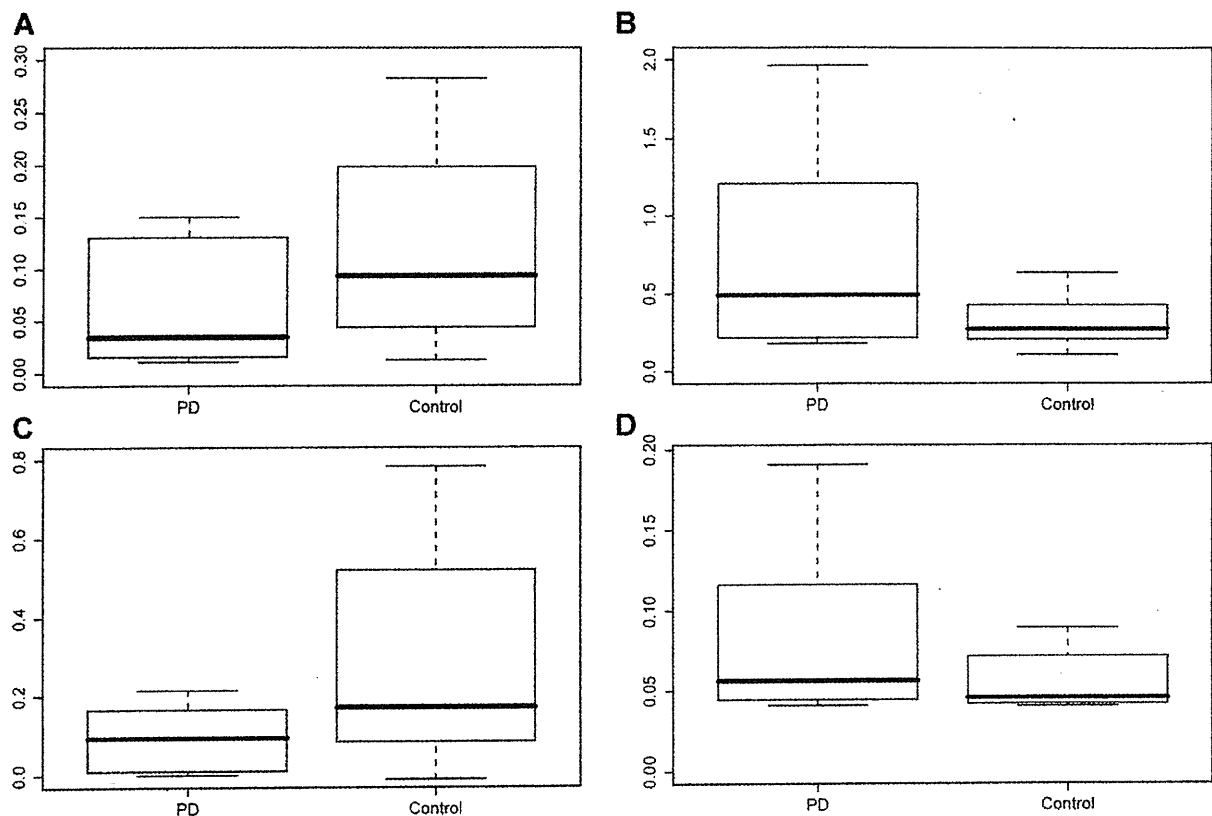


Figure 3. Box plots comparing the levels (in $\mu\text{g}/\text{ml}$) of total $\alpha\text{-syn}$ (A), pS- $\alpha\text{-syn}$ (B), oligo- $\alpha\text{-syn}$ (C), and oligo-pS- $\alpha\text{-syn}$ (D) in patients with PD compared to healthy controls. In each plot, the box extends from the lower to the upper quartile of the data, with the median indicated by a horizontal line within the box. The difference between the lower and upper quartiles is called the interquartile range (IQR). The upper and lower whiskers extend to the most extreme data values that are no more than 1.5 IQR greater than the upper quartile, and no more than 1.5 IQR less than the lower quartile, respectively.

particular sample set, this assay is less likely to have any practical value as a discriminatory diagnostic tool.

Immunoblot analysis of phosphorylated $\alpha\text{-syn}$ in plasma

To better characterize the phosphorylated $\alpha\text{-syn}$ detected in plasma, we extracted $\alpha\text{-syn}$ from individual PD plasma samples by immunocapture on magnetic Dynabeads and then analyzed the extracted proteins by immunoblotting. The beads were derivatized with the phosphorylation-independent $\alpha\text{-syn}$ antibody N-19 (Santa Cruz Biotechnology), which is the antibody used for capture in the pS- $\alpha\text{-syn}$ immunoassay. Proteins eluted from the beads were detected by immunoblotting with two different $\alpha\text{-syn}$ antibodies: phosphorylation-dependent rabbit monoclonal antibody, pS129, which is the detection antibody used in the pS- $\alpha\text{-syn}$ immunoassay (Fig. 5A), and the phosphorylation-independent rabbit polyclonal antibody, FL-140 (Fig. 5B). Rabbit anti-ubiquitin antibody FL-76 (Santa Cruz Biotechnology) was used to determine whether any of the bands represented ubiquitinated forms of $\alpha\text{-syn}$ (Fig. 5C).

The plasma samples were chosen according to the immunoassay results, with one sample giving a low signal for pS- $\alpha\text{-syn}$ (Fig. 5, lane 2) and the other a high signal (Fig. 5, lane 3). Immunoblots using the phospho- $\alpha\text{-syn}$ -dependent antibody revealed immunoreactive bands from both of these plasma samples, together with the human recombinant phospho- $\alpha\text{-syn}$ (at ~ 17 kDa), but not the nonphosphorylated recombinant protein (Fig. 5A, lane 4). The sample derived from the high-reading plasma revealed more intense bands than the low-reading sample, at ~ 21 , 24, and 50–60 kDa. FL-140 revealed both the phosphorylated and nonphosphorylated recombinant protein standards, and also a 24-kDa band in both plasma samples (Fig. 5B). The 21-kDa band detected by pS129 was absent, but an additional higher-molecular-weight smear, at >35 kDa, was present. On the basis of the size of these $\alpha\text{-syn}$ species, we hypothesize that the 24-kDa band may correspond to phosphorylated, monoubiquitinated $\alpha\text{-syn}$. The anti-ubiquitin antibody, FL-76, strongly labeled the 24-kDa band, as well as the broad “smears” at higher molecular mass, suggesting that all of these bands represent ubiquitinated forms of $\alpha\text{-syn}$ (Fig. 5C). Control samples of 100 ng human IgG and albumin, the N-19 immunocapture antibody, and a control immunoprecipitation

