The diagnostic discrimination of cutaneous α -synuclein deposition in Parkinson disease

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ABSTRACT

Objective: To determine the diagnostic discrimination of cutaneous α -synuclein deposition in individuals with Parkinson disease (PD) with and without autonomic dysfunction on autonomic testing, in early and late stages of the disease, and of short and long duration.

Methods: Twenty-eight participants with PD and 23 control participants were studied by skin biopsies at multiple sites, autonomic function testing, and disease-specific scales.

Results: Skin biopsies provide >90% sensitivity and >90% specificity to distinguish PD from control participants across all biopsies sites with quantification of either pilomotor or sudomotor α -synuclein deposition. All individuals with PD have significantly higher cutaneous α -synuclein deposition than control participants, even those individuals with PD and no evidence of autonomic dysfunction. Deposition of α -synuclein is most prominent in sympathetic adrenergic nerve fibers innervating the arrector pili muscles, but is also present in sudomotor (sympathetic cholinergic) nerve fibers. α -Synuclein is present even in the early stages of disease and disease of short duration. α -Synuclein ratios were higher in individuals with autonomic failure, with more advanced stages of disease and disease of longer duration.

Conclusions: The α -synuclein ratio provides a sensitive and specific diagnostic biomarker of PD even in patients without autonomic failure.

Classification of evidence: This study provides Class III evidence that cutaneous α -synuclein deposition accurately identifies patients with PD. *Neurology*® 2016;87:505-512

GLOSSARY

H&Y = Hoehn & Yahr; **IENTFD** = intraepidermal nerve fiber density; **MDS-UPDRS** = Movement Disorders Society Unified Parkinson's Disease Rating Scale; **PD** = Parkinson disease; **PD-AF** = Parkinson disease with autonomic failure; **PD-NAF** = Parkinson disease with no autonomic failure; **PGP 9.5** = protein gene product 9.5; **PMNFD** = pilomotor nerve fiber density; **ROC** = receiver operating characteristic; **SGNFD** = sweat gland nerve fiber density.

Parkinson disease (PD) is characterized clinically by motor manifestations that include tremor, rigidity, and bradykinesia and pathologically by the deposition of α -synuclein within Lewy bodies and Lewy neurites.¹

We, and others, have recently reported that deposition of α -synuclein can be detected within cutaneous autonomic nerve fibers using the punch biopsy technique.²⁻⁵ In a prior study, using an antibody that measured total (both phosphorylated and native) α -synuclein, we observed high amounts of α -synuclein deposition in all individuals with PD and low amounts of α -synuclein deposition in healthy control participants. The α -synuclein deposition correlates with measures of sympathetic adrenergic and cardiac parasympathetic function.^{2,3} But it is still not known whether the utility of cutaneous α -synuclein deposition as a biomarker in PD is restricted to those patients with PD who have autonomic dysfunction. Further, because clinically significant autonomic dysfunction usually (although not always) occurs late in the course of PD,^{6,7} we also sought to define the relationship between cutaneous α -synuclein deposition disease severity and disease duration. We therefore studied a new cohort of participants with PD with a broad range of disease duration, severity, and autonomic dysfunction with the following prospective aims: to determine the diagnostic discrimination of cutaneous α -synuclein deposition in individuals with

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PD (1) with and without autonomic dysfunction on autonomic testing; (2) in early and late stages of the disease; and (3) of short and long duration. We also used this new cohort of participants as a confirmatory dataset to validate our previously reported findings.

METHODS Participants. Twenty-eight participants with PD and 23 control participants were recruited for participation into a prospective longitudinal study with yearly visits. Eligible participants with PD had a diagnosis confirmed by a movement disorders specialist (L.C.S.) using all 3 steps of the UK PD Brain Bank Criteria for Parkinson's disease, applying exclusion criteria and incorporating supportive features such as asymmetric onset, durable dopaminergic medication responsiveness, and presence of motor fluctuations.⁸ The current data are from the initial visit of the study.

Standard protocol approvals, registrations, and patient consents. The Beth Israel Deaconess Institutional Review Board approved the study and all participants signed an informed consent.

Examination. All participants underwent a detailed history and neurologic examination. Parkinson disease severity was rated by the Movement Disorders Society Unified Parkinson's Disease Rating Scale (MDS-UPDRS) and the modified Hoehn & Yahr scale (H&Y).^{9,10} Examination scores were measured off PD medications for at least 16 hours.

