

RESEARCH ARTICLE

Parkinson disease and progressive supranuclear palsy: protein expression in skin

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Abstract

Objective: This study characterizes the expression of tau (p-tau) and α -synuclein (α -syn) by immunohistochemistry in the skin of three different populations: healthy control (HC), Parkinson disease (PD), and progressive supranuclear paralysis (PSP) subjects, with the purpose of finding a biomarker that could differentiate between subjects with PD and PSP. **Material and Methods:** We evaluated the presence of p-tau and α -syn in a pilot study in the skin of three distinct groups of patients: 17 healthy subjects, 17 patients with PD, and 10 patients with PSP. Four millimeters punch biopsies were obtained from the occipital area and analyzed by immunohistochemistry using antibodies against α -syn and phosphorylated species of tau. PHF (paired helical filaments) antibody identifies p-tau in both normal and pathological conditions and AT8 recognizes p-tau characteristic of pathological conditions. Differences between the three groups were assessed by quantification of immunopositive areas in the epidermis. **Results:** The immunopositivity pattern of p-tau and α -syn was significantly different among the three groups. Healthy subjects showed minimal staining using AT8 and α -syn. The PD group showed significantly higher α -syn and AT8 immunopositivity, while the PSP group only expressed higher AT8 immunopositivity than HCs. **Conclusion:** These data suggest that the skin reflects brain pathology. Therefore, immunohistochemical analysis of p-tau and α -syn in the skin can be useful for further characterization of PD and PSP.

Introduction

Neurodegenerative disorders seem to share a common pathway with the presence of misfolded proteins in the central nervous system (CNS). Patients who present with neurodegenerative forms of parkinsonism can be broadly classified as suffering from either synucleinopathies or tauopathies.¹ Synucleinopathies are characterized by the presence of stable unfolded oligomers or multimers of α -syn, the main protein component of Lewy bodies and Lewy neurites that represent the defining hallmark of

Parkinson disease (PD).² Tauopathies are disorders associated with intracellular deposition of abnormally phosphorylated tau (p-tau), found as neurofibrillary tangles (NFT), neuropile threads, or abnormal tau filaments. Progressive Supranuclear Palsy (PSP) is a tauopathy, and the most common form of atypical parkinsonism (AP).³

PD is a clinical condition with the four cardinal manifestations of parkinsonism (tremor, rigidity, akinesia/bradykinesia, and postural instability) together with other nonmotor manifestations (hyposmia, sleep disorders, dysautonomia, and neuropsychiatric symptoms).⁴ Frequent

falls, supranuclear vertical gaze palsy, and executive cortical frontal dysfunction characterize PSP. Cognitive or behavioral deficits may be present at onset, but the main manifestation is parkinsonism. Clinically, PD and PSP can often be difficult to distinguish particularly early in the course of the disease when potential disease modifying agents may be more likely to be effective.⁵ Thus, potential biomarkers that would allow for a precise diagnosis of proteinopathies have been investigated in body fluids and peripheral tissues (salivary glands, peripheral nervous system, and skin).^{6–8} To date, there is no universally accepted diagnostic marker. Therefore, we sought to analyze the presence of p-tau and α -syn in the skin of patients with PSP and PD.

Material and Methods

Patient selection

The Ethics and Research Committee from the Central Hospital of San Luis Potosí, Mexico approved the study and the consent forms. The patients were recruited from the Neurology Department of Central Hospital in San Luis Potosí, Mexico and from the Instituto Nacional de Neurología y Neurocirugía in Mexico City. The inclusion criteria were patients with a diagnosis of PSP according to the National Institute of Neurological Disorders and Stroke and Society for Progressive Supranuclear Palsy (NINDS-SPSP) criteria⁹ or PD according to the Queen Square Brain Bank criteria.⁴ Previously, we reported the presence of α -syn inclusions in the skin of 34 PD patients and 33 parkinsonism patients, including three PSP patients.¹⁰ Only one of the PSP patients who participated in that study was biopsied again for this work, and from the previously studied PD patients, four provided new samples. The age-matched control group consisted of neurologically healthy subjects that agreed to participate in the study and all patients signed the informed consent. Neuroimaging was performed for all the patients. Patients with vascular lesions, hydrocephalus, or other form of parkinsonism were excluded from the protocol. The participants were biopsied with a 4 mm punch, 3 cm behind the ear insertion, after local anesthesia and aseptic cleaning of the region, followed by compression for hemostasis. The skin samples were processed for immunohistochemistry. All the patients were evaluated in the Hoehn–Yahr and UPDRS scales without previous levodopa administration and all the participants were measured by mini-mental state examination (MMSE).

