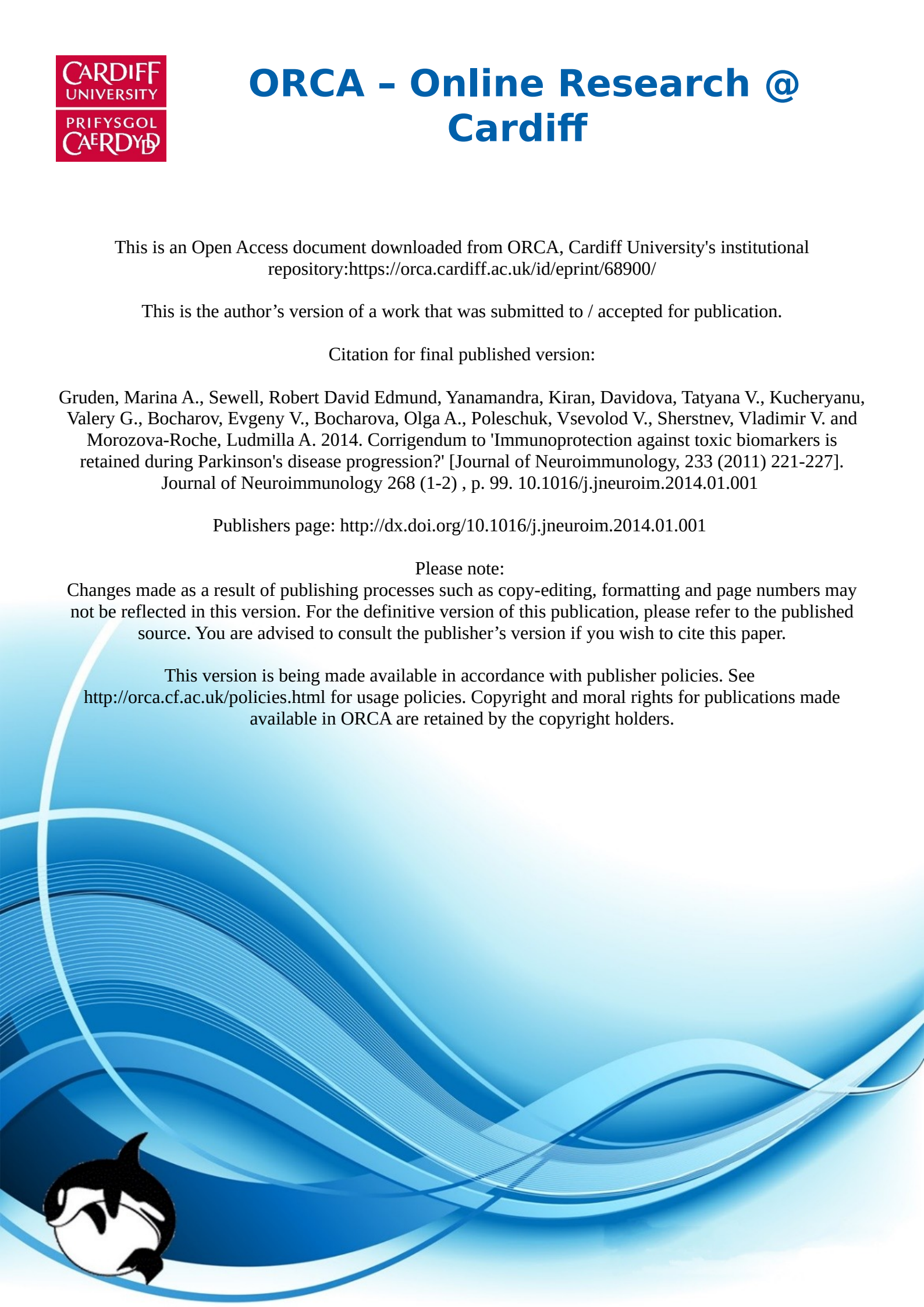

Immunoprotection against toxic biomarkers is retained during Parkinson's disease progression

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Keywords: Parkinson's disease, α-synuclein, amyloid toxicity, autoantibodies, T- cells, B-cells.

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ABSTRACT:

The aim was to ascertain any possible linkage between humoral immune responses to principal biomarkers (α-synuclein monomers, its toxic oligomers or fibrils, dopamine and S100B) and cellular immunity in Parkinson's disease development. There were elevated autoantibody titers to α-synuclein monomers, oligomers plus fibrils in 72%, 56%, and 17% of Parkinsonian patients respectively with a 5-year disease duration. Additionally, there were increased titers to dopamine and S100B (96% and 89%) in the 5-year patient group. All of these values subsided in 10-year sufferers.

Furthermore, CD3+, CD4+, CD8+ T-lymphocyte and B-lymphocyte subsets declined in the patient cohort during Parkinsonism indicating disease associated reductions in these lymphocyte subsets.