Symptom assessment. Autonomic symptoms were quantified using the Boston Autonomic Symptom Questionnaire; symptoms ranged from 0 (none) to 10 (maximal), as previously reported.^{2,11}

Autonomic function testing. All study participants underwent autonomic function testing that included tests of cardiovascular parasympathetic function (the heart rate response to deep respiration, the Valsalva maneuver, and active standing) and cardiovascular sympathetic function (the beat-to-beat blood pressure response to a Valsalva maneuver, tilt-table testing to 60° for 10 minutes, and active standing for 5 minutes) as previously described.² Medications known to alter autonomic function (including antihypertensive medications and PD medications) were held starting the evening prior to testing.

Autonomic failure. All participants with PD were divided into 2 groups: those with or without autonomic failure (PD with autonomic failure [PD-AF] and PD with no autonomic failure [PD-NAF]). Autonomic failure in this study was defined as dysfunction of both the sympathetic adrenergic and parasympathetic systems: (1) a fall in systolic blood pressure of >20 mm Hg within 3 minutes of upright tilt and (2) evidence of reduced parasympathetic function on Valsalva ratio or heart rate variability testing.

Human skin biopsy and fixation. Three-millimeter punch skin biopsies were taken from the lateral distal leg, distal thigh, proximal thigh, and mid-volar forearm.¹² Skin biopsies were placed in Zamboni fixative solution for 18 hours and then placed in cryoprotection.¹³

Immunohistochemistry. Biopsy tissues were cut by freezing microtome into 50- μ m sections. An average of 20 tissue sections was analyzed per biopsy. Fluorescent immunohistochemical staining was performed using our previously published methods for covisualizing all nerve fibers (with the panaxonal marker protein gene product 9.5 [PGP 9.5]) and α -synuclein-positive nerve

fibers.^{2,13} Complete details of our immunohistochemical methods have been reported previously.^{2,13} The primary antibodies used to visualize total nerve fibers included the panaxonal marker PGP 9.5 (UltraClone, Isle of Wight, UK). The primary antibody used to detect α -synuclein was polyclonal and recognized multiple binding sites from amino acids 111–131 (Chemicon; EMD Millipore, Gibbstown, NJ).^{2,14} Costaining with PGP 9.5 and α -synuclein was performed in all tissue sections.

Confocal imaging. All tissue sections were imaged by confocal microscopy (Zeiss LSM5 Pascal Exciter; Carl Zeiss, Thornwood, NY). A series of images of optical sections was acquired at 3- μ m intervals throughout the depth of the 50- μ m section as a z-stack (Lens Plan-Apochromat $\times$ 20/0.8; Carl Zeiss).

Quantification of sensory and autonomic nerve fibers.

Intraepidermal nerve fiber density (IENFD). Biopsies underwent blinded counting of intraepidermal nerve fibers, stained with PGP 9.5, as previously described using standard methodology.^{2,15} The results were expressed as number of fibers crossing the dermal–epidermal junction per millimeter.¹⁵

Sweat gland nerve fiber density (SGNFD). Sweat gland innervation was quantified in a blinded fashion using our previously described manual unbiased stereologic technique.^{16,17} The number of nerve fibers intersecting the circles within the area of interest was counted. Results are reported as the percentage of total circles with nerve fiber crossing.¹⁸

Pilomotor nerve fiber density (PMNFD). Pilomotor innervation was quantified in a blinded fashion as previously published.^{19,20} The average number of nerve fibers intersecting 5 horizontal lines across the width of the pilomotor muscle in 3- μ m-thick confocal images were reported in fibers/mm.

α -Synuclein nerve fiber density ratio (α -synuclein ratio). As described previously, we normalized the amount of α -synuclein within each subtype of cutaneous autonomic nerves to account for any peripheral autonomic neuropathy.² The deposition of α -synuclein within PGP 9.5–positive nerve fibers was confirmed by merged confocal images. The percent of nerve fibers that contain α -synuclein were expressed as a percent of the total nerve density (as measured by PGP 9.5) within each nerve fiber subtype (sympathetic cholinergic or sympathetic adrenergic).² Raters were blinded to all clinical data and diagnosis.