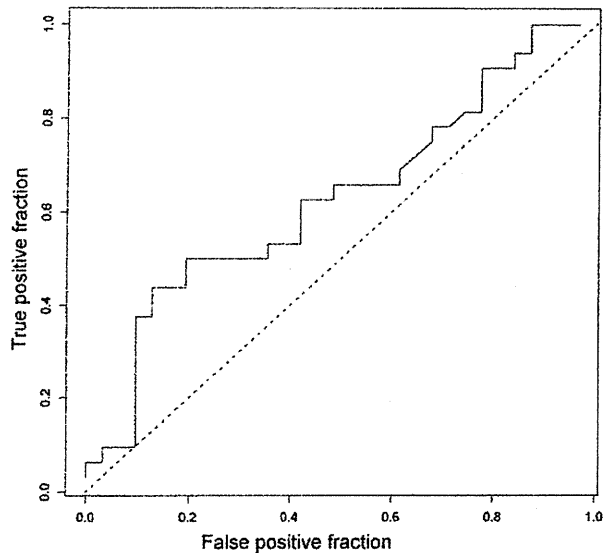


Figure 4. ROC curve showing the ability of the pS- α -syn levels to discriminate between patients with PD and healthy controls (AUC=0.68).

using PBS rather than plasma, gave no immunoreactive bands (data not shown).

DISCUSSION

α -Syn has been linked directly to the etiology of the α -synucleinopathies by mutations in and multiplication of its gene (*SNCA*) that result in familial forms of either PD or DLB. We have reported previously that α -syn is

released from cells and is present in human body fluids, including CSF and blood plasma (26). This has led to considerable interest in α -syn in these body fluids as a potential biomarker for the α -synucleinopathies (30–37, 42–44). However, most of these studies have relied on immunoassays that cannot distinguish between monomeric/oligomeric and nonphosphorylated/phosphorylated forms of the protein, apart from two previous studies of oligomeric α -syn in human CSF or blood plasma (37, 44). Here, we have set up individual sandwich immunoassays that can distinguish between total α -syn (MAb 211 capture/ FL-140 detect); oligo- α -syn (Mab 211 capture and detect); pS- α -syn (N19 capture/pS129 detect); and oligo-pS- α -syn (pS129 capture and detect). Our assays for oligomeric forms of α -syn use the double-antibody approach, where the same monoclonal antibody is used for both antigen capture and detection (37, 41). This type of assay cannot detect monomers because the capture antibody occupies the only antibody-binding site available, but it can detect oligomers, because they have multiple binding sites. Our assays for phosphorylated α -syn rely on the specificity of the monoclonal antibody pS129 to α -syn phosphorylated at Ser-129 (45). As anticipated, the recombinant nonphosphorylated α -syn gave no signal in these assays. Although the absorbance/fluorescence values for each of the 4 assays were converted to protein concentrations using the relevant standard curve, due to the nature of these assays, this may only represent an estimate of concentration for that particular assay since the precise nature of the α -syn species detected in plasma has not been not determined for each assay and the native species are likely to differ from the standards prepared from the recombinant

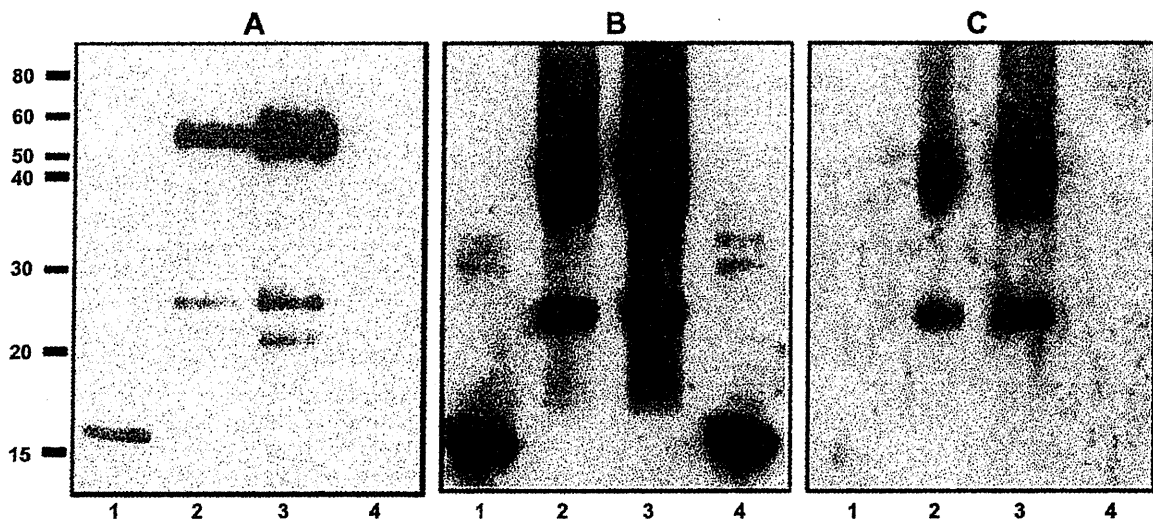


Figure 5. Immunoblot analysis of phosphorylated α -syn from plasma samples. Proteins immunocaptured from one plasma sample giving a low immunoassay signal for pS- α -syn protein (lane 2), and the other a high signal (lane 3), were immunoblotted along with recombinant phosphorylated α -syn (lane 1), and the recombinant nonphosphorylated standard (lane 4). *A*) Analysis with the phospho-dependent α -syn rabbit monoclonal antibody pS129 (Epitomics). *B*) Analysis with the rabbit polyclonal α -syn antibody FL-140 (Santa Cruz Biotechnology). Analysis with the rabbit polyclonal antiubiquitin antibody FL-76 (Santa Cruz Biotechnology).

protein. Nonetheless, the data can be compared within each individual immunoassay, although not necessarily across assays.