1. Introduction

How the immune system protects Parkinson's disease (PD) sufferers from toxic misfolded proteins requires resolution. It has been shown that α-synuclein protein, as a major PD biomarker (Lee et al., 2009) may be present not only as monomeric but also oligomeric forms in the substantia nigra or as aggregates in Lewy bodies (Yu and Lubchenko, 2009). It has also been demonstrated that prefibrillar aggregates of α-synuclein, as well as other amyloid protein species, constitute generic toxins in higher organisms including humans (Baglioni et al, 2006). Correspondingly, El-Agnaf et al. (2006) reported raised levels of oligomeric forms of α-synuclein in PD patient plasma confirming α-synuclein misfolding during neurodegeneration in vivo. Furthermore, as an accompaniment to neurodegenerative processes, natural autoimmunity may well be perturbed by generation of toxic protein species in humans (Orr et al., 2005; Gruden et al., 2007; Roodveldt et al., 2008). Previously we found that peripheral immune responses to amyloid structures are a sensitive indicator of the early onset of neurodegenerative diseases (Gruden et al 2004; Gruden et al., 2007). We have also evaluated autoimmune reactions to insulin amyloidogenic species as an endocrine biomarker in PD (Wihelm et al., 2007). Moreover, it is a distinct possibility that dopamine (DA) released from degenerating neurons may influence the process of protein misfolding in PD (Pham et al., 2000). Immunological consequences associated with inflammation and oxidative stress, have been proposed from the etiological pathogenic factors involved in the initial degenerative stages of parkinsonism (Czionkowska et al., 2002). One of these crucial factors is raised neurotrophin expression and stimulated release from cells by activated microglia. As a result, substantially increased concentrations of neurotrophins, for instance S100B, concurrently with toxic olgomeric species of α-synuclein continue to promote apoptosis and subsequent phagocytosis of DA neurons (Yu

and Lyubchenko, 2009; Nagatsu et al., 2000; Rothermundt et al., 2003). In connection with this, it is a possibility that synchronized autoimmune responses towards elevated DA and neutrotrophic factors, in parallel with autoimmune reactions to toxic α-synuclein species, may also limit α-synuclein cytotoxicity. Therefore, the aim of this study is to ascertain any potential interrelationship between autoimmune responses to selected PD biomarkers and a range of other immune system parkinsonian parameters.

2. Materials and methods

2.1 Patients

PD patients (n=32; 20 males and 12 females; 60.8 ± 2.0 years) were recruited from the Center of Neurology, RAMS. Patients underwent neurological examination and were diagnosed with PD according to disease severity by the UPDRS (Fahn and Elton, 1987). Patient scores ranged from 1 to 4, the majority being of grade 2 on the Hoehn and Yahr scale (Hoehn and Yahr, 1967). No opportunistic infections were observed either in PD patients or age-matched control subjects.

Both patient and control demographics are shown in Table 1. Sixteen PD patients presented primarily with tremor accompanied by rigidity, fifteen possessed rigidity predominantly along with tremor and one individual was diagnosed with akinetic rigid PD (Table 2). Patients were treated with dopaminergic-based antiparkinsonian therapy: the L-DOPA dopamine precursor agents L-Dopa + carbidopa (Sinemet CR®, Nacom®) or L-Dopa + benserazide (Madopar®), the non-ergot D2/D3 agonists Piribedil (Pronoran®) or pramipexole (Miropex®). The mean group dose for L-Dopa over 5.2 ± 0.7 treatment years was 411.5 ± 134.6 g/patient.

Following ethical approval subjects gave informed consent for study participation which was conducted in accord with the provisions of the World Medical Association Declaration of Helsinki (2000).

2.2 Protein samples

S100B and DA were purchased from Sigma, USA, α-synuclein - Millipore, USA. After 1 h of coagulation, blood (5ml) was centrifuged (15 minutes, 3,500g, 4^{o}C). The sera were aliquoted into Eppendorf tubes, frozen, stored at -80^{o}C, defrosted immediately prior to ELISA.

2.3 Production of α-synuclein amyloidogenic species

α-Synuclein (3 mg/ml) was incubated in 10 mM Na_2HPO_4 at pH 7.4 and 37°C with continuous agitation (300 rpm) for 7 days to produce oligomers and 14 days for fibrils.