Sensitivity and specificity. The α -synuclein ratio was calculated by nerve fiber type for each individual at every biopsy site and standard receiver operating characteristic (ROC) plots traced the tradeoff of sensitivity and specificity at different thresholds by disease (PD or control). Results are reported in aggregate and by individual biopsy site location. Data from the ROC curves were used to establish cutoff thresholds; the thresholds were not prespecified.

Statistical analysis. Statistical data analysis was performed using SPSS v17.0 (IBM; Chicago, IL). Data are presented as mean $\pm$ SD. Differences in total nerve fiber density, α -synuclein ratios, and autonomic test results are reported by group (control or PD with or without autonomic failure). Continuous variables were measured by Kruskal-Wallis tests, with Mann-Whitney *U* tests for post hoc analysis. Correlations between tests are expressed as Pearson correlations. A 2-sided *p* value of 0.05 was used to define statistical significance for all datasets (with corrections for multiple comparisons).

RESULTS Demographic information. Parkinson participants.

Twenty-eight participants with PD participated in the study. Fifteen individuals were classified as

PD-AF, 13 as PD-NAF. The demographic information for these individuals by test group is shown in table 1. Individuals with PD had a disease duration ranging from 4 months to 13 years; those with autonomic failure had longer average disease durations (4.3 years vs 8.6 years, table 1).

Control participants. Twenty-three healthy controls participated in the study. The demographic results are summarized in table 1.

Standards for reporting diagnostic accuracy studies. The flow of the patients and a summary of the biopsy and study outcomes is highlighted in figure 1.

Examination scores. Participants in this study had modified H&Y scores ranging from 1 to 3. Total MDS-UPDRS scores ranged from 19 to 105 and MDS-UPDRS motor scores ranging from 14 to 68. There was no difference in H&Y scores between those with and without autonomic failure, although MDS-UPDRS total and motor scores were higher in those individuals with autonomic failure.

Intraepidermal nerve fiber density. There was no difference in mean IENFD between participants with PD, with or without autonomic failure, and healthy control participants. However, 10 participants with PD met criteria for a small fiber neuropathy with reduced IENFD at the distal leg (5 participants in each group with and without autonomic failure). None of the control participants met criteria for small fiber neuropathy ($p < 0.001$, χ^2); results are reported in table 1.

Pilomotor nerve fiber density. There was a reduction in the PMNFD in the PD-AF group compared to control participants. The differences in PMNFD were seen at all biopsy sites ($p < 0.01$). Results are reported in table 1.

Sweat gland nerve fiber density. There was a reduction in the sudomotor nerve fiber density in the PD-AF group compared to control participants. The reductions in SGNFD were seen at the distal leg and distal thigh ($p < 0.01$), but not the proximal thigh or forearm. Results are reported in table 1.

Diagnostic discrimination of α -synuclein with and without autonomic failure. α -Synuclein was detected in all skin biopsy samples of all participants. Examples of α -synuclein deposition are shown in figure 2. The individual data points for all participants and all biopsy sites are shown in figure 3 with group data shown in table 2. Those individuals with PD-AF had the highest α -synuclein ratios ($p < 0.05$ vs controls and PD, for all biopsy sites, table 2). The PD-NAF group had greater α -synuclein deposition, and α -synuclein ratios, than control participants ($p < 0.05$ for all biopsy sites, table 2).

Sensitivity and specificity of cutaneous α -synuclein deposition. ROC curves were plotted for individual biopsy sites and with combined biopsy data. With a sudomotor α -synuclein ratio of 0.14 as a diagnostic cut-off for all biopsy sites, there was 86% sensitivity and 100% specificity to distinguish PD from control participants. By decreasing the sudomotor α -synuclein ratio threshold to 0.10, there was 94.9% sensitivity and 91.1% specificity to distinguish PD from control participants. The results for individual biopsy sites are shown in table e-1 on the *Neurology*[®] Web site at Neurology.org and the ROC curves in figure e-1.

With a pilomotor α -synuclein ratio threshold of 0.45, there was 93.9% sensitivity and 100% specificity to distinguish PD from control participants. By decreasing the pilomotor α -synuclein ratio threshold to 0.40 there was 98% sensitivity and 93% specificity to distinguish PD from control participants. A summary of all ROC curve results are included in table e-1 and figure e-1.

