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ORIGINAL ARTICLE

Skin Nerve Phosphorylated α-Synuclein Deposits in Parkinson Disease With Orthostatic Hypotension

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Abstract

This study aimed to investigate phosphorylated α-synuclein (p-syn) in autonomic skin nerves of Parkinson disease (PD) patients with and without orthostatic hypotension (OH). We studied 28 PD patients with normal corrected Mini-Mental State Examination including 14 patients with neurogenic OH (PD + OH) and 14 matched patients did not complain of OH (PD − OH); 7 of whom were re-evaluated over a follow-up period (4 ± 2 years). Skin biopsy was performed in proximal and distal sites. PD + OH patients showed a higher p-syn deposition than PD − OH, with widespread autonomic cholinergic and adrenergic skin nerve involvement. Over the follow-up period, PD − OH patients showed an increase in motor dysfunction scores without autonomic symptoms and a slight increase of skin p-syn deposition but still lower than PD + OH, mainly restricted to adrenergic fibers of skin vessels (SV). In summary, PD + OH patients showed a wide involvement of p-syn deposits in autonomic cholinergic and adrenergic skin nerves compared with PD − OH, and PD − OH patients showed a lower load of skin p-syn restricted to adrenergic fibers of SV still persisting over the follow-up period. The data supported a different pathogenesis between PD + OH and PD − OH and may help to identify a specific diagnostic trait for PD + OH.

Key Words: Idiopathic Parkinson disease, Orthostatic hypotension, Phosphorylated α-synuclein, Skin biopsy.

INTRODUCTION

Parkinson disease (PD) is a frequent neurodegenerative disorder characterized by neuronal misfolded α-synuclein (α-syn) deposits usually showing motor symptoms but often presenting nonmotor symptoms. Among nonmotor symptoms, neurogenic orthostatic hypotension (OH) is a major determinant of disability and is associated with increased mortality (1). OH occurs in ∼30%–40% of PD patients independent of medication (2), and is usually an early finding in the disease, developing either before, concurrent with, or soon after the onset of the motor dysfunction (3, 4). It is more likely found in older patients (5). Furthermore, a higher incidence of REM sleep behavior disorder (RBD) has been reported in PD with autonomic dysfunctions (6). OH is defined as a persistent, rather than episodic decrease in systolic blood pressure of at least 20 mmHg or diastolic blood pressure 10 mmHg within 3 minutes of standing or head-up tilt to at least 60° on a tilt table (7). Symptoms of OH may vary across patients with light-headedness, generalized weakness or fatigue, altered vision, pain in the shoulders and back of the neck ("coat hanger" phenomenon) during standing, and the rapid disappearance of these symptoms when lying down.

Because OH in PD has been reported to be more common with increasing disease duration, disease severity and L-dopa equivalent dose, the question whether PD + OH is a variant of PD or an independent clinical variant of synucleinopathy is still debated (8). Establishing whether PD + OH is an independent clinical variant (and not a possible unpredictable evolution of PD) may help the early recognition of this condition with better clinical management (e.g. identifying PD + OH may prevent falls and other autonomic complications). It may also be a predictor of survival of the underlying disorder. In fact, survival of PD + OH patients is shorter than in PD − OH patients (9). Skin biopsy is a promising and minimally invasive diagnostic tool for the in vivo detection of phosphorylated α-synuclein (p-syn) in skin autonomic nerves (10–16). P-syn is particularly expressed in α-syn deposits of patients with synucleinopathies, and its detection allows normal α-syn to be distinguished from abnormal α-syn (17). P-syn may differ in patients with and without OH, reflecting a distinct involvement of postganglionic autonomic skin fibers. Therefore, the specific aim of this study was to investigate the distribution of p-syn deposits in skin nerves and the clinical characteristics of idiopathic PD patients with OH and a matched group of PD patients without dysautonomia to ascertain possible distinct findings supporting a different pathogenesis.