As far as we are aware, this is the first report to look at α -syn levels in repeat blood samples taken from individual patients with PD (collected here over an initial 3-mo period, from the first 32 patients enrolled into an ongoing longitudinal study) and also the first to detect phosphorylated forms of α -syn in blood plasma. One of the most important and novel results arising from this study is that although it is clear that the concentrations of α -syn vary greatly between individuals (for reasons that are, as yet, unknown), they remain remarkably consistent (at least over 3 mo) within the vast majority of individuals. This was a general finding for all four of the immunoassays. This lack of fluctuation of α -syn levels within individuals is a prerequisite for establishment of any viable biomarker. It has been reported that only a very small proportion of the α -syn in whole blood is present in peripheral blood mononuclear cells, platelets, and plasma, with the majority being present in red blood cells (46). Considering the abundance and fragility of red blood cells, α -syn levels in plasma, or other bodily fluids, such as CSF, could be artificially elevated in some samples by contamination with intact or lysed red blood cells (32). However, this type of contamination cannot be a confounding factor here, given the very high degree of consistency of α -syn concentrations within the plasma samples prepared from blood taken on 4 different occasions from the same individual. Moreover, it is unlikely that conditions, such as anemia would confound the results because all recruits with PD had undergone regularly blood screening for hemoglobin levels, and if anemia had been detected, it would have been treated.

The results of the 4 different immunoassays reveal that there was, statistically, no difference between the levels of total α -syn, oligo- α -syn, or oligo-pS- α -syn when comparing the 32 patients with PD with the healthy (nondiseased) control group of 30 individuals. This is not consistent with our previous findings for oligo- α -syn (37), which was elevated in PD. However, in our previous study, the control samples were obtained from individuals with serious medical conditions, such as stroke, heart disease, and cancer, and they were also taken at a different institution from the PD samples. In our current study, the controls were from healthy people, and variables such as the collection, separation and storage of the blood samples were more stringently controlled for. It should be noted that the oligo- α -syn immunoassay is the same as that reported in our previous publication (37), except for the detection system, which was changed from alkaline phosphatase (absorbance-based assay) to streptavidin-europium (time-resolved fluorescence). This has improved assay sensitivity and has allowed us to dilute the plasma samples to 1:25, whereas previously (37), the samples were not diluted. It is possible that this has also contributed to the different findings reported here. We now find that only the levels of pS- α -syn were higher in

the PD group than in the control group ($P=0.053$), suggesting that the total phosphorylated protein may be the more useful diagnostic marker in plasma, and this is reflected in the ROC analysis for pS- α -syn (AUC=0.68). It should be noted that we did not make any formal adjustment for multiple testing (Bonferroni correction), because this is only a small-scale study, and this question will be addressed more fully in later work, when we have acquired and analyzed data from many more subjects.

The immunoblot results confirm that immunoreactive protein bands, with an intensity compatible with the immunoassay results, are detected when the protein from plasma is immunocaptured with N-19 and detected on immunoblots with pS129 (*i.e.*, the same capture and detection antibodies as those used for the pS- α -syn immunoassay). Some of these bands seem to represent ubiquitinated forms of the protein, since they also reacted with an antiubiquitin antibody. Whether these phosphorylated and ubiquitinated forms of α -syn originate from a cellular component in the blood itself or whether they originate from a peripheral tissue source elsewhere in the body, or from the brain (*via* CSF) is currently unknown.

Phosphorylation and ubiquitination are important secondary modifications of α -syn, with the protein deposited in LBs being predominantly phosphorylated at serine 129 (3, 47). Ubiquitination is a means of targeting a protein for destruction *via* the proteasome, and a defect in the ubiquitin-proteasome system is likely to be fundamental to the molecular pathogenesis of PD (48), although, recently, it has been suggested that α -syn phosphorylated at Ser-129 is targeted to the proteasome in a ubiquitin-independent manner (49). Phosphorylation of α -syn has been found to promote fibril formation, suggesting that hyperphosphorylation of α -syn might be a contributing factor in the pathogenesis of PD (47, 50). It has also been suggested that α -syn phosphorylated at serine 129 and its aggregation are involved in pathway responsible for α -syn toxicity in oligodendrocytes (51, 52). Given this pathological role for phosphorylated and ubiquitinated forms of α -syn, the levels of these modified proteins in body fluids, including blood plasma, are more likely to reflect the fundamental neuropathology of PD than the normal protein (33). This inference is borne out by the results of the present study. Observations that 10–37% of aged, neurologically healthy controls display some α -syn pathology in their brains (53, 54), with about half of such subjects showing abundant α -syn pathology (53), could explain why the levels of pS- α -syn did not better discriminate between patients with PD and healthy controls.

It is also worth noting that the levels of the nonphosphorylated protein in plasma showed a weak but positive correlation with sampling age (of the patients with PD) in the present study, whereas the phosphorylated protein showed no such correlation. Age has already been noted as a confounding variable for total α -syn levels in CSF (32). This lack of correlation between

phosphorylated α -syn and age could be an additional advantage in its development as a potential molecular biomarker.

In summary, we have validated some novel assays for assessing α -syn levels in blood plasma; shown that these levels are highly consistent in repeat blood samples taken over 3 mo from patients with PD; presented evidence for the presence of pS- α -syn (phosphorylated at Ser-129) in blood plasma; and found that the mean level of pS- α -syn was marginally significantly higher ($P=0.053$) in the PD samples than in the controls. We accept that the latter result is preliminary and will need to be confirmed in larger-scale studies. Nevertheless, on the basis of the data presented here, further study of phosphorylated α -syn as a potential biomarker for PD and related α -synucleinopathies is clearly warranted. Moreover, whether any of the different forms of α -syn can be used to monitor the progression of PD cannot be determined from the present study with longitudinal sampling over 3 mo only and must await data from our ongoing longer-term longitudinal studies. **[F]**

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In vitro recapitulation of aberrant protein inclusions in neurodegenerative diseases

New cellular models of neurodegenerative diseases

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A aberrant protein aggregates in affected brain cells of patients with neurodegenerative diseases are a well-known hallmark, but although the formation of these inclusions is an important pathogenic event, the mechanism involved remains unclear. We have recently established a simple method to introduce protein fibrils into cultured cells as seeds for protein aggregation, and we showed that intracellular soluble α -synuclein or tau can aggregate in cultured cells dependently upon seeds introduced in this way. Seeded aggregation of α -synuclein induced necrotic cell death, which was suppressed by the addition of various polyphenols. Our cellular models are expected to be valuable tools not only for elucidating the molecular mechanisms of onset of neurodegenerative diseases, but also for drug discovery.