Immunohistochemistry

All the reagents were analytical grade and molecular grade water (18.2 Ω /cm) was employed. The biopsies were immersed in 0.1 mol/L phosphate buffer containing

4% paraformaldehyde during 24 h and embedded in paraffin. Coronal 5 μ m sections were collected in electro-charged slides (Biocare Medical LLC, Concord, CA). Then, the sections were dewaxed by heating (60°C, 10 min), followed by xylene and ethanol rinses, and rehydrated. For epitope recovery, slides with three tissue sections each were submerged in DIVA decloaker solution (Biocare Medical, LLC, Concord, CA) and placed in a pressure cooker during 30 min followed by a 20 min cooling for 20 min at room temperature. After three rinses with distilled water, endogenous peroxidase was depleted incubating the sections 15 min with 3% hydrogen peroxide. Sections were then subjected to subsequent incubation steps (15 min each) in a humidity chamber at room temperature to block nonspecific background staining (Background sniper; Biocare Medical, LLC, Concord, CA) and endogenous biotin and biotin-binding proteins (avidin/biotin blocking kit; Vector Laboratories Inc, Burlingame, CA) followed always by rinses with TBS-tween.

The antibodies selection was based in previous literature reports and earlier work from our group.^{10,11} Two phosphorylated species of tau were tested. PHF (paired helically filaments) monoclonal antibodies identify aminoacids around the phosphorylation site Ser396 (ABCAM, Cambridge, MA), which is immunopositive in both normal and pathological conditions. AT8 recognizes the site Ser202/Thr205 (Thermo Scientific, Rockford, IL), which is phosphorylated mostly in pathological conditions. Also, a polyclonal and a monoclonal anti α -syn antibody were used (Thermo Scientific, Rockford, IL and Becton, Dickinson and Co. Franklin Lakes NJ, USA). Primary antibodies were incubated for 1 h, followed by the streptavidin–biotin marked secondary antibody (DAKO, Carpinteria, CA) for 15 min. Peroxidase activity was visualized by incubating the sections with amine-ethylcarbazole to obtain a red coloration, and counterstained with Harris hematoxylin. Negative controls consisted of tissue sections treated without the primary antibody. The sections were viewed with an Olympus microscope equipped with a digital camera (AmScope, Irvine, CA). For immunofluorescence, the primary antibodies were incubated overnight at 4°C, and the secondary antibodies used were a goat anti-mouse IgG antibody marked with Alexa Fluor 488 and a goat anti-rabbit IgG antibody marked with Cy5 (Molecular Probes, Eugene, OR). Nuclei were visualized with an orange nucleic acid stain, Sytox (Molecular Probes, Eugene, OR). The samples were analyzed with a confocal microscope (LEICA TCS SP2; Leica Microsystems GmbH, Heidelberg, Germany). Brain tissue from patients with clinical and pathological diagnosis of PD (Lewy bodies) or Alzheimer Disease (NFT) was employed as positive control of immunohistochemical analysis.

Western blot

A quantity of 50 mg of rat brain, human brain obtained from a parkinsonism patient (normal pressure hydrocephalus), and human skin were processed by homogenization with a lysis buffer (0.025 mol/L Tris, 0.15 mol/L NaCl, 0.001 mol/L Ethylenediamine tetraacetic acid (EDTA), 1% NP-40, and 5% glycerol) at pH 7.4 and supplemented with a protease and phosphatase inhibitor cocktail (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Supernatants were recovered by centrifugation at $12,000 \times g$ 10 min. Total protein was quantified by Bicinchoninic acid protein assay (BCA) assay obtaining 150–500 μg of total proteins. About 50 to 80 μg were diluted 1:1 in 2X Laemmli buffer supplemented with 10%

Table 1. General characteristics of the population.

	Control	PD	PSP
Males/females	11/6	13/4	3/7
Age, years (mean \pm SD)	71 \pm 14	68 \pm 9	71 \pm 8
Time of evolution (mean \pm SD)	–	6.1 \pm 3.8	5.7 \pm 3.1
MMSE score (mean \pm SD)	30	25 \pm 5* [†]	16.7 \pm 7*
Hoehn–Yahr (mean \pm SD)	–	3.5 \pm 0.8	4.3 \pm 0.9 [‡]
UPDRS (mean \pm SD)	–	68 \pm 35	97 \pm 41

PD, Parkinson disease; PSP, progressive supranuclear palsy; MMSE, mini-mental state examination.