PD duration. The α -synuclein ratios were higher in individuals with longer duration of disease across all

Table 1 Demographic information

Characteristic	Control	PD-NAF	PD-AF	p Value (ANOVA) ^a
No.	23	13	15	
Sex, F/M	10/13	3/10	5/10	
Age, y	61.9 ± 7.4	60.1 ± 8.4	66.3 ± 7.2	NS
BMI	24.7 ± 4.5	26.2 ± 4.1	24.1 ± 2.8	NS
Disease duration	N/A	4.3 ± 5.1	8.6 ± 7.3	<0.01 ^a
MDS-UPDRS total score	0	42.3 ± 17.5	60.8 ± 21.9	<0.01 ^a
MDS-UPDRS motor (part 3)	0	28.4 ± 10.8	38.6 ± 14	<0.01 ^a
Modified Hoehn & Yahr	0	2.0 ± 0.3	2.2 ± 0.4	NS
IENFD distal leg	10.9 ± 2.6	8.3 ± 4.6	8.0 ± 4.5	NS
IENFD distal thigh	14.5 ± 3.1	13.5 ± 4.9	13.4 ± 4.2	NS
IENFD proximal thigh	20.4 ± 5.1	17.9 ± 6.5	18.3 ± 5.6	NS
IENFD forearm	18.4 ± 4.8	17.1 ± 6.2	18.2 ± 5.5	NS
SGNFD distal leg	12.5 ± 4.1	11.3 ± 3.9	9.2 ± 3.8	<0.05
SGNFD distal thigh	12.8 ± 2.5	12.0 ± 3.1	10.4 ± 2.6	<0.05
SGNFD proximal thigh	14.3 ± 4.0	15.1 ± 3.9	13.2 ± 3.7	NS
SGNFD forearm	13.4 ± 4.1	13.7 ± 5.2	12.6 ± 4.4	NS
PMNFD distal leg	52.0 ± 13.5	50.1 ± 13.6	42.0 ± 17.4	<0.01
PMNFD distal thigh	74.1 ± 17	68.3 ± 18.2	61.3 ± 12.7	<0.01
PMNFD proximal thigh	71.7 ± 19.8	66.8 ± 14.7	56.9 ± 13.2	<0.01
PMNFD forearm	81.5 ± 16.1	76.2 ± 15.8	68.0 ± 17.3	<0.01

Abbreviations: ANOVA = analysis of variance; BMI = body mass index; IENFD = intraepidermal nerve fiber density; MDS-UPDRS = Movement Disorders Society Unified Parkinson's Disease Rating Scale; PD-AF = Parkinson disease with autonomic failure; PD-NAF = Parkinson disease with no autonomic failure; PMNFD = pilomotor nerve fiber density; SGNFD = sweat gland nerve fiber density.

^aUnpaired 2-sided t tests were performed when the control group was not applicable (i.e., Unified Parkinson's Disease Rating Scale scores).

Figure 1 Standards for reporting diagnostic accuracy studies diagram

A total of 52 participants were screened and 51 participants were eligible, all of whom completed the study. One individual had a diagnosis of multiple system atrophy (MSA), not PD, so was not included in this study. The average α -synuclein (ASN) ratio across all 4 biopsy sites is reported. The results of the ASN ratio for sudomotor and pilomotor nerve fibers are reported separately.

biopsy sites in both pilomotor and sudomotor nerve fibers. The duration of PD is plotted against the pilomotor and sudomotor α -synuclein ratios in figure e-2. Seven patients with PD without AF had a diagnosis of PD for 0.5–3 years. All 7 had α -synuclein present at higher levels than control participants in sympathetic adrenergic nerve fibers (pilomotor innervation) from all 4 biopsy sites. The sudomotor nerve fibers contained elevated α -synuclein in 75% of the biopsies from this early diagnosis group.

PD severity. Among individuals with PD, the severity of PD correlated with the α -synuclein ratio. The average pilomotor α -synuclein ratio (average of all biopsy sites) had increased with the MDS-UPDRS total ($r = 0.46$, $p < 0.01$, figure e-2C), MDS-UPDRS motor subscore ($r = 0.49$, $p < 0.001$), and H&Y score ($r = 0.45$, $p < 0.05$), while the individual biopsy sites had nonsignificant correlations (correlation coefficients 0.24–0.36). The sudomotor α -synuclein ratios did not correlate with examination scores (figure e-2D).