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TABLE 1. Clinical and Demographic Data of Patients

PD + OH	Age (Years)	Sex Male: Female	DD (Years)	UPDRS-III	H&Y	Aut. Symp Start* (Years)	Aut. Symp	MMSEc	L-Dopa mg/die		RBD	DatScan	MIBG	M.Sub.
1	76	F	6	30	2	1	OH	27	400		Present†	Ab	Ab	T
2	74	M	13	30	2	6	OH, UI, SL	27	500		Present	Ab	NP	both
3	81	M	22	45	5	3	OH	28	650		Present	Ab	NP	A
4	82	M	4	16	1	1	OH	24.4	300		Present	Ab	Ab	both
5	71	F	6	25	2	1	OH	24.7	600		–	Ab	NP	both
6	78	M	5	30	2	3	OH	26	250		Present	Ab	Ab	A
7	75	M	3	20	1	5	OH, UI	28	0		Present	Ab	Ab	A
8	73	M	12	30	2	2	OH, UI	25.4	800		Present	Ab	Ab	A
9	75	M	15	40	3	5	OH	24.7	200		Present	Ab	NP	A
10	68	F	9	23	1, 5	4	OH	26.2	750		Present	Ab	NP	A
11	72	M	14	40	2, 5	2	OH	26.4	300		–	Ab	Ab	A
12	82	M	12	35	2	3	OH, ID, UI, SL	27	750		Present	Ab	Ab	Both
13	72	M	8	25	1, 5	0	OH	25	600		Present†	Ab	Ab	T
14	66	F	8	35	2, 5	2	OH	26.2	750		Present	Ab	Ab	A
Mean ± SD	75 ± 5	10:04	10 ± 5	30 ± 8	2 ± 1	3 ± 2		27 ± 1	489 ± 249	%	**86**§	100	100	57‡
PD – OH														
1	64	M	2	14	1	–	None	27.5	100		–	NP	Ab	A
2	59	M	1	15	1.5	–	None	27	100		Present	Ab	Ab	T
3	79	F	10	22	2	–	None	25	800		–	Ab	NP	A
4	72	F	2	33	2	–	None	26	400		Present	Ab	Ab	T
5	78	M	3	14	1.5	–	None	26.5	200		–	Ab	Ab	T
6	60	M	13	16	2.5	–	None	29	750		Present	Ab	Ab	T
7	64	M	3	11	1	–	None	27.5	300		Present	Ab	Ab	A
8	82	M	17	28	2	–	None	27.7	300		–	Ab	Ab	A
9	78	F	5	30	2	–	None	25	800		–	Ab	NP	A
10	74	M	10	41	4	–	None	25.4	650		–	Ab	NP	A
11	77	M	12	17	1	–	None	26	250		–	Ab	Ab	A
12	64	F	14	37	2.5	–	None	26.7	800		Present	Ab	NP	A
13	78	M	14	30	3	–	None	28.7	850		–	Ab	NP	T
14	73	M	25	32	3	–	None	29.3	600		–	Ab	NP	A
Mean ± SD	72 ± 8	10:04	10 ± 7	24 ± 10	2 ± 1	–	–	26 ± 2	493 ± 284	%	36	100	100	64‡

DD = disease duration; H&Y = Hoehn and Yahr stage; Aut. symp. = utonomic symptom; OH = orthostatic hypotension; ID = impotence dysfunction; SL = sweat loss; UI = urinary incontinence.

* = age from onset of motor symptoms; MMSEc = corrected Mini-Mental State Examination; RBD = REM sleep behavior disorder.

† = patients in whom RBD came first of parkinsonism; DatScan = nigrostriatal dopamine transporter ligand [123I]ioflupane-DatScan; MIBG = cardiac uptake of [123-I])-MIBG; M.Sub. = parkinsonism motor subtypes; T = tremor subtype; A = akinetic subtype; Ab = abnormal; N = normal; NP = not performed.

‡ = percentage of incidence of akinetic variant.

§ = $p < 0.001$ (PD + OH vs PD – OH) using the Mann-Whitney test and $p = 0.09$ after correction for multiple comparisons according to the Bonferroni method (14×).

MATERIALS AND METHODS

We studied 28 idiopathic PD patients showing a late-onset disorder (>45 years old), asymmetric motor signs (i.e. rigidity, bradykinesia and/or resting tremor), no family history and good control of motor symptoms by L-dopa alone or in combination with dopamine agonists (18). OH was not considered a red flag for the diagnosis of PD since this work was focused on PD with dysautonomia. The PD clinical diagnosis was supported by specific abnormal tests such as nigrostriatal dopamine transporter ligand (123I)ioflupane-DatScan and/or cardiac uptake of (123-I)-MIBG (19). Table 1 summarizes the demographic data and clinical profiles of the patients in our study. The patient group included: (i) 14 subjects with neurogenic OH (PD + OH) objectively defined by a decrease in systolic or diastolic blood pressure of at least 20 or 10 mmHg, respectively, without significant heart rate changes caused by a head-up tilt at 65° and absent blood pressure overshoot during Valsalva maneuver (7, 20). The tilt test was performed in the morning before taking L-dopa and dopamine agonists to rule out pharmacological hypotension. The other autonomic symptoms were reported by patients during the clinical interview (Table 1). PD + OH patients were prospectively recruited in 3 different Italian centers providing healthcare diagnosis and treatment of synucleinopathies: IRCCS of Bologna, IRCCS of Milano and IRCCS of Pozzilli (Isernia, Italy). Inclusion criteria were the