2.4 ELISA of antibodies to α-synuclein monomers, oligomers, fibrils, S100B and DA

The titers of serum antibodies to α-synuclein monomers, oligomers and fibrils also protein S100b were determined by ELISA (Rich et al., 2001) in 96-well polysterol plates (Costar, USA) using 6 repetitions per sample. Microtiter wells were coated with the antigens at concentrations of 15.0, 20.0, 20.0 and 10.5 μg/ml, respectively, in 50 mM phosphate buffer, pH 8.0 (4 °C) overnight. The plates were washed 3 times with phosphate-buffered saline (PBS), 0.05% Tween 20, blocked with 1% w/v BSA in PBS, pH 7.5 (Sigma, USA) for 2 h (4 °C) and washed with PBS, 0.05% Tween 20. The coated plates were incubated for 16 h (6°C) with the samples of PD patient serum using 2-fold serial dilutions in PBS, over the dilution range 1:2 up to 1:3000. The plates were then washed 6 times with PBS plus 0.05% Tween 20, and serum antibody-antigen interactions were identified during incubation for 2 hours at 37°C using horseradish peroxidase conjugated with goat antibodies to human immunoglobulins (IgG) (Amersham, UK) at a dilution of 1:1000. The plates were

subsequently washed 6 times and incubated for 30 min with *o*-phenylenediamine (Sigma, USA) as substrate in the enzyme reaction. Absorbance at 492 nm was measured using the plate reader (Flow Lab., USA). The titer of each serum sample was derived from the reciprocal of the greatest dilution at which the enzyme-substrate reaction gave an optical density value greater than that of the mean optical density of blanks. The values of titers at which we observed immunoreactivity to the antigens were represented in relative dilution ratio units.

In the ELISA experiments for evaluating DA-antibody titres, conjugated DA-BSA (Gruden et al., 2007) was used at a concentration of 20 µg/ml and two modifications of the reaction were made, namely a 5% solution of powdered milk was used as the non-specific blocking agent; the secondary horseradish peroxidase conjugated with goat antibodies to human IgG (Amersham, UK) were plated at a dilution of 1:2000.

The following ELISA validation experiments were performed: (i) α-synuclein and S100b binding to well surfaces were confirmed in the reaction using laboratory-purified (protein A-sepharose) antibodies against α-synuclein and S100b, (ii) the specificity of the antigen/antibody interaction was established by addition of equal displacing concentrations (10-20 µg/ml) of both antigens to three independent patient serum samples prior to coating the sera onto the wells and this yielded values corresponding to blank, (iii) Standard concentrations of human IgG (Amersham, UK) were used to construct the calibration curve and to confirm the reaction of goat antibodies to human IgG.

Statistical significance was assumed at $p < 0.05$, for all measurements unless otherwise specified.

2.4 PD patient lymphocyte subsets as indicators of immune status

Indicators of immune status in PD patients included the determination of total T- and B-lymphocyte content by detecting antigens of lymphocyte differentiation (CD3, CD4, CD8, CD16, CD20), antigens of activation (CD25, HLA/Dr, CD95) and adhesion molecules (CD11b,CD18) with flow cytofluorometric analysis (FACscan, Becton Dickinson, USA). Cortisol was detected using ELISA commercial kits (Roche, France).

2.5 Atomic force microscopy (AFM)

α-Synuclein amyloid species morphology was examined by PICO PLUS atomic force microscopy (Agilent, USA) in a non-contact mode using a 100 μm scanner with acoustically driven cantilevers employing imaging procedures (Malisauskas et al., 2003). A scanner with a 100 μm scan size and cantilevers carrying 10 nm diameter etched silicon probes of the TESP model (Veeco, Netherlands) were applied. Scanning was performed at a resonance frequency (312-340 kHz), scan rate of 1 Hz and a resolution of 512 x 512 pixels. For imaging in air, samples were incubated (30 min) on freshly cleaved mica, washed with MiliQ water (3 x 100 μl) and dried overnight. The distribution of dimensions of amyloid species was measured in AFM cross-sections.

2.6 Thioflavin-T amyloid binding assay

Formation of amyloid species was assessed using the thioflavin-T dye binding assay (LeVine, 1993). Fluorescence measurements were performed on a Jasco FP-6500 spectrofluorometer (Jasco, Japan). The dye was excited at 440 nm and emission spectra recorded (450-550 nm), setting excitation and emission slits at 3 nm.

2.7 Statistical analysis

Comparison between groups was carried out non-parametrically using the Mann–Whitney test and by two tailed Student's t-test for unequal variance.

3. Results

3.1 Characterization of α-synuclein oligomeric and fibrillar species

Oligomeric and fibrillar species of α-synuclein were characterized by the thioflavin-T binding assay and AFM analysis prior to ELISA. The formation of amyloid oligomers was accompanied by a 5 fold increase of thioflavin-T fluorescence (Figure 1D), indicative of cross-β-sheet formation (Krebs et al., 2000). The oligomers were assessed by AFM as round-shapes (Figure 1A) with heights 1.2-3.9 nm (Figure 1B) and a maximal population centered around 1.8-2 nm corresponding to 20-mers [16]. The mature fibrils were characterized by a 10 fold increase in thioflavin-T fluorescence and displayed typical fibrillar morphology (≤1 μm) (Figure 1C).