In patients with neurodegenerative disorders, intracellular aberrant protein inclusions are often found in the brain, including neurofibrillary tangles in Alzheimer's disease or Lewy bodies in Parkinson's disease and dementia with Lewy bodies. These aberrant protein aggregates are often observed in the most affected regions of diseased brains, suggesting they may cause neuronal cell death, leading to onset of these diseases. Tau and α -synuclein are well-known cytosolic proteins that are the main components of neurofibrillary tangles and Lewy bodies, respectively. They are soluble and natively unfolded proteins, and it remains unclear how they become aggregated in neuronal cells. Indeed, intracellular aggregate formation

of these proteins does not occur when cultured cells are transfected with expression plasmids encoding these proteins. On the other hand, many in vitro studies using recombinant proteins, such as A β , tau, α -synuclein or poly glutamine-containing protein, have shown that these proteins are readily aggregated into fibrils in the presence of seeds for aggregation. These findings prompted us to examine whether seeds-dependent aggregation would occur in cultured cells. Thus, we aimed to introduce protein fibrils into cultured cells as seeds for aggregation.

Transfection of plasmid DNA into cultured cells is conducted routinely by the use of liposomes of polycationic and neutral lipids in water, based on the principle of cell fusion. Several commercially available reagents such as Lipofectamine, Lipofectamine 2000 or FuGENE6 are available to efficiently transfect plasmid DNA into cultured cells. We tested whether these transfection reagents could transfect cultured SH-SY5Y cells not only with plasmid DNA, but also with protein fibrils. After much trial and error, we finally succeeded in transfecting α -synuclein fibrils into these cells using Lipofectamine reagent. We found that the introduced recombinant α -synuclein fibrils are phosphorylated at Ser129 in cultured cells, indicating that they had been introduced by Lipofectamine.¹ Interestingly, monomeric and oligomeric α -synuclein could not be introduced by the use of Lipofectamine. We applied for a patent covering the use of Lipofectamine for transduction of recombinant protein fibrils into cultured cells in 2005 (patents

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pending in the United States: 12/086124, the European Union: 06834541.2 and Japan: 2007-549210). Recently, other groups have also reported introduction of fibrillar protein into cultured cells with or without specific reagents.²⁻⁵

Next, we examined whether intracellular α -synuclein can be aggregated dependently upon introduced seeds. When α -synuclein fibrils mixed with Lipofectamine were introduced into cells transiently expressing α -synuclein, phosphorylated and ubiquitinated α -synuclein inclusions (~10 μ m in diameter) were observed by means of confocal laser microscopy, indicating that plasmid-derived soluble α -synuclein formed aggregates in the presence of exogenous α -synuclein fibrils in cells, and these inclusions resembled Lewy bodies in diseased brains. Others have also reported that α -synuclein fibrils seed the formation of Lewy body-like intracellular inclusions in cultured cells.⁴ On the other hand, introduced tau fibrils were also shown to act as seeds for intracellular aggregation of plasmid-derived soluble tau protein. Interestingly, we found that fibrils composed of 3-repeat tau isoform serve as seeds for intracellular aggregation of soluble 3-repeat tau, but not soluble 4-repeat tau and fibrils of 4-repeat tau seed serve as seeds for aggregation of soluble 4-repeat tau, but not soluble 3-repeat tau. Likewise, introduction of α -synuclein fibrils did not elicit intracellular tau aggregation in cells and soluble α -synuclein did not form intracellular aggregates in the presence of any tau fibrils. These results clearly indicate that intracellular protein aggregation is highly dependent on the species of protein fibril

seeds. Now, we are examining whether detergent-insoluble fractions prepared from several diseased brains can be introduced into cells by Lipofectamine and can serve as seeds for intracellular aggregate formation of soluble α -synuclein, tau or TDP-43.

Does the formation of these inclusions lead to cell death or toxicity? The answer is yes. We observed non-apoptotic cell death in cells harboring these inclusions. In these cells, proteasome activity was found to be significantly reduced. This suppression may be related to the cause of cell death. Furthermore, we showed that cell death in cells with α -synuclein inclusions is effectively suppressed by the addition of various small molecules to the culture medium; polyphenols such as exifone and gossypetin were the most effective, suggesting that these compounds may be possible new drugs for the treatment of neurodegenerative diseases.

Our study strongly supports a seed-dependent mechanism for the formation of the intracellular protein aggregates. Recently, the intercellular transfer of inclusions made of tau,^{3,6} α -synuclein^{2,7,8} and huntingtin⁹ has been reported, suggesting the existence of mechanisms reminiscent of those by which prions spread through the nervous system. It remains to be clarified whether the incorporation of amyloid seeds into neurons or glial cells, as shown in our study, also occurs *in vivo*, but our results strongly suggest that extracellular aggregates may be taken up into neurons by endocytosis or under certain specific conditions. Therefore, it may be crucial to inhibit not only the production of intracellular amyloid seeds, but also

their spread into extracellular space and their propagation. Vaccination against α -synuclein¹⁰ or tau may be an effective treatment, together with the inhibition of intracellular aggregates formation with small-molecular compounds, for the therapy of neurodegenerative diseases.