TABLE 2. Clinical Data over the Follow-up Period in PD Patients Without OH

Patient	Years	Change from baseline					
		UPDRS-III	H&Y	LDOPA (mg/die)	UPDRS-III (%)	H&Y (%)	LDOPA (%)
1	3	10	1.5	300	71	150	300
2	2	6	0.5	200	40	50	40
3	6	15	2	500	68	100	71
4	7	26	3	200	79	150	50
5	2	10	0.5	200	71	33	200
6	3	0	0	0	0	0	0
7	4	20	1.5	400	182	150	400
Mean ± SD	**4 ± 2**	**12 ± 9**	**2 ± 1**	**258 ± 162**	**73 ± 55**	**90 ± 63**	**152 ± 152**

The number of patients corresponds to PD – OH patients reported in Table 1.
H&Y = Hoehn and Yahr stage.

diagnosis of idiopathic PD, supported by DatScan and/or MIBG, and objectively defined OH. None of the selected patients for this study refused to participate; (ii) 14 patients did not report autonomic symptoms during the clinical interview and showed no OH within 3 minutes of standing measurement (PD – OH). These patients were selected from a local database (IRCCS of Bologna) to match the PD + OH group in terms of age, disease duration and motor involvement (i.e. UPDRS-III and H&Y scores); some of them have been previously described (11, 12). We had the chance to re-evaluate 7 of these patients after follow-up (Table 2). Corrected Mini-Mental State Examination (MMSE) for age, sex, and education was normal (>24) in all patients, making a global cognitive impairment less likely (21). RBD was first tested by a clinical interview and, if reported by the bed partner, was confirmed by a polysomnography showing REM sleep without atonia. Motor subtypes were defined by using UPDRS-based criteria in tremor dominant, akinetic, and mixed subtypes (22). Serum screening for diabetes, microbiological, autoimmune, paraneoplastic and thyroid disorders, and vitamin B12 deficiency was negative, excluding predisposing causes for peripheral neuropathy. The procedures used complied with the Helsinki Declaration regarding international clinical research involving human beings. The local Human Ethics Committee approved the study and all subjects gave their written informed consent to the study.

Skin Biopsy

Following a previously described protocol, 3-mm punch biopsies were taken from proximal and distal hairy skin sites (11, 12). The proximal site included the cervical C7 paravertebral area (close to the spinal ganglia), whereas distal sites were the thigh (15 cm above the patella) and distal leg (10 cm above the lateral malleolus). Two samples were taken in each skin site 3–4 cm away, which is useful to ascertain the widespread spatial distribution of abnormal α-syn deposits (10–12). According to previously published procedures (23), skin samples were immediately fixed in cold Zamboni's fixative and kept at 4°C overnight. Skin sections were obtained using a cryostat (HM550, Thermo Scientific, Waltham, MA).