* $P < 0.01$ versus controls, [†] $P < 0.01$ versus PD, ANOVA followed by Tukey test.

[‡] $P < 0.05$ versus PD, Student *t*-test.

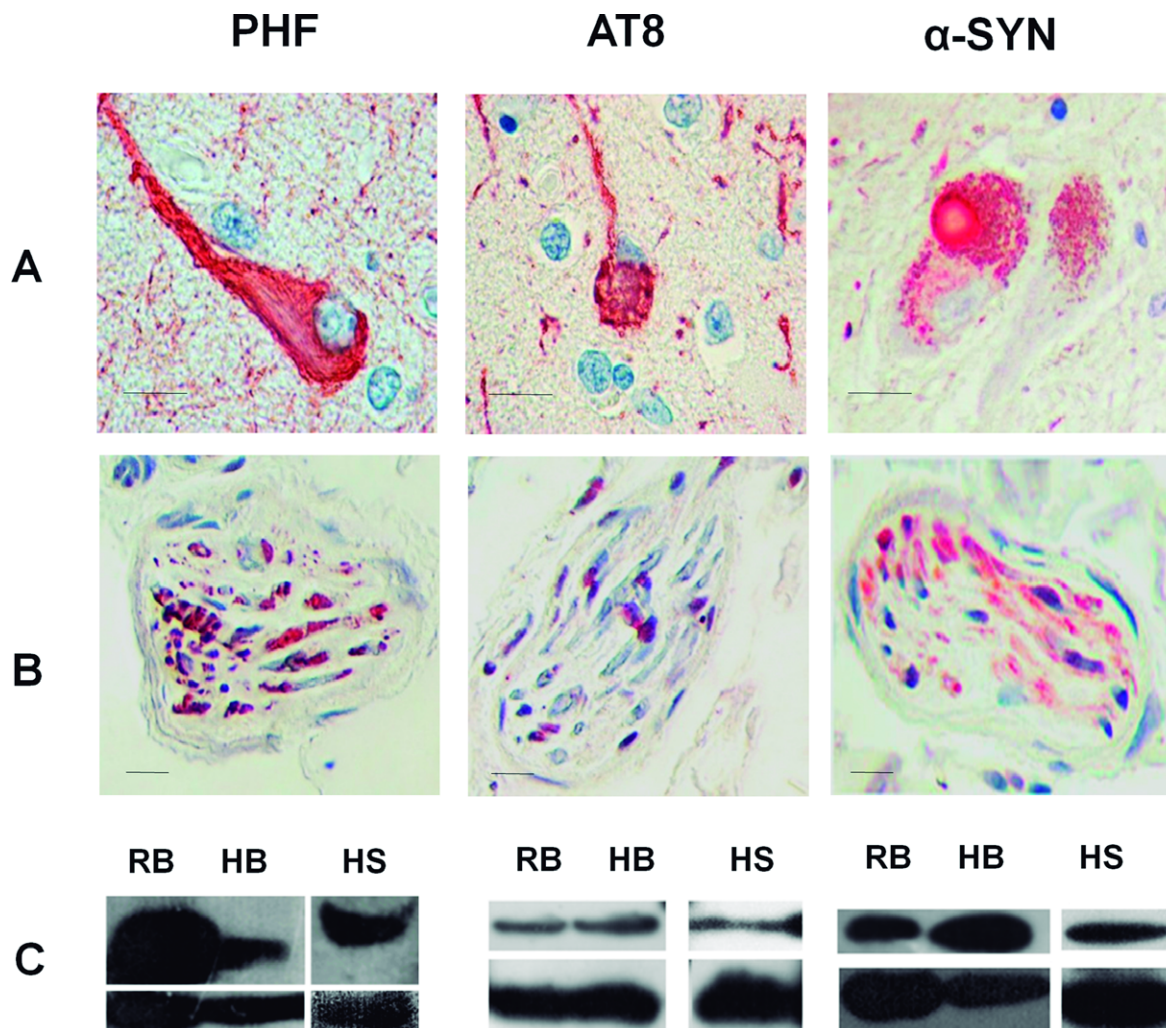


Figure 1. Identification of tau and α -syn proteins. (A) Central nervous system. Neurofibrillary tangles in hippocampal neurons immunostained with PHF and AT8 antibodies in an AD patient (1000 and 400 \times), and Lewy body immunostained with α -syn antibody in a mesencephalic neuron from a PD patient (1000 \times). Scale bar: 20 μm . (B) Immunoreactivity patterns of PHF, AT8, and α -syn antibodies in peripheral nerve terminals. Scale bar: 10 μm . (C) Western blot. Demonstration of tau (PHF and AT8) and α -syn (top) in RB, HB, and HS, Control: β -actin (bottom). PHF, paired helical filaments; RB, rat brain; HB, human brain; HS, human skin.