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Post mortem cerebrospinal fluid α -synuclein levels are raised in multiple system atrophy and distinguish this from the other α -synucleinopathies, Parkinson's disease and Dementia with Lewy bodies

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ABSTRACT

Differentiating clinically between Parkinson's disease (PD) and the atypical parkinsonian syndromes of Progressive supranuclear palsy (PSP), corticobasal syndrome (CBS) and multiple system atrophy (MSA) is challenging but crucial for patient management and recruitment into clinical trials. Because PD (and the related disorder Dementia with Lewy bodies (DLB)) and MSA are characterised by the deposition of aggregated forms of α -synuclein protein (α -syn) in the brain, whereas CBS and PSP are tauopathies, we have developed immunoassays to detect levels of total and oligomeric forms of α -syn, and phosphorylated and phosphorylated oligomeric forms of α -syn, within body fluids, in an attempt to find a biomarker that will differentiate between these disorders. Levels of these 4 different forms of α -syn were measured in post mortem samples of ventricular cerebrospinal fluid (CSF) obtained from 76 patients with PD, DLB, PSP or MSA, and in 20 healthy controls. Mean CSF levels of total and oligomeric α -syn, and phosphorylated α -syn, did not vary significantly between the diagnostic groups, whereas mean CSF levels of phosphorylated oligomeric α -syn did differ significantly ($p < 0.001$) amongst the different diagnostic groups. Although all 4 measures of α -syn were higher in patients with MSA compared to all other diagnostic groups, these were only significantly raised ($p < 0.001$) in MSA compared to all other diagnostic groups, for phosphorylated oligomeric forms of α -syn. This suggests that this particular assay may have utility in differentiating MSA from control subject and patients with other α -synucleinopathies. However, it does not appear to be of help in distinguishing patients with PD and DLB from those with PSP or from control subjects. Western blots show that the principal form of α -syn within CSF is phosphorylated, and the finding that the phosphorylated oligomeric α -syn immunoassay appears to be the most informative of the 4 assays would be consistent with this observation.

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Introduction

Idiopathic Parkinson's disease (PD) is one of several neurodegenerative disorders that can present with similar clinical symptoms, particularly parkinsonism which is a combination of tremor, rigidity and bradykinesia. Progressive supranuclear palsy (PSP), corticobasal syndrome (CBS) and multiple system atrophy (MSA) are neurode-

generative conditions that are neuropathologically distinct entities, but show clinical overlap with PD. Because of the prominent clinical features they show in addition to parkinsonism, they are often described as "atypical" Parkinsonian syndromes.

In vivo diagnosis of PD and atypical Parkinsonian disorders relies on clinical criteria (Poewe and Wenning, 2002). Although none of these disorders is currently curable, it is important to make the correct diagnosis as early as possible since the symptomatic therapeutic approaches differ, and future (causative) therapies might be targeted directly against the underlying pathological process in each of these disorders. Reliable, early clinical diagnosis is also crucial for correct classification of patients within clinical trials (Schlossmacher and

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Mollenhauer, 2010). Nonetheless, clinical diagnosis of PD is often imprecise, particularly during the early stages of the illness. Indeed, clinicopathological studies have shown that only 69–70% of people with autopsy-confirmed PD had, in life, at least two of the cardinal clinical signs of the disease, and 20–25% of people with two of these symptoms had a pathological diagnosis other than PD (Hughes et al., 1992, 2001). There is clearly an urgent need to develop a biomarker for PD and the related disorder of Dementia with Lewy bodies (DLB) which will not only distinguish these disorders from normal people, but also from patients with other parkinsonian and/or dementing syndromes. Considerable effort therefore currently goes into the development of biomarkers for PD and the atypical parkinsonian disorders that would reliably allow the clinician to distinguish between them at an early stage.

PD and DLB are both characterised pathologically by the deposition of aggregated forms of α -synuclein protein (α -syn) in the brain in the form of neuronal cytoplasmic inclusions (Lewy bodies, LBs) and dystrophic processes (Lewy neurites, LNs) (Spillantini et al., 1997, 1998). In PD, α -syn pathology is principally found in brain stem and mid brain structures (substantia nigra, locus caeruleus, dorsal motor vagus, and nucleus of Meynert) (Spillantini et al., 1997), whereas in DLB the similar α -syn changes are focussed on regions such as cingulate cortex, parahippocampal gyrus and amygdala (Spillantini et al., 1998). LBs and LNs contain a misfolded, fibrillar and phosphorylated form of α -syn (Anderson et al., 2006; Spillantini et al., 1997). In demented PD patients (PDD), there is a 'spread' of α -syn pathology into cortical structures, and PD, PDD and DLB may form a continuum of disease. Pathological changes also involving α -syn, but chiefly in glial cells, characterise MSA. Collectively, PD, DLB and MSA are often referred to as ' α -synucleinopathies' (Spillantini et al., 1998). PSP and CBS on the other hand are tauopathies.

We, and others, have previously reported that α -syn can be detected within cerebrospinal fluid (CSF) and plasma (El-Agnaf et al., 2003, 2006; Tokuda et al., 2006, 2010). This extracellular form of α -syn seems to be secreted from neuronal cells by exocytosis (Emmanouilidou et al., 2010; Lee et al., 2005) and could play an important role in cell-to-cell transfer of α -syn pathology in the brain (Angot and Brundin, 2009). Consequently, levels of α -syn within plasma and/or CSF might therefore serve as a biomarker for PD, and other α -synucleinopathies (i.e. DLB, MSA). Here, we have tested whether ventricular post mortem CSF measures of α -syn can predict the presence or amount of α -syn pathology within the brain in α -synucleinopathies, and can differentiate the α -synucleinopathies from each other, as well as from other parkinsonian disorders, such as progressive supranuclear palsy (PSP), which are characterised by tauopathy. Moreover, because pathological investigations have demonstrated that the aggregated α -syn within LBs and LNs is phosphorylated (at Ser 129) (Anderson et al., 2006; Fujiwara et al., 2002; Obi et al., 2008), we have argued (Foulds et al., 2010) that these modified, pathological forms of the protein ought to more accurately reflect the fundamental neuropathology of PD, and that measurements of phosphorylated α -syn within CSF might provide a more direct marker of α -syn pathology in the brain (akin to measurement of tau phosphorylated at Ser 181 (ptau-181) as an index of neurofibrillary pathology in AD (see Blennow and Hampel, 2003 for review)), than the more straightforward measures of 'total α -syn' which most previous assays (for example, El-Agnaf et al., 2003, 2006; Tokuda et al., 2006, 2010) have been limited to.