Skin Innervation

Fifty-micrometer-thick sections were obtained during the cryostat session. Twelve free-floating sections were incubated overnight with a panel of primary antibodies, including the pan-neuronal marker protein gene product 9.5 (rabbit PGP, 1:500; Abcam, Cambridge, UK, cat. no. ab108986 or mouse PGP, 1:750; Abcam, cat. no. ab72911), mouse collagen IV (ColIV, 1:800, Chemicon, Temecula, CA, cat. num. MAB1910) and autonomic markers like rabbit tyrosine-hydroxylase ([TH], 1:1000, Novus Biologicals, Littleton, CO, cat. no. NB300-109) to identify the noradrenergic fibers and rabbit vasoactive intestinal peptide ([VIP], 1:1000, Incstar, Stillwater, MN) colocalized in sudomotor cholinergic fibers (23). Sections were then washed and secondary antibodies labeled with mouse Alexa Fluor(R) 488 (1:400; Jackson ImmunoResearch, West Grove, PA, cat. no. 715-545-150) and rabbit cyanine dye fluorophores 3.18 (1:200 when double-stained with p-syn or 1:800 double-stained with the remaining primary antibodies, Jackson ImmunoResearch; cat. no. 711-165-152) were added for overnight incubation. Sections were initially viewed under a Zeiss fluorescent microscope (model Axioskop 40; Jena, Germany). Autonomic innervation density was quantified using the previously described automated technique known as the "unsharp mask filter," which creates a composite image by subtracting the background color in the out-of-focus image from the base image expressing the autonomic innervation staining (Image Pro Plus, Media Cybernetics, Rockville, MD) (23). Target autonomic structures included skin vessels (SV) and muscle arrector pilorum (MAP) mainly expressing adrenergic nerve fibers and sweat glands (SG) expressing cholinergic fibers (23). Because of the highly variable pattern of innervation in SV, the autonomic innervation was only quantified in SG and MAP by using the PGP signal providing a stronger staining. This was easier to quantify than the specific autonomic markers, but it was correlated with the innervation quantified by the specific autonomic markers (23). The autonomic innervation score was usually expressed as the percentage area of PGP staining in 2 or 3 different cholinergic or adrenergic target structures identified by ColIV staining for each skin site. Intraepidermal nerve fiber density was calculated by considering a single epidermal

TABLE 3. Skin Innervation Scores

	Skin Innervation Scores								
	Leg			Thigh			Cervical		
Patient	*ENFs*	*SG*	*MAP*	*ENFs*	*SG*	*MAP*	*ENFs*	*SG*	*MAP*
	mm	FD %	FD %	mm	FD %	FD %	mm	FD %	FD %
PD + OH	**4 ± 2***	**7 ± 2***	**6 ± 3***	8 ± 4	11 ± 3	11 ± 3	19 ± 6	10 ± 1	14 ± 3
PD – OH	7 ± 2	9 ± 2	8 ± 2	10 ± 4	11 ± 2	10 ± 3	19 ± 8	10 ± 1	13 ± 3

ENFs = Epidermal nerve fiber density; SG = sweat glands; MAP = muscle arrector pilorum; FD = fiber density.
*$p < 0.05$ (PD + OH vs PD – OH) using the Mann-Whitney test but the significant value was lost after correction for multiple comparisons according to the Bonferroni method (14×).
In bold values showing a significant difference between PD + OH and PD − OH.

nerve fiber marked by PGP crossings of the dermal-epidermal junction stained by ColIV.

P-syn Deposits

Additional 10-μm-thick sections from the same skin sample were obtained to evaluate α-synuclein deposits (10–12). They were double-immunostained overnight with a panel of primary antibodies including rabbit monoclonal p-syn at Ser 129 (p-syn; 1:500, Abcam, cat. num. ab-51253) or mouse p-syn (1:4000, BioLegend, San Diego, CA, cat. no. 825701), mouse or rabbit PGP, rabbit TH and rabbit VIP. Sections were then washed and secondary antibodies (i.e. anti-mouse Alexa Fluor(R) 488 or rabbit cyanine dye fluorophores 3.18) were added for a 1-hour incubation at room temperature. The microscope analysis and criteria to determine p-syn positivity were previously described (10–12). Shortly, sections were initially viewed and analyzed under a Zeiss fluorescent microscope. The correspondence between rabbit p-syn and mouse PGP staining helped to verify the intraneuronal deposits excluding possible non-specific staining arising from the background. The analysis was made in a blinded fashion by 2 authors with expertise in immunofluorescent analysis (D.V. and I.A.). P-syn staining was rated in each skin site as the percentage of autonomic structures or nerve bundles showing a positive staining at high magnification (400×). For 3D colocalization analysis of mouse p-syn with specific autonomic markers (i.e. rabbit TH or VIP), digital images were also acquired using a laser-scanning confocal microscope (Leica DMIRE 2, TCS SL, Leica Microsystems, Heidelberg, Germany). Each image was collected in successive frames of 1–2-μm increments on a Z-stack plan at the appropriate wavelengths for the fluorophores coupled with secondary antibodies with a 200× or 400× plan apochromat objective and subsequently projected to obtain a double-stained 3D digital image by a computerized system (LCS lite, Leica Microsystems).

