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Journal Name

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ARTICLE

An impedimetric assay of α-synuclein autoantibodies in early stage Parkinson's disease

Cite this: DOI: 10.1039/x0xx00000x

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/x0xx00000x

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α-synuclein (α-Syn), a protein synthesized by neurons, as the major protein component of Lewy body inclusions, undoubtedly has a prominent role in the pathogenesis of Parkinson's Disease (PD). In an attempt to enable pre-symptomatic and definitive diagnosis, numerous attempts have been made to align assayed total α-Syn levels in serum (where there is a native presence) to PD disease status. Results have been conflicting. The status of circulating and potentially neuroprotective α-Syn autoantibodies in PD subjects is also unclear. In previous work we demonstrated that electrochemically assayed autoantibody levels were higher in PD patients compared to controls and, significantly, noted that this differentiation was most marked early in disease. Herein we report a robust (coefficient of variation 3.0%) single step and label free analysis of 60 patients, with a mean disease duration of 1.4 years and 29 control subjects. In this cross sectional cohort we observe a statistically significant ($p<0.05$; Mann-Whitney U test) difference in autoantibody levels in PD patients versus controls, although there was no resolved scaling with symptomatic disease stage ($p>0.05$; Kruskal-Wallis test).

Introduction

Parkinson's disease, like other neurodegenerative disorders, is devastating, incurable and increasingly prevalent. There remains a critical need to understand pathological progression, to improve early diagnosis and to discover therapeutics. Although the major pathology of PD arises from the death of dopaminergic neurons in the substantia nigra, a detailed map of pathogenic progression remains unclear. To date, there is no accepted clinical diagnostic test for PD based on a blood or cerebrospinal fluid (CSF) analysis – diagnosis remains invariably underpinned by symptoms and clinical examination, being inherently made relatively late in disease progression (if onset is regarded as the point at which a patients physiological status deviated from norm). There is a well-known long latency between the onset of cellular damage and the onset of clinical symptoms[1]. Distressingly, the latter, and thus associated diagnoses, are often not evident until some 80% of dopaminergic neurons have been lost.[2] The rapidly scaling cost and social burden of neurodegenerative care brings with it an acute need to diagnose earlier in order to facilitate earlier intervention and clinical management. There has been, understandably, considerable effort invested in exploring potential serum or CSF circulating biomarkers. Although a number of markers have been recognized as capable of separating Alzheimers patients from healthy controls (HC),[3] this has been notably less so for PD where the most accepted disease markers have been neuroimaging based (such as dopamine transporter (DAT) and vesicular monoamine transporter (UMAT)).[4] These have not been shown to underpin either early diagnosis or to separate overlapping Parkinson plus disorders. Much focus has been based around α-Syn. This 14kDa protein is expressed at high levels in nervous tissue and comprises the major component of Lewy bodies and Lewy neuritis characteristic in the brains of patients with PD. Several diseases, in fact, are associated with α-Syn deposition – the "synucleinopathy disorders". Although several studies have reported that CSF or serum levels of synuclein correlate with PD status, observations have by no means, been consistent. Several studies have also scrutinized serum / CSF levels of synuclein autoantibodies and, again, observations have been mixed. One contributing problem has been both the lab-to-lab variance associated with the default sandwich assays employed (assays requiring both two independent binding events and a robustly behaving antibody-enzyme conjugate) and the diverse nature of patient cohorts analyzed. In most cases, cohort numbers are <40 persons and are strongly biased towards mean disease durations in excess of ten years.[5] It is well

documented, now, that autoimmune responses are likely to diminish through disease progression.[5] In preliminary work we were able to demonstrate the assaying of α-Syn autoantibodies by impedance at synuclein modified electrode confined films. Herein we extend this analysis to a larger (60 patients) well-characterised, early stage cohort (Oxford Parkinson's Disease Centre (OPDC) Discovery cohort: http://opdc.medsci.ox.ac.uk).