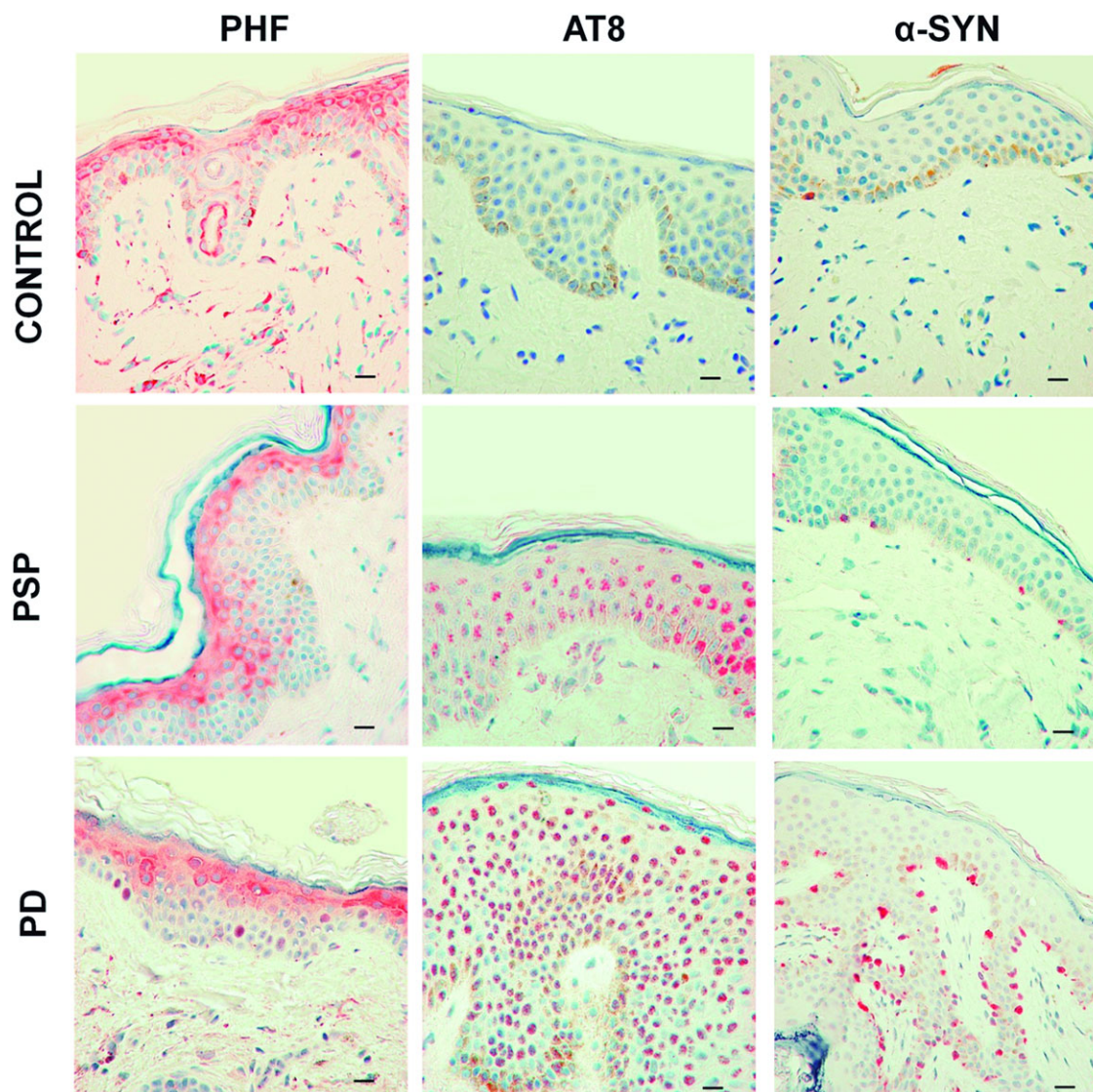


Figure 2. Immunoreactivity patterns in epidermis. Control subject, PSP and PD patients with PHF, AT8, and α -syn antibodies. Amine-ethyl-carbazole staining. Scale bar: 10 μ m. PSP, progressive supranuclear paralysis; PD, Parkinson disease; PHF, paired helical filaments.