Autonomic function tests. A detailed summary of all autonomic function testing results is provided in table 2. There were correlations noted between the α -synuclein ratio and autonomic function: the fall in systolic blood pressure on tilt-table testing was correlated with the pilomotor α -synuclein ratio at the

distal leg ($r = 0.56$, $p < 0.001$), distal thigh ($r = 0.51$, $p < 0.01$), proximal thigh ($r = 0.49$, $p < 0.01$), and forearm ($r = 0.44$, $p < 0.001$). There was a greater fall in systolic blood pressure during phase 2 of the Valsalva maneuver with greater pilomotor α -synuclein ratio at the distal leg ($r = 0.37$, $p < 0.01$), distal thigh ($r = 0.40$, $p < 0.01$), proximal thigh ($r = 0.35$, $p < 0.05$), and forearm ($r = 0.36$, $p < 0.05$). Similarly, there was diminished phase 4 blood pressure overshoot during the Valsalva maneuver with greater pilomotor α -synuclein ratio at the distal leg ($r = -0.61$, $p < 0.01$), distal thigh ($r = -0.59$, $p < 0.01$), proximal thigh ($r = -0.64$, $p < 0.01$), and forearm ($r = -0.58$, $p < 0.01$).

Autonomic symptoms. Individuals with PD-AF had greater symptoms of autonomic dysfunction than control participants: more postural lightheadedness (3.4 ± 1.8 vs 0.4 ± 0.3 controls, $p < 0.05$), constipation (6.4 ± 3.2 vs 0.8 ± 1.4 controls, $p < 0.05$), urinary frequency (4.5 ± 3.1 vs 1.2 ± 1.5 controls, $p < 0.05$), urinary urgency (4.7 ± 3.4 vs 1.7 ± 1.2 controls, $p < 0.05$), nocturia (5.9 ± 3.4 vs 2.0 ± 1.7 controls, $p < 0.05$), urinary incontinence (2.9 ± 2.2 vs 0.4 ± 0.3 controls, $p < 0.05$), erectile dysfunction (4.8 ± 2.9 vs 1.7 ± 1.7 controls, $p < 0.05$), and sweating dysfunction (4.5 ± 2.9 vs

Figure 2 α -Synuclein images

Confocal images from control participants are seen in (A-F), and from individuals with Parkinson disease (PD) in (G-L). The images in the left column are stained with the panaxonal marker protein gene product 9.5 (PGP 9.5). The center column of images is stained with α -synuclein. The right column of images is merged PGP 9.5 and α -synuclein. In (A-C), a sweat gland from a control subject is shown. In (D-F), pilomotor innervation from a control participant is seen. In (G-I), the sweat gland from an individual with PD is shown. In (J-L), the pilomotor muscle from an individual with PD is shown. The amount of α -synuclein in the PD participants (H, K) is greater than that of control participants (B, E).

1.2 ± 1.1 controls, $p < 0.05$). Patients with PD-NAF had no differences in postural lightheadedness, urinary dysfunction, sexual dysfunction, and sweating dysfunction compared to control participants. Constipation was greater in participants with PD-NAF compared to controls (3.1 ± 2.8 vs 1.2 ± 0.4 controls, $p < 0.05$).

Details of the correlations between autonomic symptoms and the α -synuclein ratios are reported in table e-2. Pilomotor α -synuclein ratios correlated more closely with orthostatic symptoms while sudomotor α -synuclein ratios correlated more closely with bowel, bladder, and sweat function.

Figure 3 α -Synuclein ratio across biopsy sites and dermal organelles

In (A), the pilomotor α -synuclein ratio (ASN) is shown across all 4 biopsy sites. Individual data points are plotted as individual circles. The 3 groups of participants include controls, Parkinson disease with no autonomic failure (PD-NAF), and Parkinson disease with autonomic failure (PD-AF). In (B), the same data are shown for the sudomotor α -synuclein ratio.