Materials and methods

All CSF samples and brain tissues had been collected with full Ethical permission, following donation by next of kin, and were kindly provided by the Parkinson's Disease UK Brain Bank (PDUKBB) and Queen Square Brain Bank (QSBB), except for one MSA case from Manchester Brain Bank (MBB). Clinical diagnoses had been made

locally by the referring specialist Neurologist in care of the patient. Nonetheless, in all instances, the clinical diagnosis had been confirmed pathologically by Neuropathologists within their respective tissue banks. For PDUKBB cases, clinical information and neuropathological reports were available on PDUK web site. For QSBB and MBB cases relevant information was available locally. All clinical and pathological diagnoses were made in accordance with internationally recognised criteria.

Samples of CSF were obtained at post mortem from 96 individuals (Table 1), 85 were provided by the PDUKBB, 10 by QABB and one from MBB. CSF was drawn directly at post mortem from the subarachnoid space and/or lateral ventricles and immediately frozen and stored at -80°C pending analysis. The post mortem delay time between death and obtaining/freezing CSF was variable, ranging from 2 to 96 h, though 62% of samples had been collected within 24 h of death and only 15% after 48 h of death.

Of the 85 samples from PDUKBB, 39 were from patients clinically diagnosed as having PD, 17 patients had DLB, 7 had PSP, 4 had MSA and 18 were controls. Of the 10 samples from QSBB, 5 had PSP, 3 had MSA and 2 were controls. The sample from MBB had MSA. Twenty six of the PD patients were anecdotally reported in their clinical histories as suffering from dementia (PD Dem) and/or cognitive impairment (PD Cog), whereas no evidence of cognitive impairment or dementia had been reported in the other 13 patients who were therefore considered to be cognitively unimpaired (PD nonD). All patients with PSP had classical Steele–Richardson syndrome. All patients with MSA had striatonigral degeneration (SND) subtype except one with a mixed subtype. Formal neuropsychological testing had not been performed for most of the PD and DLB cases, and MMSE scores were therefore generally not available. One of the QSBB PSP cases scored 23/30 on MMSE and 3 others from PDUKBB were reported as suffering from dementia; but the remaining PSP cases, and all the MSA and control cases had been considered to display no cognitive impairment, or had normal MMSE scores (where available).

Although, overall, age at onset, age at death and duration of illness differed significantly between PD, DLB, PSP, MSA and control groups ($F_{3,72}=2.95$, $p=0.039$, $F_{4,95}=2.48$, $p=0.05$, $F_{3,72}=4.55$, $p=0.006$, respectively) (Table 1), post hoc Tukey test showed no significant differences in any of these measures between any of the diagnostic groups, probably because of the small sample sizes involving PSP and MSA groups, particularly in respect of disease duration (Table 1). There were no significant differences between PD, PDD or DLB groups ($F_{2,52}=0.43$, $p=0.654$; $F_{2,53}=2.40$, $p=0.100$; $F_{2,52}=0.845$, $p=0.435$, respectively).

Paraffin sections (6 μm) of frontal and cingulate cortex, hippocampus and temporal cortex, amygdala and parahippocampus, and substantia nigra were obtained from the PDUKBB and QSBB from the same PD, DLB, PSP and MSA patients, wherever possible. However, sections were only available from 6 of the 20 control subjects (4 from PDUKBB and 2 from QSBB (Table 1)).

Table 1
Selected clinical and demographic details of cases studied.

Group	Gender	Age at onset (year)	Age at death (year)	Duration (year)
All PD (n=39)	29M, 13F	64.2 \pm 11.8	78.4 \pm 6.7	14.2 \pm 7.8
PD (n=13)	10M, 3F	66.1 \pm 11.7	79.0 \pm 6.5	12.9 \pm 6.6
PDD (n=26)	19M, 10F	63.3 \pm 12.0	78.1 \pm 6.9	14.8 \pm 8.4
DLB (n=17)	14M, 3F	62.4 \pm 8.2	74.0 \pm 7.5	11.8 \pm 6.9
PSP (n=12)	10M, 2F	73.5 \pm 6.9	80.7 \pm 7.9	6.6 \pm 3.8
MSA (n=8)	4M, 4F	64.3 \pm 7.6	70.9 \pm 7.4	7.6 \pm 2.9
Controls (n=20)	13M, 7F	na	77.9 \pm 12.1	na
*Controls (n=6)	5M, 1F	na	73.3 \pm 12.4	na

^a Those 6 of the 20 control cases for which paraffin sections were available.

Biochemical methods

Preparation of recombinant α -syn

Recombinant α -syn (without any purification tag) was prepared at Lancaster University from *E. coli* using the following protocol. pJEK2 was used to transform FB850, a *rec A*⁻ derivative of BL21 (DE3) pLysS. FB850 carrying this plasmid was grown in an 800 ml batch culture and protein expression was induced through the addition of isopropyl- β -D-thiogalactopyranoside (IPTG). A protein with a molecular weight of ~17 kDa started to accumulate in the cells 30 min after induction and reached maximum levels after 150 min. Immunoblot analysis identified this protein as α -syn using an anti- α -syn mouse monoclonal antibody (MAb 211, from Santa Cruz Biotechnology, Santa Cruz, CA, USA). After a 3 h induction, the suspension was centrifuged, and the cells resuspended in buffer. The cells were lysed by sonication, and then cell debris and insoluble material was removed by centrifugation at 4 °C for 1 h at 30,000 rpm. α -Syn was extracted from the supernatant by ammonium sulphate precipitation, then purified using two chromatography columns; mono Q and Superose 6. After purification, 5 μ g of protein ran as a single band when observed on a Coomassie blue-stained SDS gel, corresponding to monomeric α -syn.

Preparation of phosphorylated α -syn

Phosphorylated α -syn was prepared from recombinant α -syn as described previously (Sasakawa et al., 2007).

Preparation of oligomeric forms of α -syn

To prepare a standard for the oligomeric α -syn immunoassay, the recombinant protein was oligomerised by incubation at 45 μ M in phosphate-buffered saline (PBS) in an orbital shaker at 37 °C for 5 days, and the monomer and oligomer were separated by size exclusion chromatography. A sample (0.5 ml) of pre-aggregated α -syn was loaded onto a Superose 6 column (44 × 1 cm) connected to a fast protein liquid chromatography (FPLC) system (Atka Purifier, GE Healthcare) and eluted with running buffer (PBS) at a flow rate of 0.5 ml/min. Absorbance of the eluate was monitored at 280 nm and fractions of 1 ml were collected and protein concentration determined.