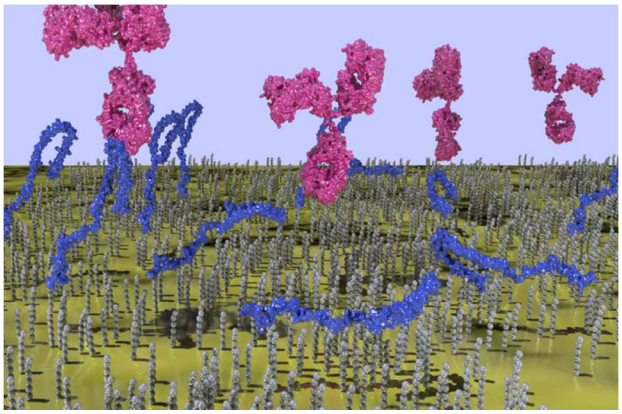

Fig. 1. Schematic representation of the autoantibody-recruiting electrode interface. Clean gold electrodes are initially passivated with a PEG SAM on which recombinant human synuclein (blue) is covalently tethered. The interfaces, thereafter, are selectively responsive to the synuclein autoantibodies (red).

Experiment section

Materials and sample preparation

A consecutive sample of 60 early PD patients (mean (SD) disease duration from diagnosis 1.40 (1.44) years) and 29 control subjects were prospectively recruited as part of the OPDC Discovery cohort study from 11 hospitals across the Thames Valley covering an estimated population of 2.1 million. Patients with PD were diagnosed according to clinically probable diagnostic criteria of the UK Parkinson's Disease Society Brain bank.. Controls had no family history of PD, had a normal formal neurological examination and negative genotyping for rare monogenic variants relevant to PD (LRRK2, GBA mutations). The demographic and clinical characteristics of investigated groups are shown in Table 1, with full descriptions of the study protocol detailed elsewhere (Rolinski M, Ben-Shlomo Y, Szewczyk-Krolikowski K, Tomlinson P, Nithi K, Murray C, Talbot K, Hu MT**.** JNNP 2014, PMID: 24187013). There were no significant differences in age between PD and control subjects overall (mean (SD) age (yrs): 69.4 (10.8) PD vs 67.1 (7.8) controls). Patients were subdivided according to motor disease severity into Hoehn and Yahr (H&Y) stages, where stage 1 (n=20) is unilateral early disease, H&Y stage 2 (n=20) is bilateral disease with preserved postural reflexes, and H&Y stage 3 (n=20) is bilateral disease with impaired postural reflexes. Subject blood samples were collected in BD Vacutainer SSTII blood collection tubes prior to serum separation. Briefly, 5 mL of blood was inverted 5 times and then stand for 10 min at room temperature for the clot to form. The clotted samples were then centrifuged at 3500 RPM/13000g for 10 min. The acquired serum samples were stored at -80 °C prior analysis.

Serum samples were stored at -80 oC before analysis. α-Syn Autoantibodies (mouse monoclonal IgG) were purchased from Santa Cruz Biotechnology, Inc. (USA). Recombinant human syn was expressed in E. coli and provided by Umea University, Sweden.

Table 1 Demographic of investigated groups

	Control	H&Y Stage I	H&Y Stage II	H&Y Stage III
Numbers of subjects	29	20	20	20
Female	18	10	08	9
Mean age at study ± SD (y)	67.1±7.8	68.9±9.6	65.5±11.3	73.6±10.2
Age range (y)	55-88	51-85	46-84	46-88
Mean duration of disease (y)	N/A	1.1±0.8	1.4±1.2	1.7±2.0

Measurement of serum α-Syn Ab

Each patient serum sample was analysed by EIS on individual 3 precalibrated independent electrodes. The electrode polishing and surface modification are as prior reported[6]. Briefly, pre-cleaned gold electrodes were immersed in HS-C11-$(EG)_3$-OCH_2-COOH in ethanol overnight in 4°C fridge to form a self assembled monolayer (SAM) on the gold interface. The supporting PEG SAM layer film was characterized by Faradaic impedance (increase in baseline impedance from 160 ± 9 Ω cm^{-2} for clean gold to 4 ± 0.04 MΩ cm^{-2}), ellipsometry (film thickness 1.9±0.1 nM) and thiol stripping voltammetry in 0.5 M KOH (film molecular density 1.1×10-10 ± 0.15×10^{-10}; error bar from triplicate repeat, value consistent with expectations for films of this type). The carboxyl terminated SAM modified electrode was activated by 0.4 M N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 0.1 M N-Hydroxysuccinimide (NHS) in PBS for 40 min, and subsequently incubated with 1 μM α-Syn for 3 hours to form the α-Syn Ab sensory interface.

Data processing

Statistical variations between patient groups were analysed by SPSS (version 19.0.0, SPSS, Chicago, IL). A Mann-Whitney U test was specifically utilized to analyze the differences between two groups (HC/PD stage I, HC/PD stage II, or HC/PD stage III). To analyze for correlation of response across all patient groups a Kruskal-Wallis test was used. To investigate the correlation of the demographic characteristics and α-Syn Ab level in PD patients, a Spearman rank correlation was used. A significance value of $p < 0.05$ was set for all analysis. Receiver operating characteristic (ROC) curves were constructed (SPSS) to assess diagnostic potency.