DISCUSSION In the present prospective study, we extend previously reported findings of elevated cutaneous α -synuclein deposition in individuals with PD. First, individuals with PD have significantly greater amounts of cutaneous α -synuclein deposition than control participants, even those individuals with PD and no evidence of autonomic dysfunction. α -Synuclein is deposited prominently in sympathetic adrenergic nerve fibers innervating the arrector pili muscles, but is also present in sudomotor (sympathetic cholinergic) nerve fibers, but is not detected in sensory fibers. Second, α -synuclein is present even in the early stages of disease and disease of short duration. Third, the α -synuclein ratio provides a sensitive and specific diagnostic biomarker of PD. Finally, we confirm the findings of our previous study on a new cohort.

Autonomic manifestations such as orthostatic hypotension, gastrointestinal dysfunction, and genitourinary disturbances are common in PD. While some autonomic features may precede the diagnosis, clinically evident autonomic failure (particularly orthostatic hypotension) usually occurs in the later stages of the disease. This clinical course raised concerns that cutaneous α -synuclein deposition, which occurs in the autonomic nerves in the skin, would be present only in those patients with autonomic dysfunction and in the later stages of the disease. The present data alleviate these concerns. While α -synuclein deposition is greater in patients with autonomic failure as documented by autonomic testing, it is present in patients without autonomic failure as well.

Similarly, α -synuclein deposition is present in the early stages of the disease and in PD of short duration. Indeed, the present data indicate that cutaneous α -synuclein deposition occurs in the earliest stages of the disease and, by extrapolation, suggest that deposition may be present in the premotor stages. These findings are consistent with reports of reduced cardiac uptake of meta-iodobenzylguanidine early in PD, studies that suggest the degeneration of cardiac sympathetic nerves occurs in the early stages of PD.^{6,21,22}

As reported previously,² α -synuclein deposition correlated with autonomic test results. We supplement these objective test results with questionnaire-derived autonomic symptoms. While the association of α -synuclein deposition with autonomic symptoms was not as strong as the association with autonomic test results, this is not surprising; autonomic symptoms are subjective and system-specific and may not closely reflect local biopsy results. Nevertheless, α -synuclein deposition in sympathetic adrenergic nervous system innervated piloerector muscles tended to be more strongly associated with adrenergic symptoms whereas the association with α -synuclein deposition in sympathetic cholinergic nervous system innervated sweat glands tended to be more strongly associated with cholinergic symptoms.

The present data demonstrate high sensitivity and specificity of the total α -synuclein ratio in distinguishing individuals with PD from healthy control participants. A recent systematic review reported 19% sensitivity and 80% specificity using skin biopsy

Table 2 Autonomic function and α -synuclein ratios

Test	Control	PD-NAF	PD-AF	Significance
Supine systolic blood pressure, mm Hg	124 $\pm$ 20	129 $\pm$ 11	140 $\pm$ 18 ^{a,b}	<0.01
Supine diastolic blood pressure, mm Hg	77 $\pm$ 9	78 $\pm$ 12	83 $\pm$ 13	NS
Supine heart rate, beats/min	62 $\pm$ 10	66 $\pm$ 7	73 $\pm$ 12 ^a	<0.05
Upright tilt systolic blood pressure, mm Hg	119 $\pm$ 10	115 $\pm$ 11	103 $\pm$ 26 ^{a,b}	<0.01
Upright tilt diastolic blood pressure, mm Hg	76 $\pm$ 7	74 $\pm$ 8	58 $\pm$ 12 ^{a,b}	<0.01
Upright tilt heart rate, beats/min	78 $\pm$ 11	81 $\pm$ 13	84 $\pm$ 12	NS
Fall in systolic blood pressure on tilt, mm Hg	6 $\pm$ 8	14 $\pm$ 6 ^a	37 $\pm$ 22 ^{a,b}	<0.001
Expiratory to inspiratory ratio	1.36 $\pm$ 0.10	1.19 $\pm$ 0.11 ^a	1.12 $\pm$ 0.08 ^{a,b}	<0.01
Heart rate variability to paced breathing, beats/min	15.2 $\pm$ 6.2	11.7 $\pm$ 6.6	9.1 $\pm$ 5.7 ^{a,b}	<0.05
Valsalva ratio	1.44 $\pm$ 0.20	1.65 $\pm$ 0.31 ^a	1.31 $\pm$ 0.19 ^{a,b}	<0.01
Valsalva phase 2 systolic blood pressure drop, mm Hg	-23 $\pm$ 12	-46 $\pm$ 13 ^a	-55 $\pm$ 18 ^a	<0.001
Valsalva phase 4 systolic blood pressure overshoot, mm Hg	24 $\pm$ 11	18 $\pm$ 10	6 $\pm$ 11 ^{a,b}	<0.001
Sudomotor α -synuclein ratio: distal leg	0.04 $\pm$ 0.03	0.20 $\pm$ 0.09	0.28 $\pm$ 0.12	<0.001
Sudomotor α -synuclein ratio: distal thigh	0.03 $\pm$ 0.03	0.21 $\pm$ 0.09	0.36 $\pm$ 0.18	<0.001
Sudomotor α -synuclein ratio: proximal thigh	0.03 $\pm$ 0.02	0.19 $\pm$ 0.08	0.32 $\pm$ 0.1	<0.001
Sudomotor α -synuclein ratio: forearm	0.04 $\pm$ 0.03	0.22 $\pm$ 0.11	0.33 $\pm$ 0.12	<0.001
Pilomotor α -synuclein ratio: distal leg	0.24 $\pm$ 0.09	0.71 $\pm$ 0.20	0.82 $\pm$ 0.13	<0.001
Pilomotor α -synuclein ratio: distal thigh	0.19 $\pm$ 0.08	0.74 $\pm$ 0.13	0.81 $\pm$ 0.18	<0.001
Pilomotor α -synuclein ratio: proximal thigh	0.24 $\pm$ 0.08	0.73 $\pm$ 0.16	0.78 $\pm$ 0.15	<0.001
Pilomotor α -synuclein ratio: forearm	0.22 $\pm$ 0.11	0.75 $\pm$ 0.14	0.79 $\pm$ 0.14	<0.001