β -mercaptoethanol, boiled 10 min at 95°C and run on 10–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrophoretically transferred to 0.22 μ m Polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences, Sunnyvalley, CA, USA, GE Healthcare, Amersham Biosciences, Los Angeles, CA, USA) and blocked with 3% bovine serum albumin (BSA) or 5% nonfat dry milk (Bio-Rad, Laboratories, Hercules, CA) in Tris-buffered saline (pH 7.6) containing 0.1% Tween (TBST). Membranes were probed overnight at 4°C with PHF antibody (1:500 in TBST 5% nonfat dry milk), AT8 antibody (1:250 in TBST 3% BSA), and anti- α -syn (1:500 in TBST 5% nonfat dry milk). In addition, all membranes were probed with anti β -actin as a control

protein in whole lysates. After rinses with TBST, the membranes were incubated with the secondary antibodies HRP-conjugated (1:5000) during 1 h 30 min. After subsequent rinses, bands were revealed by chemiluminescence (Pierce, Rockford, IL) followed by autoradiography.

Evaluation and statistical analysis

The analyzed structure was the epidermis and the analyst reading the specimens was blind to the patient's clinical diagnosis. Five fields per section were captured at the same magnification (40 \times) and digitally analyzed as previously reported using the Image Pro Plus 7 program.¹¹ A