Statistical Analysis

Statistical analyses were performed using SPSS 24.0 for Windows. To test whether significant intergroup differences occurred, we used Mann-Whitney *U* test for the analysis of continuous variables and χ^2 test for categorical variables. The resulting p values were corrected for multiple comparisons according to Bonferroni's method (17×). For all analyses, significance was assumed with $p < 0.05$. As it was an exploratory analysis, we searched for correlation between the change in p-syn deposits and clinical scores during the follow-up using Spearman test.

RESULTS

Clinical Characteristics

PD + OH patients developed autonomic symptoms after a mean of 3 years from the onset of motor symptoms; the disease started with OH in only 1 patient. PD + OH showed a higher incidence of RBD than PD – OH, whereas motor subtypes did not differ between these 2 groups with akinetic as prevalent subtype, although PD + OH more frequently presented with a mixed subtype (Table 1). No significant differences of L-dopa dosage were found between the 2 groups. The mean duration of follow-up in PD – OH patients (4 years) was comparable to the time window needed to develop autonomic symptoms in the PD + OH patients. Over the follow-up period, PD – OH patients showed a motor worsening with increase of UPDRS-III and HY scores and L-dopa dosage (Table 2) without occurrence of autonomic symptoms. Recruited patients did not complain of hallucinations, drug sensitivity or fluctuating attention or alertness.

Skin Innervation

Leg somatic and autonomic innervations were decreased in PAD+OH compared with PD – OH, although they did not reach statistical significance using Bonferroni's correction. No differences were found in the thigh and cervical sites between the 2 groups (Table 3). The re-evaluation of PD – OH patients over the follow-up showed a slight decrease of distal epidermal (leg: 7 ± 3 vs 5 ± 2 mm; thigh: 10 ± 6 vs 9 ± 4 mm) and autonomic (leg SG: 9 ± 3 vs 8 ± 2 FD%; leg MAP: 9 ± 2 vs 6 ± 5 FD%; thigh SG: 9 ± 3 vs 8 ± 2 FD%; thigh MAP: 10 ± 2 vs 7 ± 2 FD%) innervations. No differences were found in C7 for both somatic (19 ± 9 vs 17 ± 6 mm) and autonomic (GH: 10 ± 1 vs 10 ± 1 FD%; MAP: 13 ± 1 vs 15 ± 6 FD%) innervations.

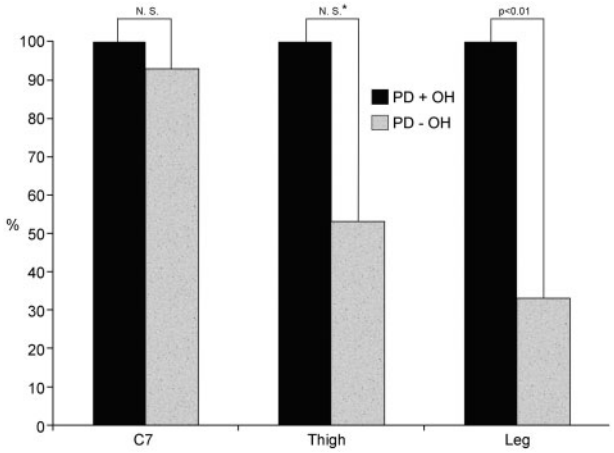

FIGURE 1. Distribution of phosphorylated α-synuclein among different skin sites in PD + OH and PD – OH patients. Percentage of 2 nearby p-syn-positive skin samples in PD + OH and PD – OH patients. The 2 groups of patients showed pronounced differences in the p-syn staining between proximal and distal skin sites. PD + OH patients showed a homogeneous distribution of p-syn deposits between proximal and distal skin sites whereas a proximal-distal gradient (with highest positivity in C7) was evident in PD – OH. After Bonferroni's correction, only the leg site presented a significant difference, whereas a significant difference in the thigh (asterisk, $p = 0.006$) was lost after the correction. These data supported a centrifugal spread of p-syn along peripheral nerves in PD – OH but a widespread involvement of skin samples in PD + OH.