3.2 Autoimmune responses to α-synuclein monomers oligomers, fibrils, DA and S100B

There was a significant ($P<0.05$) increase in autoimmune reactivity towards α-synuclein monomers. Autoantibody titers to oligomeric α-synuclein species in the 5-year patient sera were 4 fold higher than controls whereas sera from 10-year PD patients displayed lower autoantibody levels (Figure 2). There was a discernable peak in antibody titers to both forms of α-synuclein at the 5-year disease duration and this subsided during disease progression up to 10 years. A doubling of titer values to fibrillar α-synuclein was seen in less than 20% of patients versus controls and antibody titers to these protein aggregates remained generally low throughout (Figure 2). PD patients were screened for serum immune responses towards DA (Figure 2), the mean value being 25 fold higher than age

matched controls at 5-years and this markedly fell by the 10-year disease phase. The level of immune reactivity to S100B, as reflected by antibody levels in the 5-year PD patients, was 4.0 fold higher ($P<0.01$) than controls. Overall, it is clear that all PD patients displayed elevated levels of serum autoantibodies to the markers studied and they reached a zenith 5 years after disease diagnosis. Moreover, there was no correlation between the autoimmune reactivity to any of the biomarkers with respect to age ($P<0.02$) or gender ($P<0.1$).

3.3 Indicators of immune status in PD patients

There were no statistically significant differences in the content of T and B lymphocytes in the blood sera of PD patients with a 5- and 10-year disease duration, though this does not exclude the possibility of divergence in later stages of the disease. In drug-treated PD patients there were reduced counts of total CD3+ lymphocytes (< -21%), helper/inducer CD4+ T-cells (-23%), and CD20 positive B-lymphocytes (-16%) (Table 3). There were also mildly enhanced HLA-DR cell counts (+16%) and elevated counts of CD11b (+241%), CD18+ (+44%), CD25+ (+43%) and CD95+ (+296%) lymphocytes. Additionally, CD50+ cells were increased by 20% in PD patients.

3.4 Serum cortisol content in PD patients

The concentration of cortisol in PD patients was 893.5 ± 86.3 nM/l compared to a value of 735.0 ± 46.0 nM/l in controls (i.e. +21.5%, $P<0.05$).

4. Discussion

We simultaneously analyzed the spectrum of autoantibodies generated to native and amyloidogenic α-synuclein species, neurotrophic factor S100B and DA as major PD biomarkers in patient blood (Graeber, 2009) along with regulatory T- and B-cells. In altered conditions *in vitro* and *in vivo*, α-synuclein may self assemble forming ordered fibrils (Bertoncini et al., 2005) characterized by cross β−sheet structures similar to Lewy body aggregates (Foltynie, 2008). The initial phase of α-synuclein aggregation is thought to involve formation of oligomeric species possessing a higher degree of cytotoxicity than their mature fibrils (Stefani et al., 2003). The biological role of α-synuclein remains somewhat obscure but it may have a regulatory influence on synaptic vesicular pools such as DA (Perez and Hastings, 2004). Aggregation of α-synuclein exacerbates oxidative stress and cellular damage (Giasson et al., 2000) which might be a primary cause of immunological abnormalities in PD. In order to elucidate potential links amongst the above mentioned processes, both clinical and non-clinical experimental approaches were adopted. Immune response to α-synuclein amyloid species during PD progression have not previously been studied relative to the stages of amyloid structure formation. There was a substantial increase in autoantibodies to monomeric α-synuclein in 72% of PD patients with a 5-year disease duration. Fifty-six percent of patients displayed higher levels of autoantibodies to α-synuclein oligomers and only 17% to fibrillar α-synuclein. At the 10-year disease stage, all autoantibody titers had returned to control levels, indicating that there was a rise in autoantibodies up to 5 years and then a decline (Figure 3). In prior studies, multi-epitopic autoantibodies to α-synuclein were disclosed in 65% of patients with inherited PD (Papachroni et al., 2007). Moreover, α-synuclein immunization decreased the accumulation of aggregated α-synuclein in neurons and synapses associated with reduced neurodegeneration in mice (Masliah et al., 2005). S100B was selected as another indicator