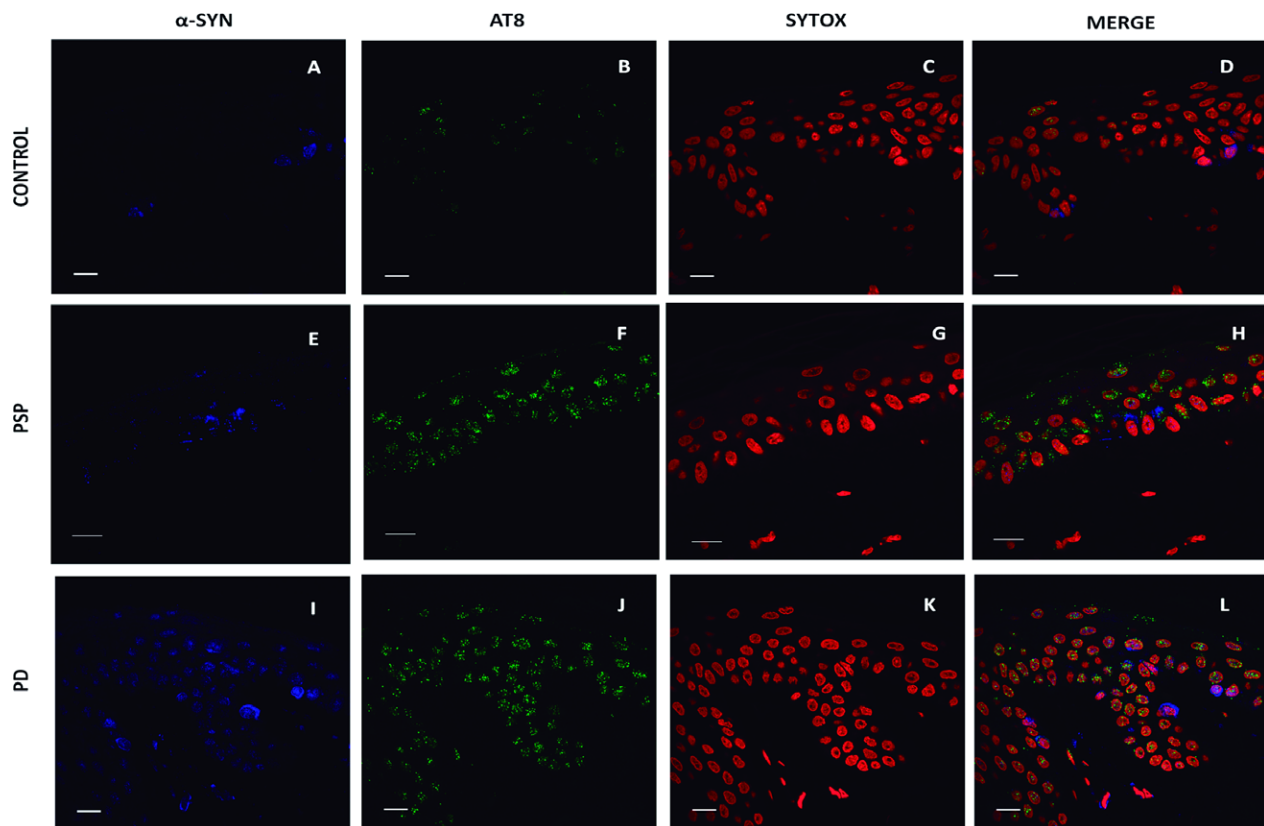


Figure 3. Skin immunofluorescence. Confocal microscopy. α -syn (Cy5) and AT8 (Alexa Fluor 488) antibodies. Control subject (A–D), PSP patient (E–H) and PD patient (I–L). Cell nuclei stained with SYTOX. Scale bar 10 μ m. PSP, progressive supranuclear paralysis; PD, Parkinson disease.

parametrical statistical test was applied in order to compare age, MMSE, and HY scores among the three analyzed groups, considering values of P less than 0.05 as being significant. Scores of immunopositivity were subjected to a nonparametric analysis by means of Kruskal–Wallis test followed by Mann–Whitney U test to make the comparison between groups.

Results

A total of 17 patients with PD, 10 patients with PSP and 17 healthy controls (HCs) were included. The proportion of male patients was higher compared to female in control and PD groups, contrary to PSP group (Table 1). The MMSE and Hoehn–Yahr scores were significantly different in the groups of patients, while the difference in UPDRS scores did not reach statistical significance ($P = 0.07$). The PSP group cohort showed greater cognitive deficit ($P < 0.01$) and disability scores ($P < 0.05$) compared with the PD group (Table 1).

The immunohistochemical technique and the antibodies against p-tau (PHF and AT8) and α -syn were assayed first in autopsied brain tissue from definitive AD and PD

patients, confirming the detection of NFT and Lewy bodies (Fig. 1A). Then, p-tau and α -syn expression were demonstrated in peripheral nerve terminals of the skin. PHF and α -syn immunopositivity was mainly axonal, while AT8 staining was observed in Schwann cells nuclei (Fig. 1B). The identity of the proteins of interest was further demonstrated through their molecular weight by western blot, in samples of rat brain and human biopsies from the human brain and skin (Fig. 1C).

Using the validated immunohistochemical technique and antibodies, we looked for the presence of PHF, AT8, and α -syn in skin biopsies from the three experimental groups. PHF immunopositivity was present along the epidermis, including basal cells and keratinocytes, and its localization was mostly cytoplasmic and less frequently nuclear; its presence was very similar among the three groups (Fig. 2, left column). In contrast, AT8 immunopositivity was inside or around nuclei of epidermis cells in the PSP and PD groups, whereas it was almost absent in the control group (Fig. 2, central column). α -syn immunopositivity in the control and PSP groups was present in scattered basal cells of the epidermis, while in the PD group, immunopositivity was abundant and intense in