To prepare a standard for the phosphorylated oligomeric α -syn immunoassay, the phosphorylated protein was allowed to aggregate by incubation at 50 μ M in PBS in an orbital shaker at 37 °C for 5 days. Aggregation of the protein was confirmed by thioflavin T assay (see Supplementary data). In this case, the amount of sample available was too small to fractionate by size-exclusion chromatography.

Immunoassays

We have already established immunoassays for the measurement of 'total' and 'soluble oligomeric' forms of α -syn in human biological fluids, including blood plasma and CSF (El-Agnaf et al., 2003, 2006; Tokuda et al., 2006), but these methods have been further optimized here.

Total α -syn

An ELISA plate (Iwaki) was coated with 100 μ l/well of anti- α -syn monoclonal antibody 211 diluted 1:1000 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (0.2 μ g/ml) in 50 mM NaHCO₃, pH 9.6, and incubated at 4 °C overnight. The wells were then washed 4 times with PBS containing 0.05% Tween-20 (PBS-T), and incubated for 2 h at 37 °C with 200 μ l/well of freshly prepared blocking buffer (2.5% gelatin in PBS-T). The plate was washed again 4 times with PBS-T and 100 μ l of the assay standard or CSF samples were added to each well, (each CSF sample was diluted 1:40 with PBS), and the assays were performed in triplicate. Following this, the plate was incubated at 37 °C for 2 h. After a repeat washing with PBS-T, 100 μ l/well of the

detection antibody, anti- α / β / γ -synuclein FL-140 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), dilution 1:750 (0.27 μ g/ml) in blocking buffer was added, and the plate was incubated at 37 °C for 2 h. After another wash with PBS-T, the plate was incubated with 100 μ l/well of secondary antibody (goat anti-rabbit HRP (Sigma), dilution 1:10,000 in blocking buffer at 37 °C for 2 h. The plate was then washed again with PBS-T before adding 100 μ l/well of Sure Blue TMB Microwell Peroxidase Substrate (KPL, USA) and leaving the colour to develop for 30 min at room temperature. Finally 100 μ l/well of stop solution (0.3 M H₂SO₄) was added and absorbance at 450 nm was determined. Recombinant monomeric α -syn was used to create a standard curve (Fig. 1a).

Oligomeric α -syn

The microtitre plate was coated and blocked using the same method as the assay for 'total α -syn'. The wells were then washed 4 times with PBS-T and 100 μ l of the CSF samples (diluted 1:25 with PBS) or assay standard (oligomeric α -syn) was added to each well, in triplicate. Following this, the plate was incubated at 37 °C for 2 h. After a repeat wash with PBS-T, 100 μ l/well of the detection antibody, biotinylated anti- α -synuclein 211 (diluted 1:1000 in blocking buffer) was added, and the plate was incubated at 37 °C for 2 h. After another wash with PBS-T, the plate was incubated with 100 μ l/well of streptavidin-europium, diluted 1:500 in streptavidin-europium buffer (Perkin Elmer) and shaken for 10 min. After a further 50 min agitation on a rotating platform, the plate was washed again with PBS-T, before adding 100 μ l/well enhancer solution (Perkin Elmer). Finally, the plates were read on a Wallac Victor² 1420 multi-label plate reader, using the time-resolved fluorescence setting for europium.

Oligomeric α -syn was used to create a standard curve (Fig. 1b). The specificity of this assay towards aggregated forms of α -syn was confirmed (the α -syn monomer gave no significant signal).

Phosphorylated α -syn

The antibody-sandwich immunoassay for 'total' α -syn was modified to detect only phosphorylated forms of the protein by replacing the 211 phospho-independent capture antibody with polyclonal anti- α -synuclein N-19 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), diluted 1:3000 (0.07 μ g/ml). The phospho-dependent rabbit monoclonal antibody, Phospho (pS129) Antibody (Epitomics Inc., CA, USA), used at a dilution of 1:3000, was the chosen detection antibody. This antibody only detects α -syn phosphorylated at Ser129. The preferred secondary antibody was human serum absorbed goat anti-rabbit HRP, 1:3000 (KPL, USA, rehydrated in 1 ml H₂O). This assay did not detect non-phosphorylated recombinant α -syn.

Oligomeric, phosphorylated α -syn

The antibody-sandwich ELISA for 'oligomeric' α -syn was modified to detect only phosphorylated, oligomeric forms of the protein, by replacing the 211 phospho-independent capture antibody with the phospho-dependent rabbit monoclonal antibody, Phospho (pS129) (Epitomics, Inc., CA, USA), used at a dilution of 1:3000. The detection antibody was biotinylated Phospho (pS129) at a dilution of 1:400. Recombinant, oligomerised, phosphorylated α -syn (oligo-pS- α -syn) was used to generate a standard curve (Fig. 1d). This assay did not detect the monomeric form of pS- α -syn.

Immunoblotting

According to the measures of total and oligomeric α -syn within CSF, cases with relatively high and low concentrations of α -syn were