of PD pathology since it has been associated with neurodegenerative processes (Donato et al., 2009). Immunological studies in mice have additionally shown that S100B expression is increased in glial cells after neurotoxin treatment which is known to cause parkinsonism (Muramatsu et al., 2003). Thus, assessment of synchronized immuno-reactivity towards our studied proteins in PD may be valuable in exposing on-going neurodegenerative processes. In 5-year PD patients, S100B autoantibodies were 4 times higher than controls but then dropped to control levels by the 10-year disease stage and this biphasic pattern reflects the progression of neurodegeneration. Autoantibodies to boosted DA, similar to the other autoantibodies investigated, peaked in 5-year disease sufferers and fell during the subsequent progression of PD (Figures 2 and 3). Thus, the overall diminution of the immunological pattern of autoantibodies to various disease biomarkers confirmed that activity of humoral immunity was diminished during later stage disease progression. The biphasic profile of idiotypic antibody generation to functionally important disease proteins and DA, plus the fact that constituents of the classical or antibody triggered complement cascade occur in Lewy bodies (Yamada et al., 1992), substantiate the finding that PD pathology engages humoral immunity (Orr et al., 2005). Thus, in the PD brain, there is a detectable level of immunoglobulin G which binds to DA neurons in a concentrated distribution at neuronal surfaces or on Lewy bodies co-localized with α-synuclein (Orr et al., 2005). Moreover, initiation of humoral immunity in early onset PD may also be concomitant with inflammation (Hirsch and Hunot, 2009) which plays a fundamental role in parkinsonism (Stolp and Dziegielewska, 2009). Increased production of disordered α-synuclein species is conducive towards activation of the innate immune system generating antibodies to the main molecular participants in disease pathology, the levels of which are determined by the stage of PD progression. It might be hypothesized from the current findings that there is a protective role for idiotypic autoantibodies to toxic oligomeric α-

synuclein species with the purpose of abolishing toxic effects of aggregates towards dopaminergic substantia nigra cells and in other brain regions pertinent to pathology. Moreover, elevation of S100B expression in PD relevant brain structures (Rothermundt et al., 2003; Fahn and Elton, 1987) and release of DA (Pham et al., 2000), may activate microglia to generate pro-inflammatory cytokines and install changes in cellular immunity via the antigens expressed by T- lymphocytes. The overall reduction in cell counts for T-lymphocytes (CD3+), helper-inducer (CD4+), B-lymphocytes (CD20+) and also cells expressing HLA-DR antigen signified PD associated decrements in these cellular subsets. In contrast, the patients had raised counts for CD11b+, CD18+, CD25+ and CD95+ lymphocytes which was characteristic of pro-inflammatory activation of the immune system and inflammatory reactions in microglial cells (McGeer and McGeer , 2008). Thus, there are two major arms of the immune system, one which functions through antibodies produced by plasma or B-cells, and the other which operates via immune cells including CD4+ T-cells. It is the cell-mediated arm of the immune system that is depressed in patients diagnosed with PD. Accordingly the current data concur with reported aberrations in PD peripheral lymphocyte subsets indicating that T lymphocytes, as well as T-helper and T-suppressor elements are partially decreased relative to the control ratio of CD4/CD8 lymphocyte subsets (see Stone et al., 2009). A selective loss of CD4+ lymphocytes has also been detected in the early stages of multiple sclerosis and Down's syndrome suggesting a common immunological abnormality in such neurological disorders (Fiszer et al., 1994).

Furthermore, the reduced lymphocyte subsets observed in our study inevitably relates to augmented blood cortisol which would weaken the activity of the immune system by prevention of T-cell proliferation (Stypuia et al., 1996). At the asymptomatic stage of PD (≤30% of neuronal loss) when oxidative stress initiates formation of α-synuclein amyloidogenic species, humoral immunity actively protects dopaminergic neurons from

oligomeric toxicity by producing a variety of antibodies to the complex of disease biomarkers which includes α-synuclein oligomers themselves. (see Figure 3 at the 5-year disease stage). As PD progresses (≥ 50% neuronal loss), diminishing autoantibody binding to DA yields a relative lack of antioxidant activity, so autoimmunity fails to cope with oxidative stress and cytotoxicity. During the decline in lymphocyte subsets in PD patients (Figure 3) free DA influences mitochondrial respiration causing release of cytochrome C and in so doing, this initiates neuronal apoptosis (Berman and Hastings, 1999). Similarly, overexpression of S100B, along with neurodegenerative processes (Rothermundt et (al., 2003), blood-brain barrier dysfunction (Stolp and Dziegielewska, 2009) and impaired autoantibody protection at the progressive stages of PD may be conducive to DA cell loss. In summary, it has become accepted that processes of α-synuclein misfolding and the appearance of toxic aggregates may invoke humoral immunity in the pathological gambit causing dopaminergic neuronal death in PD. Also, during PD progression, T-cell immunity subsides together with microglial activation to become increasingly involved in the cascade of neurodegenerative processes. The current findings suggest possible links between α-synuclein toxicity elimination by autoantibodies to its oligomers in early onset PD pathology (Figure 3) and decay of lymphocyte subsets which reflects the influence of inflammatory and oxidative stress reactions. Moreover, as PD can be difficult to diagnose in the initial stages, determination of the autoantibody profile to disease associated biomarkers may be a supplementary diagnostic tactic (Hawa et al., 2004). Hence, humoral immunity is directed to protect and prevent the brain from substantial cytotoxicity and neurodegeneration and thus, potential PD treatment strategies should attempt to maintain patient immune status.