Results and discussion

Electrochemical characterization of α-Syn Ab

The impedance of the so formed sensory interface was initially calibrated in $[Fe(CN)_6]^{3-/4-}$ by incubation with specific concentrations (0.05 nM to 2 nM) of α-Syn Ab spiked PBS. The interfacial impedance change was observed to be linear with logarithmic concentration of α-Syn Ab from 0.05 nM to 1 nM

(equivalent to 7.5 ng/mL to 0.15 µg/mL), saturating at 1 nM with a detection limit of 21±4 pM (equivalent to ~3.1 ng/mL).

Electrochemical impedance spectroscopy measurements for investigated subjects

In order to bring patient sample autoantibody levels into assay linear range a ten fold sample dilution was carried out in PBS. To investigate the impact of serum based background ex-situ signal analyses were carried out in 10%, 20%, 50% and 100% dilutions of serum were initially measured; interfacial impedance response at the 10% dilutions utilized with all patient samples was 4.2 ± 0.3 % of that observed for 0.1 nM α-Syn Ab response (the middle of the quantified clinical range analysed). Assay selectivity was additionally analysed by monitoring interfacial response to BSA. Changes in Rct were observed to be <2% even at BSA concentrations some three orders of magnitude higher (impedance of 0.1 nM α-Syn Ab 15.4±0.7 MΩ cm^{-2}; impedance of mixture of 0.1 nM α-Syn Ab and 100 nM BSA 15.7±0.4 MΩ cm^{-2}) than the autoantibody levels being sampled (Fig. 2).

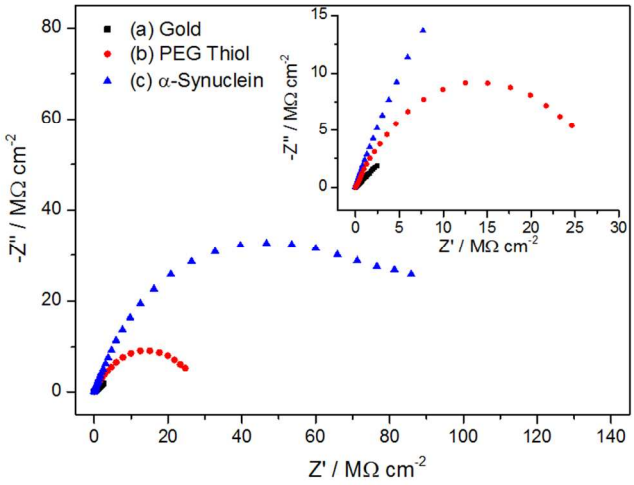

Fig. 2. Typical Nyquist plots of different films, recorded in PBS (10 mM, pH 7.4) solution containing 1.0 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl. (a) Bare gold surface (R_{ct} ~ 160 ± 9 Ω cm^{-2}), (b) PEG SAM interface (R_{ct} ~ 4 ± 0.04 MΩ cm^{-2}), (c) α-Syn modified interface (Rct ~ 6 ± 0.01 MΩ cm^{-2}); Error bars represent the standard deviations of 3 measurements on three individual electrodes. Inset is a magnified view of the high frequency response, showing the relative impedance of the underlying bare electrode (black curve).

Each sample quantification process was repeated in triplicate at the same electrode and then again across three different (pre-calibrated) electrodes. For each analysis 10 µL of serum was spiked into 90 µL freshly prepared PBS then incubated with pre-prepared synuclein-PEG film interfaces for 20 minutes. Faradaic impedance analyses were then carried out in 1.0 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl across a sampling frequency of 0.01 Hz to 1000000 Hz. All patient samples were incubated with precalibrated electrodes for 20 min before rinsing with PBS and analysis.