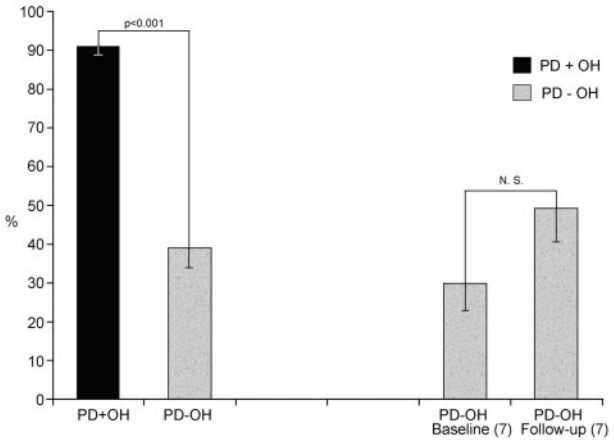

FIGURE 2. Phosphorylated α-synuclein deposits load in PD + OH and PD – OH both at baseline and during the follow-up. The mean amount of p-syn deposits in all analyzed skin samples was markedly higher in PD + OH than PD – OH (still significant after Bonferroni's correction). During the PD – OH follow-up, p-syn deposits were slightly increased in skin samples but the final mean amount of deposits was far less than that of PD + OH (the number in brackets indicates the number of patients included in the analysis). Bar = standard error.

P-syn Deposits

Abnormal p-syn deposits in skin nerves showed pronounced differences between the 2 groups of PD patients. PD + OH patients displayed a homogeneous distribution of p-syn deposits between proximal and distal skin sites, whereas PD – OH patients showed a proximal-distal gradient (with highest positivity in C7) as already described in PD (Fig. 1). The percentage of skin samples showing p-syn deposits was markedly higher in PD + OH (90% of all analyzed skin samples) than PD – OH (38%; corrected $p < 0.001$) (Fig. 2). Furthermore, p-syn deposits were differently expressed in autonomic annexes in the 2 groups of PD patients since PD + OH showed a widespread involvement of cholinergic and noradrenergic autonomic fibers (i.e. SG, MAP and skin plexuses; PD + OH vs PD – OH corrected $p < 0.05$), whereas PD – OH presented a prevalent involvement of adrenergic fibers to SV comparable to PD + OH ($p > 0.8$; Figs. 3, 4). Over the follow-up period the mean amount of skin samples positive for p-syn slightly increased (from 34% to 52%) in PD – OH but never reached the level of PD + OH (Fig. 2). The p-syn increase was not correlated with UPDRS-III, HY or L-dopa changes, and abnormal deposits were still mainly found in adrenergic fibers around SV.

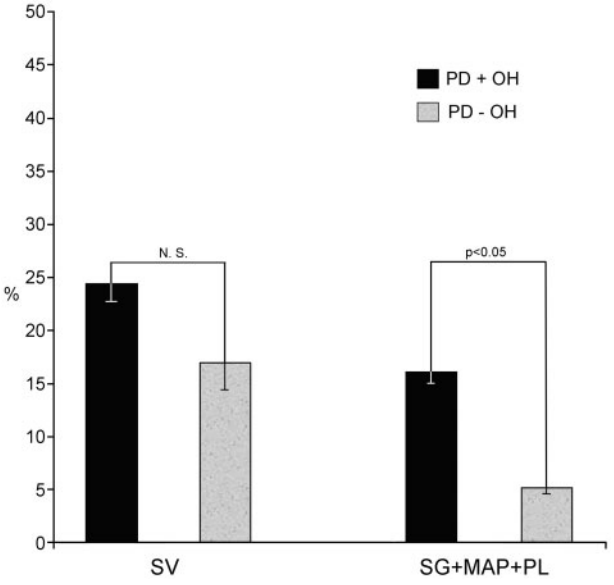

FIGURE 3. Distribution of intraneural p-syn deposits in skin annexes. The pattern of p-syn distribution among skin annexes disclosed a similar mean amount of deposits around skin vessels (SV) in PD + OH and PD – OH (corrected $p > 0.8$). In contrast, mean abnormal p-syn deposits were significantly higher in sweat glands (SG), muscle arrector pilorum (MAP), and skin plexuses in PD + OH compared with PD – OH. These results underlined how PD + OH was characterized by the widespread extension of deposits in cholinergic and adrenergic autonomic nerves differently from PD – OH demonstrating a prevalent restricted involvement of SV adrenergic fibers. Bar = standard error.