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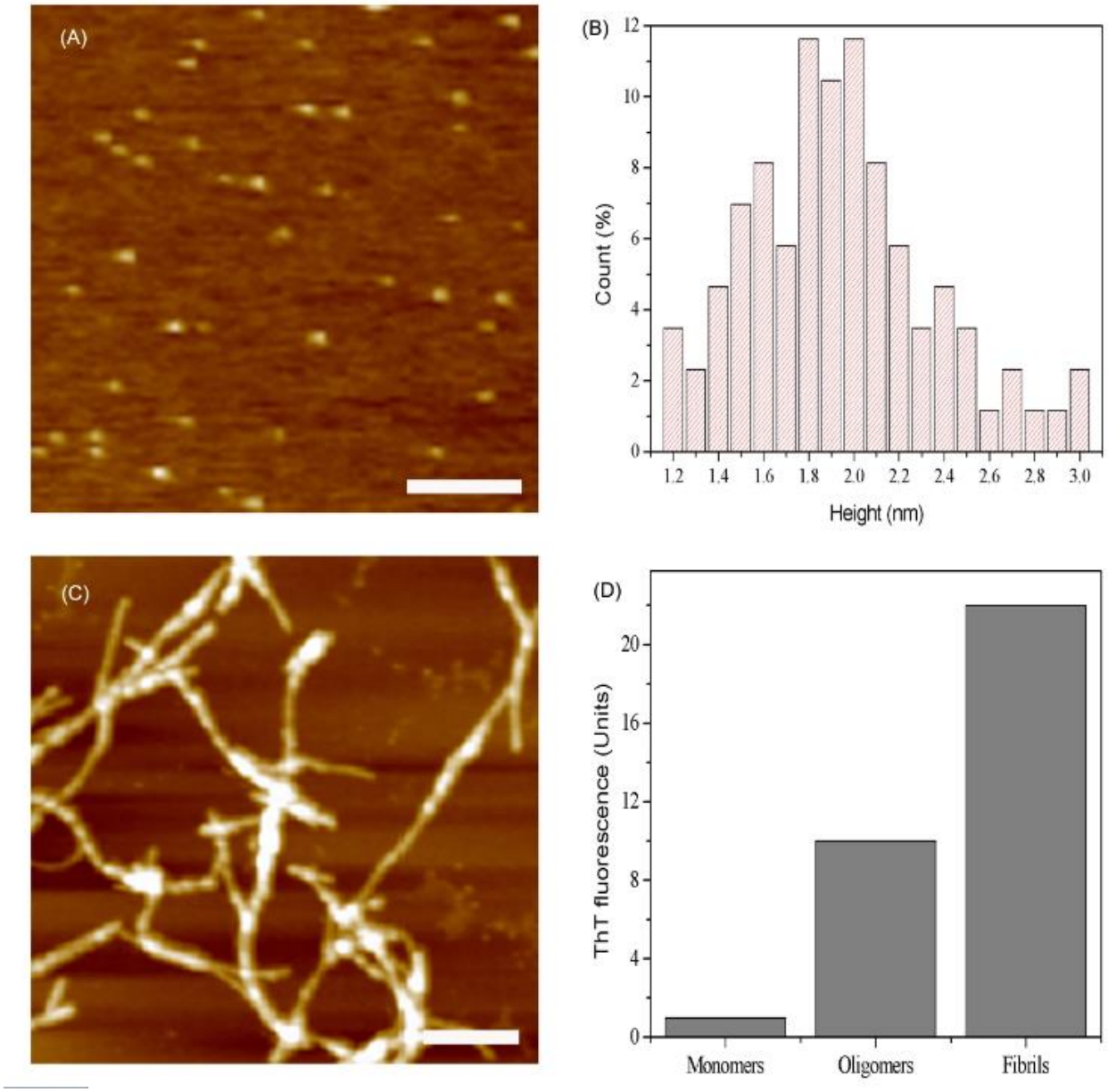

Figure 1. AFM imaging and thioflavin-T fluorescence assay of α-synuclein amyloid species produced in sodium phosphate buffer pH 7.4 at 22^0C with agitation. (A) AFM height image of amyloid oligomers formed after 7 days of incubation; (B) distribution of the heights of oligomeric species measured by AFM cross-section analysis; (C) AFM height image of amyloid fibrils of α-synuclein produced under the same conditions after 14 days of incubation. (D) Thioflavin-T fluorescence intensities measured at 480 nm in the presence of monomeric α-synuclein, amyloid oligomers and fibrils, respectively.