Statistic analysis of patient samples

Herein 89 serum samples have been tested with 20 PD subjects at each of stages I-III. There were 29 healthy subject samples analysed (ages 55 to 88 years). The mean age of all studied subjects was 68.6 years with 53% of these being female. 97% of the patients have been PD symptomatic for less than 3.5 years and mean disease duration across the PD group as a whole is 1.4 years. A multiple repeat quantification of autoantibody levels reported mean concentrations of 1.66 ± 0.324 (mean ± SEM, standard error of mean), 1.62 ± 0.264, and 1.92 ± 0.387 nM, respectively for patients of H&Y Stage I, II and III. For the HC group, the mean concentration is 1.24 ± 0.268 nM. In analyzing potential contributions to data of assay variance, sample quantification was analysed through triplicate repeats at single assaying electrodes and then again at different electrodes (9 quantifications per sample). Note that, as expected, a statistical analysis of these results is shaped by the variance observed within these staging populations; it is, specifically, the case that the greater variance (SEM) in the Stage III subset means that, although the mean quantification is greater than for Stage I and II, the greater SEM more than offsets this (such that one has to conclude a lack of robust differentiation against HC for this subset in isolation). This is partially the case also for the Stage I subset but not for the Stage II - we suspect these observations are likely to be affect of this particular cohort. The relatively low SEM associated with the Stage II subset is sufficient to both robustly differentiate it from the HC subset and to do likewise for the pooled PD patients as a whole. The mean CV_{intra} (triplicate measurements on a same electrode) is 3.4 ± 1.1%, with a small variation across all investigated concentrations, the mean CV_{inter} is 7.5 ± 4.9% as assessed across all samples with three independent electrodes per sample. It should be noted that these values reflect responsive surface reproducibility but are, in turn, much greater than the real quantification error since single electrodes are individually calibrated and quantifications ultimately performed by the "standard addition method". It should be additionally noted that these CV's report on assay accuracy as determined through reproducible analyses – they are not affected by, for example, patient sample heterogeneity. Statistical analysis across all the summed data confirms a resolved significant difference in autoantibody levels between HC and PD patients (p=0.041; Mann-Whitney U test) (Fig. 3). No significant differentiation between PD Stages (p>0.05; Kruskal-Wallis test) is observed.

To evaluate the utility of serum α-Syn Ab detection by electrochemical immunoassay in discriminating PD group from HC, receiver operating characteristic (ROC) curve analysis was performed (Fig. 4). The area under the ROC curve (AUC) was 0.623(95% confidence interval 0.493-0.752) for PD vs Control. Naturally occurring autoantibodies are likely to act to eliminate circulate excess or foreign proteins in a manner evolved to reduce their potentially damaging impact. The degree of association between CSF and serum autoantibody levels or indeed antigen levels is unknown. There exists some evidence that such antibodies may be involved in clearing misfolded proteins [7] and, significantly, that PD patients have increased autoantibodies against amyloid beta.[8, 9] Increased levels of autoantibodies against myelin basic protein and the calcium binding protein S100B have also been noted.[10, 11] Attempts to associate serum Syn autoantibodies with PD have, however, been very mixed [5, 12] in prior ELISA and Western Blot analyses.

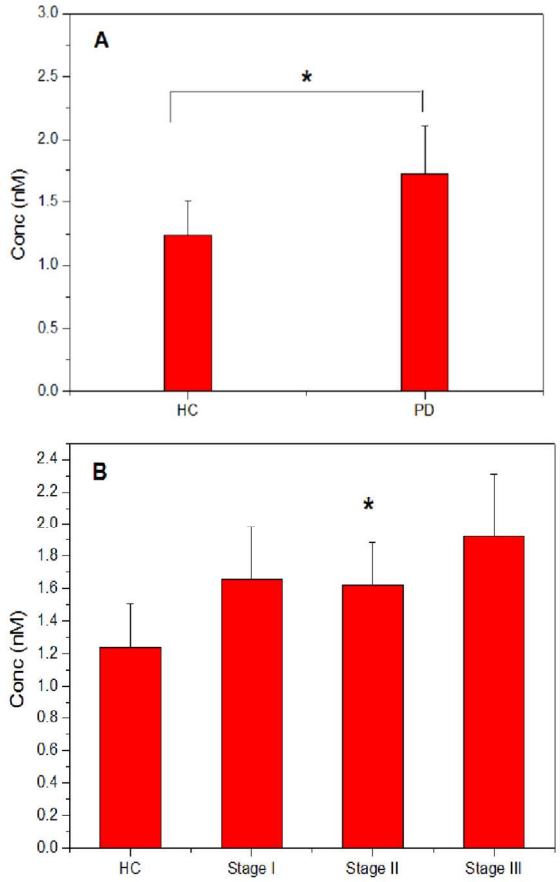

Fig. 3. Comparative levels of serum α-Syn Ab from healthy control and the PD cohort analysed herein. There is a significant difference in α-Syn Ab levels between PD and HC (*p<0.05) (A), and PD Stage II and HC (*p<0.05) (B). Error bars shown are SEM and height is the mean concentration of each group.