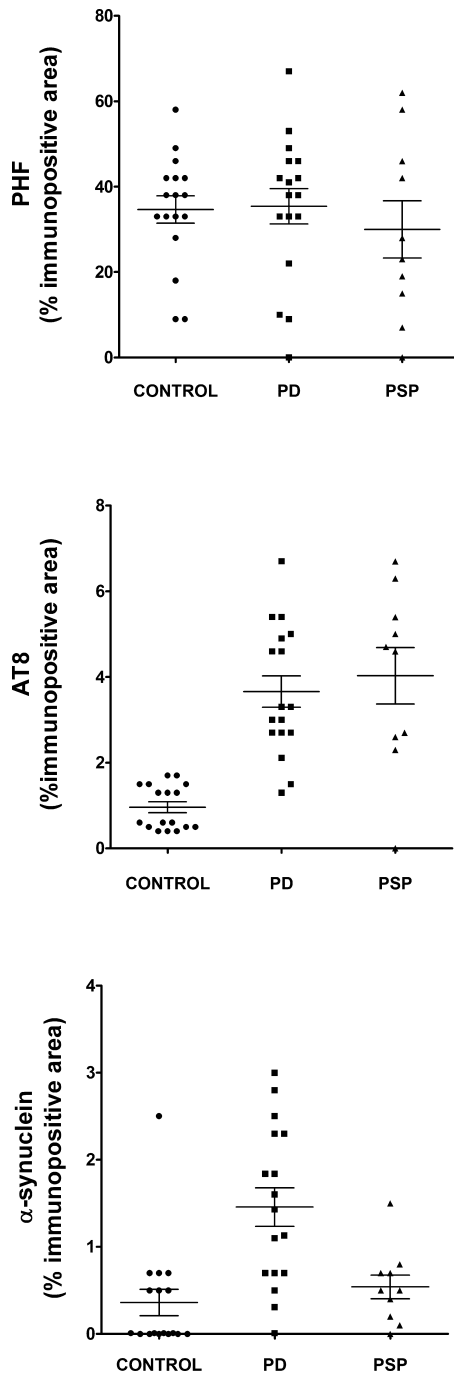


Figure 4. Immunopositivity quantification. PHF, AT8, and α -syn immunopositivity expressed as percentage of immunopositive pixels/total area. PHF immunopositivity showed a similar pattern of expression in all groups ($P = 0.72$). AT8 immunoreactivity was significantly different in PSP and PD patients as compared with the control group ($P < 0.001$). α -syn immunoreactivity in the PD group was significantly different from both control ($P < 0.001$) and PSP ($P < 0.01$) groups. Kruskal–Wallis analysis followed by Mann–Whitney U test. PHF, paired helical filaments; PSP, progressive supranuclear palsy; PD, Parkinson disease.

Table 2. Immunohistochemistry.

Immunohistochemistry (% immunopositive area: median, range)	Control	PD	PSP
PHF (phosphorylated Tau in Ser 296)	38 (18–49)	38 (9–53)	25 (0–62)
AT8 (phosphorylated Tau in Ser 202 Thr 205)	1.3 (0.4–1.7)	3.15* (2.7–6.7)	4.65* (0–6.7)
alpha-Synuclein	0.1 (0–0.7)	1.35* (0.5–2.8)	0.5 [†] (0–1.5)

PD, Parkinson disease; PSP, progressive supranuclear palsy; PHF, paired helical filaments.

* $P < 0.001$ versus controls, [†] $P < 0.01$ versus PD, Kruskal–Wallis followed by Mann–Whitney U test.

the basal cell stratum (Fig. 2, right column). Using confocal microscopy (Fig. 3), we observed greater nuclear expression of AT8 in PSP and PD (Fig. 3F and J) and higher cytoplasmic α -syn immunopositivity in PD (Fig. 3I). As compared to controls, the representative PSP image showed higher AT8 immunopositivity (Fig. 3H), while there was a clear overlap of high AT8 and α Syn immunopositivity in the image of the PD patient (Fig. 3L). Immunopositivity with α -syn was also observed in keratinocytes. The statistical comparison of the quantification of digital images quantification is presented in Figure 4 and Table 2. Although the PSP group showed a greater dispersion of data than control and PD groups for the three analyzed parameters, PHF values were similar among the three groups ($P = 0.72$), while AT8 was significantly higher in both PSP and PD groups ($P < 0.001$) as compared to controls. α -syn values were significantly higher in the PD group as compared with both control ($P < 0.001$) and PSP groups ($P < 0.01$).

Discussion

Misfolding of intraneuronal proteins in patients with PD and PSP is well known. Often, these neurodegenerative diseases can be difficult to distinguish particularly early in the course of the disease. We sought to explore the possibility that skin biopsies may differentially express forms of p-tau or α -syn and that this may help diagnose these diseases through the use of a simple punch biopsy.

A wide distribution outside the CNS is reported for α -syn; it is found in peripheral nerve terminals of the skin^{12–15} in myoenteric plexus, vagal terminal nerves,¹⁶ salivary glands, pancreas,¹⁷ adrenal medulla, urinary bladder, and cardiac epicardium.¹⁸ We previously reported a moderate expression in keratinocytes (especially in the basal layer) and skin appendages in patients with PD, minimal expression in patients with parkinsonism and no expression in HCs¹⁰ Several experimental models propose