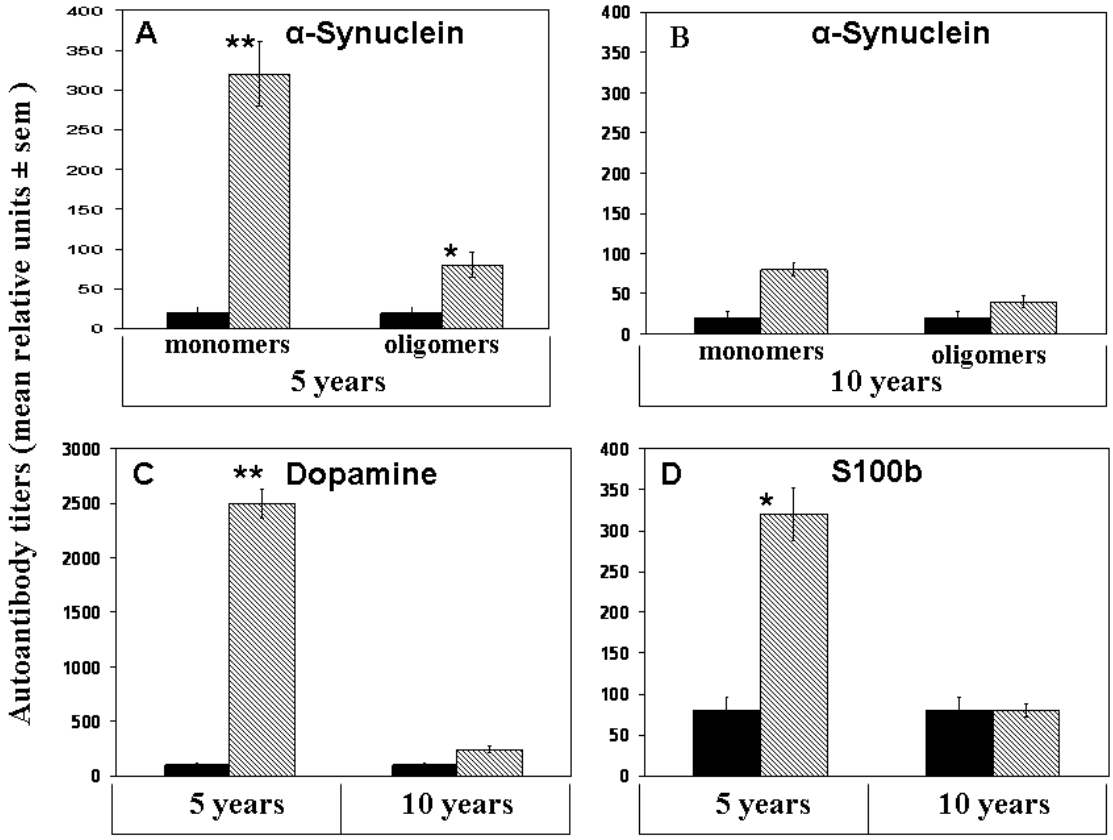

Figure 2. Titers of serum antibodies (relative dilution ratios) to α-synuclein monomer and α-synuclein amyloid oligomers in controls (black columns) and Parkinson's disease patients (hatched columns) with ≤5 year disease duration (A), to α-synuclein monomer and α-synuclein amyloid oligomers in controls (black columns) and Parkinson's disease patients (hatched columns) with ≥10 year disease duration (B), dopamine in controls (black columns) and Parkinson's disease patients (hatched columns) with ≤5 year disease duration (C) and ≥10 year disease duration (C) and protein S100B in controls (black columns) and Parkinson's disease patients (hatched columns) with ≤5 year disease duration (D) and ≥10 year disease duration (D), respectively. *p≤0.05, **p≤0.01