Prior enzyme-linked immunosorbent assay (ELISA) or Western blot analysis of serum α-Syn Ab levels in PD patient samples have been largely inconclusive. Papachroni[13] et al have also reported increased α-Syn antibody levels in the serum of familial PD (20 subjects) compared to HC (26 controls). In significant other work, however, conclusions have been rather mixed. In a study of 28 patients and 19 healthy controls, Woulfe et al.[14], for example, observed no statistical difference in sandwich ELISA analyses.

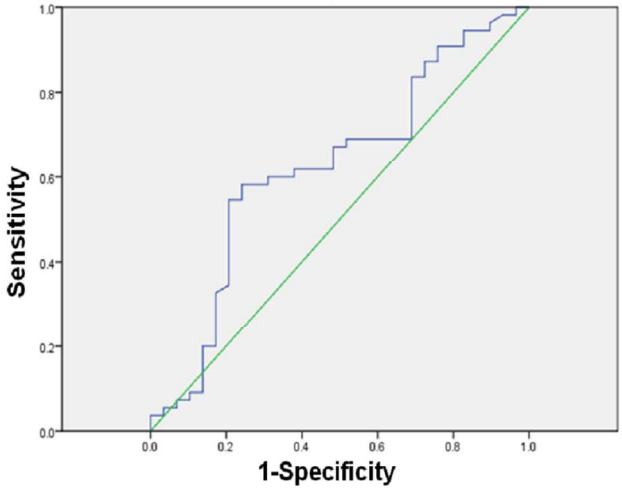

Fig. 4. Receiver operating characteristic curves to evaluate α-Syn Ab as a biomarker for Parkinson disease. ROC curve of patients with Parkinson disease (PD) vs controls. Area under the curve (AUC) 0.623 (95% confidence interval 0.493-0.752).

A similar conclusion has also been presented by Besong-Agbo et al.[15] from an ELISA assessment of 46 of HC and 62 PD subjects (mean disease duration 10.2 years).

In a contrasting analysis of 60 patients, also by ELISA and Western Blot, Yanamandra et al. [12] noted, however, significantly higher synuclein autoantibody levels in PD patients (most notably, in patients where disease duration was <5 years – a point that we will return to below). This proposed peak in autoantibody level was further observed by Gruden et al.[5] in an ELISA analysis 32 subjects. Recently Smith et al. [16] have reported, again, raised levels of synuclein antibody in the serum samples of PD patients when disease duration is less than 4 years. Koehler et al.[11] have recently reported similar observations with patients classified as having Lewy Body Dementia.

Inescapable contributions to this scatter are the lab to lab variance in quantification by these methods (a CV of 30% is entirely typical for ELISA[17]), notable issues with antibody and antibody-enzyme conjugate stability and batch to batch variation[18] and inherent patient heterogeneity. We believe it is also significant that most, if not all, of these prior works for which correlation with disease has been absent, are associated with mean disease durations generally of 8-10 years; the potentially protective role of these antibodies is likely to be dominant at much earlier staging - autoantibody levels have been previously shown to fall in PD patients at long disease duration[5, 6, 12]. Gruden et al. have suggested that these antibody levels peak some 5 years after initial clinical diagnosis.

Conclusions

In this work, we have sought to analyse a relatively large (and disease young) cross sectional cohort by an ultrasensitive label free electroanalytical methodology using native human synuclein capture antigens. Consistent with initial observations made with a different cross sectional cohort, we have, again, resolved a statistically significant (Mann Whitney U test p<0.05) increase in Syn autoantibodies in PD patients relative to age-matched controls. We have carefully analysed background and experimental error contributions to the data and deemed these to be acceptable and markedly less than the resolved quantification differences observed. We have not resolved a robust variance in autoantibody levels across H&Y stages but note that all patients herein have disease durations <4 years. Although ROC analyses of these single marker

assessments confirm that diagnostic potency is only moderate, we believe the observations within this work to be significant and the methodology readily applicable to the simultaneous quantification of multiple markers in future studies.

Acknowledgements

This study was funded by an award from Parkinsons UK (JJD, QX). The authors acknowledge additional support from the Monument Trust Discovery Award from Parkinson's UK and supported by the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre based at Oxford University Hospitals NHS Trust and University of Oxford, and the Dementias and Neurodegenerative Diseases Research Network (DeNDRoN).

Notes and references

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