Abbreviations: PD-AF = Parkinson disease with autonomic failure; PD-NAF = Parkinson disease with no autonomic failure.

^a p < 0.05 vs control.

^b p < 0.05 vs Parkinson disease (Tamhane T2 post hoc test).

analysis of α -synuclein to differentiate PD from control participants.²³ However, more recent studies using phosphorylated α -synuclein report high diagnostic sensitivity and specificity, but limited correlation with disease severity or autonomic dysfunction.^{3,24} In contrast, the α -synuclein ratio provides evidence of correlation with disease severity, duration, and autonomic dysfunction, in addition to the potential diagnostic utility. The strength of association may in part be related to the mathematical qualities of the α -synuclein ratio. It is a continuous variable whereas measures of phosphorylated α -synuclein are semi-quantitative and an ordinal variable.

We did not find an association between α -synuclein deposition and small fiber neuropathy, although small fiber neuropathy was present in a subset of both groups of individuals with PD. Similar findings have been reported by others.^{4,25,26} We have not found α -synuclein present within cutaneous sensory fibers, only autonomic nerve fibers. These data continue to support the theory that the length-dependent reduction in IENFD and the α -synuclein-associated autonomic neuropathy have different pathogenetic mechanisms.

Unlike prior investigators, we did not find a length-dependent difference in α -synuclein deposition at

proximal or distal sites. However, we normalized our values to the nerve fiber density. Therefore, it appears that α -synuclein can be reliably measured at any of the locations we tested (distal leg, distal thigh, proximal thigh, or forearm). However, a more proximal site should be included if a length-dependent autonomic neuropathy is suspected.

There are several limitations to this study. We did not measure phosphorylated α -synuclein in this study. These studies are in progress. Although we enrolled individuals with early to mid-stages of PD, we did not enroll any at-risk patients or patients with very early stage disease. This study therefore does not define the earliest stages of α -synuclein deposition in PD.

There continues to be a large medical need for a simple and repeatable biomarker for the detection of PD. The present study supports the α -synuclein ratio as a potential biomarker of PD.

AUTHOR CONTRIBUTIONS

Dr. Gibbons was involved in study design, data collection, data analysis, data interpretation, statistical analysis, and writing the manuscript. Dr. Garcia was involved in data collection, data analysis, data interpretation, and manuscript revision. Dr. Shih was involved in data collection, data analysis, data interpretation, and manuscript revision. Dr. Wang was

involved in data collection, data analysis, data interpretation, and manuscript revision. Dr. Freeman was involved in study design, data analysis, data interpretation, and manuscript revision.

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