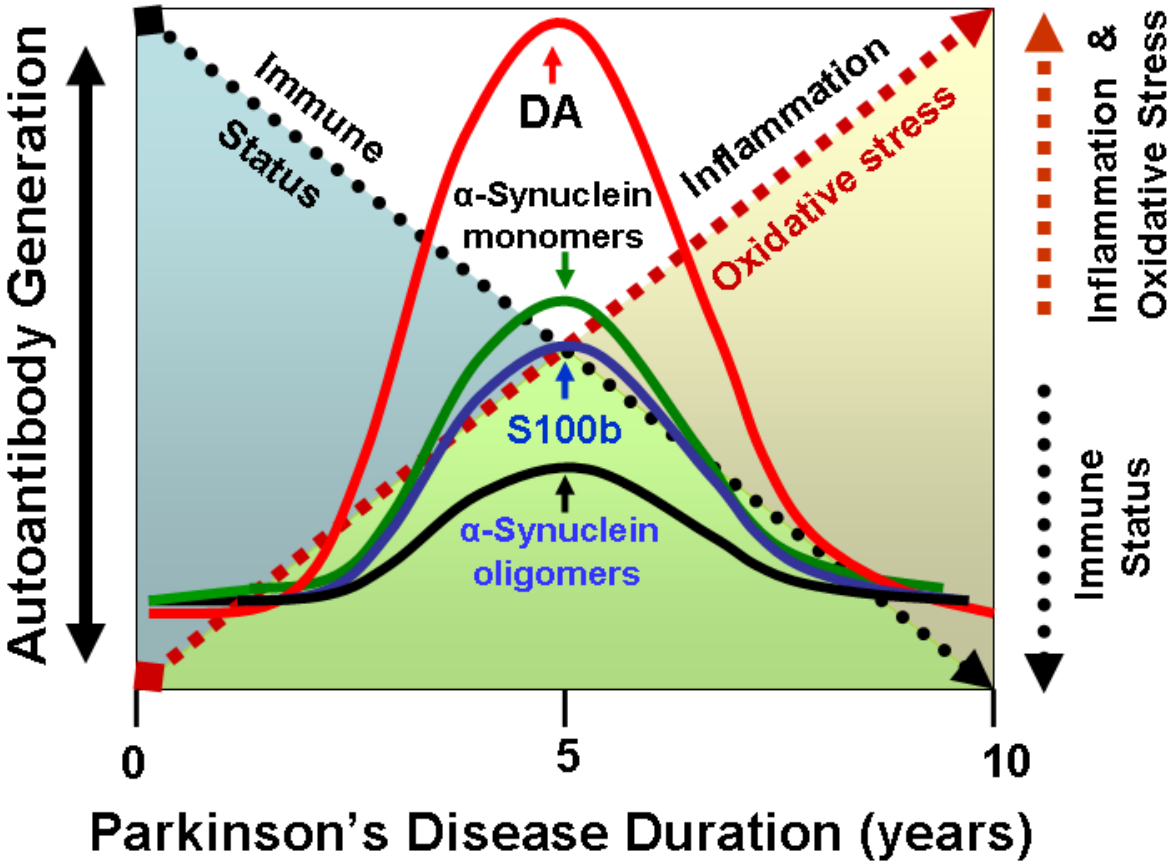

Figure 3. Schematic showing the differential generation of autoantibodies to α-synuclein monomers, oligomers, S100B and dopamine in 5- and 10-year Parkinson's disease sufferers in relation to their declining immune status and progressively increasing indicators of inflammation and oxidative stress.

Table 1. Group characteristics of the study population (group mean ± s.e.m)

Group characteristics	PD patients (n =32)	Controls (n = 26)
Age (years)	60.8 ± 2.0	63.0 ± 3.0
Gender *n* (male:female ratio)	20:12	19:7
Age at onset of PD (years)	60.8 ± 2.0	-
Duration of PD (years)	8.6± 3.4	-
[a]UPDRS motor score	23.3 ± 1.9	-
Hoehn and Yahr stage	2.1 ± 0.6	-

[a]UPDRS: Unified Parkinson's disease rating scale

Table 2. Clinical analysis of Parkinson disease patients by Unified Parkinson's disease rating scale (UPDRS)

Clinical components of UPDRS	UPDRS Score (mean ± s.e.m)
Impairment of daily activity	18.7 ± 1.6
Motor activity	23.3 ± 1.9
Tremor	2.3 ± 0.13
Rigidity	2.2 ± 0.14
Bradykinesia	2.0 ± 0.18

Table 3. Indicators of immune status of PD patients and controls (mean ± s.e.m)

Antigen	Control group (lymphocyte counts)	PD patients	
		(lymphocyte counts)	% change
CD3	61.1 ± 0.9	48.3 ± 2.9 [a]	-21
CD4	34.3 ± 0.7	26.5 ± 1.8 [a]	-23
CD8	30.0 ± 1.5	25.2 ± 1.3	-16
CD4/CD8	1.2 ± 0.1	1.1 ± 0.1	0
CD20	5.7 ± 0.8	4.8 ± 0.5 [a]	-16
HLA-DR	7.2 ± 0.5	8.4 ± 1.1 [a]	+16
CD16	11.4 ± 0.5	16.7 ± 2.3 [a]	+46
CD11b	11.9±0.8	23.0 ± 1.6 [a]	+241
CD18	56.8±1.1	81.9 ± 3.2 [a]	+44
CD50	69.4 ± 1.1	83.5 ± 4.4 [a]	+20
CD95	11.8 ± 0.4	35.0 ± 2.5 [a]	+296
CD25	3.1 ± 0.4	5.4 ± 1.1 [a]	+43

[a] P<0.05