DISCUSSION

Our main results are as follows: (i) PD + OH showed a wide involvement of p-syn deposits in cholinergic and

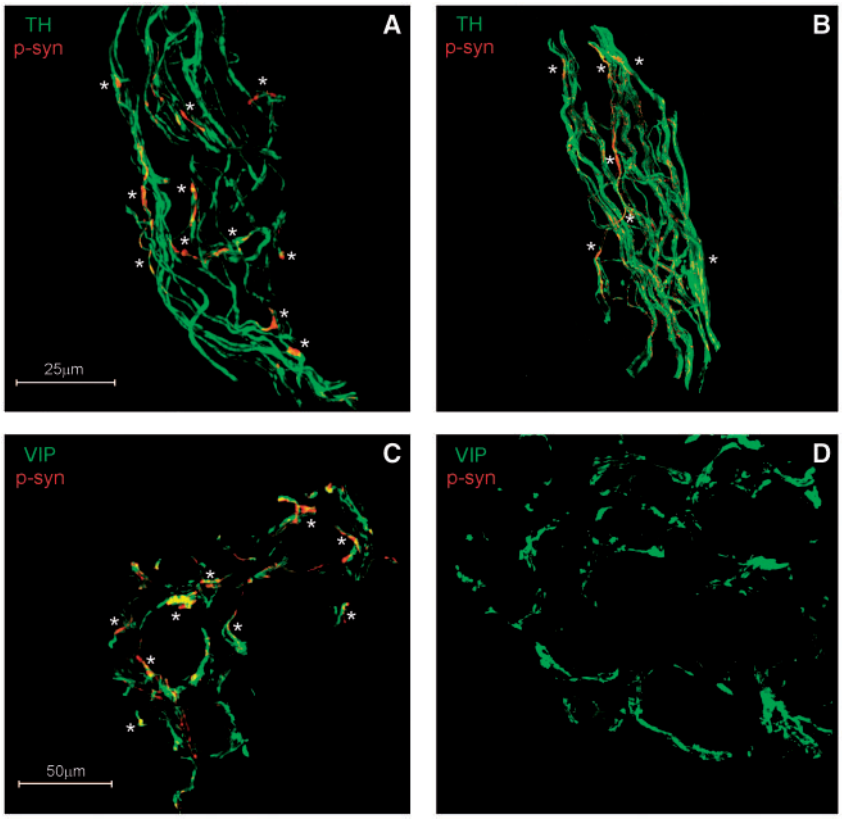

FIGURE 4. P-syn deposits in cholinergic and adrenergic autonomic nerve fibers. Confocal microscope (400×) study of p-syn deposits in autonomic nerves of skin vessels and sweat glands of a patient with PD + OH **(A, C)** and a patient with PD – OH **(B, D)**. **(A)** Adrenergic TH-positive fibers (green) around a dermal arteriole and abnormal p-syn deposits (red) in a patient with PD + OH. This merged image showed that different autonomic adrenergic fibers presented abnormal p-syn deposits (asterisks); **(B)** similar findings were found in a skin arteriole of a patient with PD – OH; **(C)** cholinergic VIP-positive fibers (green) around a sweat gland and abnormal p-syn deposits (red) in a patient with PD + OH. Most of these fibers demonstrated the presence of p-syn as neuritic inclusions (asterisks); **(D)** in contrast cholinergic VIP-positive fibers (green) around a sweat gland of a patient with PD – OH did not show neuritic p-syn inclusions.

adrenergic autonomic skin nerves and higher incidence of RBD compared with PD – OH; and (ii) PD – OH showed a lower load of skin p-syn mainly restricted to adrenergic fibers of SV still persisting over a follow-up, despite a worsening of motor performances.

PD + OH showed a different skin p-syn load and a more peculiar clinical picture than PD – OH. Our data disclosed a widespread spatial diffusion of skin nerve p-syn deposits in PD + OH patients with nearly all analyzed skin samples showing abnormal deposits without difference between proximal and distal skin sites. This pattern is markedly different from that found in PD – OH showing a classical proximal-distal gradient with higher deposits in C7, which may support a centrifugal spread of p-syn along peripheral nerves (11, 12). Furthermore, the total amount of p-syn deposits in analyzed skin samples was lower in PD – OH than PD + OH, with more than half of skin samples showing no abnormal deposits. In addition, abnormal deposits in PD + OH also showed a widespread diffusion among adrenergic and cholinergic fibers compared with PD – OH, showing abnormal deposits mainly restricted to adrenergic fibers of SV. These data supported the higher load of p-syn deposits in the peripheral innervation of PD + OH compared with PD – OH patients and may help to identify a specific diagnostic trait for PD + OH patients, although the same widespread load of p-syn deposits was found in other synucleinopathies displaying OH (10, 13). Skin autonomic innervation is not likely functionally relevant for the blood pressure regulation and its failure is not sufficient to explain the functional deficit underlying OH, but our data underlined how a widespread inclusion of p-syn in skin nerves likely reflects a diffused involvement of other peripheral sympathetic branches, that is, splanchnic, cardiac and renal, explaining the functional deficit yielding to OH (24). In agreement with this conclusion, baroreflex sympathoneural failure is usually more marked in PD + OH than PD – OH (5). In addition, even the clinical picture of PD + OH patients showed peculiar characteristics differently from PD – OH. The incidence of RBD is higher in PD + OH than PD – OH, supporting previous studies demonstrating a strong association between RBD and the autonomic failure in synucleinopathies (6, 25). This finding underlined how the pathophysiology of RBD and autonomic dysfunctions are linked although the underlying