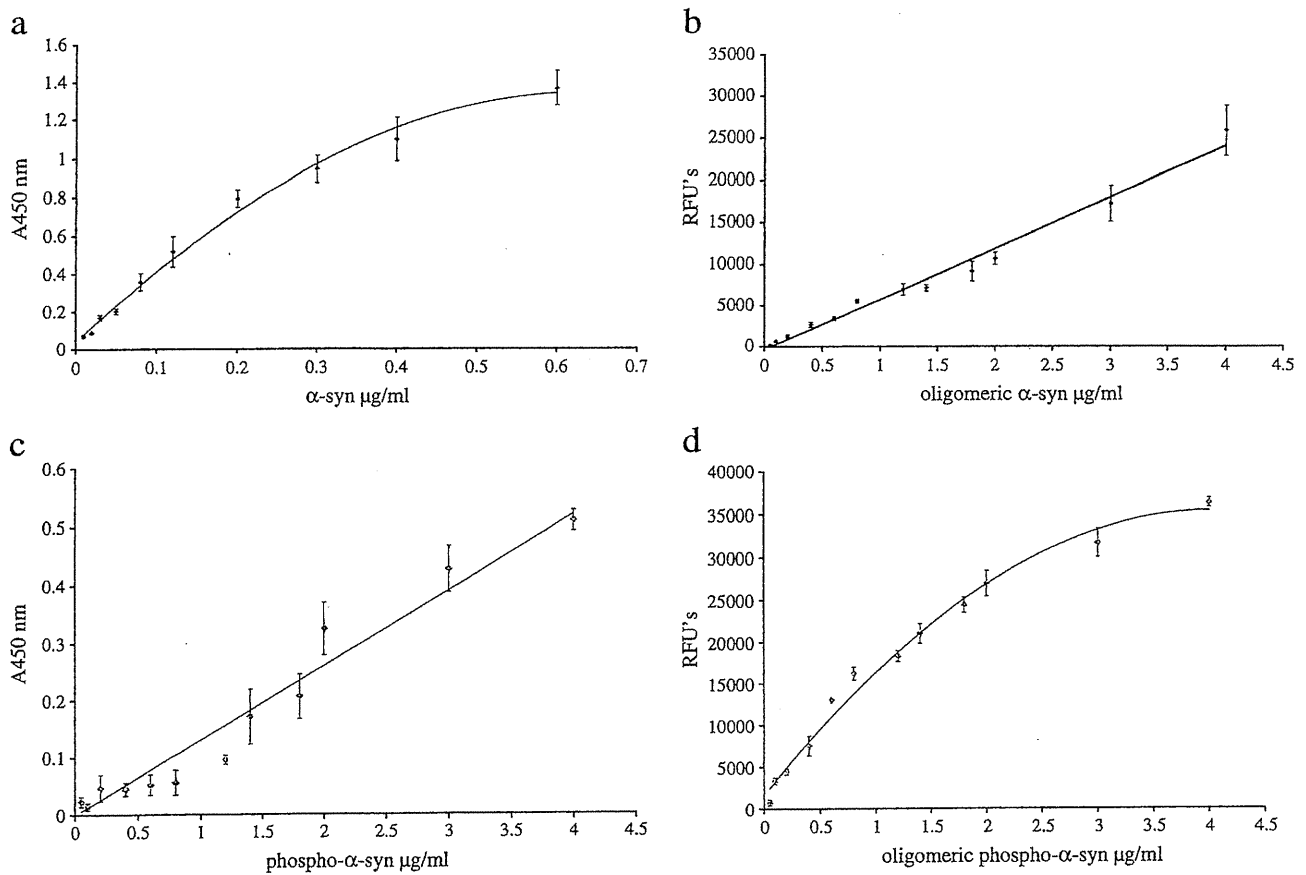


Fig. 1. Standard curves for immunoassays. Total α -syn (a), oligomeric α -syn (b), phosphorylated α -syn (c) and oligomeric phosphorylated α -syn (d).

selected for immunoblotting in order to characterise the molecular properties of α -syn within CSF.

For SDS-PAGE, these samples were run on 12.5% polyacrylamide gels and the separated proteins were electrotransferred onto nitrocellulose membranes (0.45 μ m, Invitrogen), at 25 V, 125 mA for 75 min, which were then blocked with 5% powdered, skimmed milk dissolved in PBS-T for 1 h. Membranes were incubated overnight with (a) polyclonal antibody, anti- $\alpha/\beta/\gamma$ -synuclein (FL-140) (Santa Cruz, USA), dilution 1:3000 or (b) phosphorylated anti- α -synuclein pS129 (Epitomics, USA), dilution 1:3000. The membranes were washed three times in PBS-T, followed by incubation with HRP-conjugated rabbit anti-mouse or goat anti-rabbit (Sigma), as appropriate, at 1:5000 in PBS-T, for 1 h. The protein bands were visualised using ECL reagents (Pierce, Rockford, IL) as described by the manufacturer.

Haemoglobin assays

Because previous studies have suggested that contamination of CSF samples by blood, either at lumbar puncture or at post mortem, might contribute through lysed red cells to α -syn measures (Hong et al., 2010), we assayed our CSF samples for haemoglobin levels using an immunoassay. The haemoglobin levels in CSF samples were measured using a Human haemoglobin ELISA Quantitation Kit from Bethyl Lab Inc (Montgomery, TX, USA) according to the manufacturer's instructions.

Histological methods

Wax sections were immunostained for α -syn pathology using the rabbit polyclonal antibody #1175 with microwave pretreatment in 0.1 M citrate buffer pH 6.0. This antibody recognises both phosphorylated and non-phosphorylated forms of α -syn, but in PD and DLB

generates identical patterns of immunostaining as pSer129 – an antibody specific to forms of α -syn phosphorylated at Ser129 (Obi et al., 2008).

The severity of α -syn pathology (ie overall density of Lewy bodies and Lewy neurites) within each brain region was rated on a 4 point scale (0 = absent, 1 = occasional/mild, 2 = common/moderate, 3 = numerous/severe), and a composite score across all 5 regions obtained by summation of individual scores, with a maximum score of 20 possible.

Statistical analysis

All data analysis was performed using SPSS v 16.0. For normally distributed data, two-sample Student's *t*-test for independent samples or one-way ANOVA were applied in comparing means of CSF α -syn measures between two or more groups, respectively. Alternatively, for non-normally distributed data, Kruskal–Wallis analysis of variance with post hoc Mann Whitney *U* test was used. Similarly, when testing the correlations between CSF α -syn measures and age at onset or death, or duration of illness, or haemoglobin concentration Spearman's first rank correlation or Pearson's correlation tests were used as appropriate. All levels of significance were two-tailed and set at $p < 0.05$.

Results

There were no significant differences in mean CSF levels of total α -syn (Fig. 2a), oligomeric α -syn (Fig. 2b) or phosphorylated α -syn (Fig. 2c) between PD, DLB, PSP, MSA and control groups ($F_{4,89} = 1.36$, $p = 0.255$, $F_{4,89} = 1.37$, $p = 0.249$, $F_{4,87} = 1.21$, $p = 0.313$, respectively) (Table 2). In contrast, mean CSF levels of oligomeric phosphorylated α -syn (Fig. 2d) were highly significantly different between PD, DLB, PSP, MSA and control groups ($F_{4,84} = 22.4$ $p < 0.001$) (Table 2). Post