mechanisms are still unclear. A possible causative relationship could be related to the regulation of the REM sleep muscle atonia circuits by the autonomic neural systems since important autonomic central network centers such as hypothalamus, thalamus and basal forebrain are also able to modulate REM sleep regulation (25). Alternatively, RBD in PD promotes autonomic dysfunctions by sharing a common anatomical pathway with the widespread deposition of α-syn in nearby brainstem nuclei controlling RBD, that is, coeruleus/subcoeruleus complex, and autonomic functions, that is, reticular formation (26). This last hypothesis was strengthened by the disclosure of a greater amount of α-syn deposits in the brain of PD patients with RBD suggesting that RBD may indicate a diffuse synuclein-driven pathophysiology (27). Our PD + OH patients showed normal cognition supporting the independence of autonomic failure from cognitive decline in PD, unlike previous studies showing that both dysfunctions may coexist (28). The diagnosis of dementia with Lewy bodies in our PD + OH patients was unlikely because of normal corrected MMSE and absent fluctuating attention or alertness, hallucinations and drug sensitivity considered the diagnostic core of this disease. Multiple system atrophy was also unlikely because abnormal cardiac innervation and a good control of motor symptoms by L-dopa and dopamine agonists treatments. Unlike a previous study (28), we did not find a prevalent motor subtype in PD + OH but our investigated group of patients is rather small. To this end, a skin biopsy study involving a larger cohort of PD + OH patients is needed before establishing a prevalent motor subtype in PD + OH or a possible causal relationship between autonomic and cognitive dysfunctions in PD.

However, taken together, our results demonstrated that PD + OH patients showed pronounced differences in p-syn distribution with a different RBD incidence compared with PD − OH, underlining that these 2 disorders likely presented a different pathogenesis. Accordingly, PD + OH could not be included in the spectrum of idiopathic PD, possibly representing an independent clinical variant of synucleinopathy, although a larger study involving a higher number of patients is needed to support this conclusion. In addition, reproducibility data of the method used (i.e. immunofluorescence analysis of p-syn deposits) are currently lacking; however, we have recently demonstrated excellent intra and the interlaboratory reproducibility in 2 laboratories with a major expertise in this analysis supporting the reliability of this technique (Donadio et al, manuscript submitted).

PD − OH showed a lower load of skin p-syn without autonomic symptoms over follow-up, despite a worsening of motor performances. A group of PD − OH patients were followed for a period sufficient to develop autonomic symptoms in PD + OH. Nevertheless, the PD − OH demonstrated a slight increase of p-syn load in skin samples over the follow-up period that was much lower than the load displayed by PD + OH. Skin autonomic fibers involved by abnormal deposits in PD − OH were mainly restricted to the adrenergic fibers of SV even during the follow-up. In addition, PD − OH patients did not complain of autonomic symptoms or OH over the follow-up. These data agree with the hypothesis (see above) that OH is associated in PD with a widespread p-syn involvement of adrenergic and cholinergic autonomic skin fibers (10, 12). During the follow-up period, the progression of motor dysfunctions was not correlated to the increase of p-syn deposits in skin nerves, suggesting that central nervous system involvement independently progresses from dysfunctions of the peripheral nervous system in idiopathic PD − OH, as previously described (29). In addition, motor scores worsening over the follow-up (∼70%–90%; Table 2) were higher than the increase of p-syn deposits in skin samples (18%; Fig. 2), supporting the prevalent involvement of the central nervous system and a relative sparing of peripheral innervation in PD − OH differently from PD + OH. Once again, these data underlined that PD − OH likely shows a different pathogenesis than PD + OH. The underlying mechanisms are poorly understood but could be related to a specific genetic profile of the patients (i.e. host) predisposing the deposition of misfolded aggregates of α-syn (30), or the specific deposition in more vulnerable neurons according to the recent "threshold theory" (31).

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