that synucleinopathies could start outside the CNS. It has been shown that α -syn inoculation in the sciatic nerve could propagate to the CNS and result in synucleinopathy in mice.¹⁹ Also, the propagation of synucleinopathy from the gut to the CNS has been demonstrated,²⁰ supporting the possibility that the applied protein would trigger and spread α -syn pathology.²¹ Besides, it is clear now that there is a “cell to cell” transmission of α -syn in the CNS, a theory that has gained significant support since the report from Olanow and Prusiner about the presence of synucleinopathy in the dopaminergic embryonic neurons previously transplanted for PD treatment, and the recent demonstration of synucleinopathy transmission to mice from the human brain extracts of deceased multiple systems atrophy patients.^{22,23} The release into the extracellular space and propagation could also be extended to include other cells like the keratinocytes, although we still do not know whether nonneuronal cell types are also susceptible to cell damage through α -syn aggregates.

Since it is now known that PSP is a taupathy, it is possible that abnormally phosphorylated aggregates of tau may be present outside of the CNS.⁷ It is intriguing that in nonneuronal cell types such as lymphocytes, fibroblasts, and neoplastic cells, tau is frequently expressed in cell nuclei, while in the brain, it is a microtubule-associated protein related with several cellular functions. In PSP, tau is misfolded into NFT, globular glial inclusions in the astrocytes (“tufted astrocytes”) and in the oligodendroglia (“coiled bodies”) in both cortical and subcortical regions. Although the AT8 antibody identified NFTs in the brain tissue, in epidermis it showed a predominant nuclear localization that could be related to other functions of tau besides cytoskeletal support. An explanation for this would be found in recent studies performed *in vitro* and in animal models indicating that the nuclear translocation of tau plays an important role in DNA and RNA protection in conditions of cellular stress.^{24–26} In this scenario, NFTs would mediate a loss of tau functions at nuclear levels leading to DNA and RNA damage in neurons.

Synucleinopathy and taupathy overlap in neurodegenerative diseases and have been reported in Lewy pathology in the nervous tissue.^{27–29} The clinical manifestations of PD and PSP are associated with the distribution of the α -syn and tau pathology as well as the time of evolution. For instance, the presence of dementia in PD seems to correlate with cortical involvement and the association with the presence of β -amyloid plaques and tau NFTs, implying a potential interaction between PD and AD.³⁰ Postmortem studies showed increased accumulation of p-tau in the striata of PD patients.³¹ Indeed, we found the presence of both α -syn and p-tau in the skin of PD patients. In contrast to previous reports that looked for α -syn expression in nervous peripheral terminals from

somatic or autonomic fibers in the skin of PD patients, we have found it not only in the nervous tissue, but also in the keratinocytes of the epidermis.^{32–36} The co-occurrence of both tau and α -syn in nervous tissue has been reported previously; both proteins appear to promote the fibrillation and solubility of each other either *in vitro* or *in vivo*. Hence, while PSP is a recognized taupathy and PD is a synucleinopathy, though the latter is also frequently associated with a taupathy. In fact, families with mutations in the microtubule-associated protein tau or SNCA may have both dementia and parkinsonism.³⁷ At a molecular level, the simultaneous expression of both proteinopathies occurs in PD and AD, as well as other pathologies like Lewy bodies dementia, Lewy variant of AD, Guam–Parkinson–ALS–dementia complex, and Down’s syndrome.³⁸

Finally, concerning the dispersion of the obtained data, since our study was made with the clinical, not histopathological diagnosis of PD and PSP the outliers are possible, a factible explanation could be the variation among different batches of commercial antibodies, a common problem that is increasingly gaining attention in scientific reports.³⁹

In summary, immunohistochemical analysis of p-tau and α -syn in the skin can be used to distinguish PD and PSP, though not as a unique and reliable peripheral biomarker in clinical routine.²⁸ In addition, these data add to previous findings suggesting that the skin reflects clinically suspected brain pathology.^{10,11} These results suggest that we may be able to study the pathophysiology of neurodegenerative diseases through an analysis of skin. Further, because of the ease of obtaining 4 mm punch biopsies, these techniques could be added to the analysis of α -syn in body fluids and peripheral tissues as a potential biomarker for PD.⁴⁰

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Author Contributions

I. R.-L., MD, FAAN: Study conception, leader of the clinical part of the study, recruitment and diagnosis of patients, analysis of results, and manuscript redaction. E. G. C.-A., BSc: Immunohistochemistry. J. C., MD: Software design and digital quantification of immunostaining. M. R.-V., MD: Recruitment and diagnosis of patients; critical revision of manuscript. S. V.-O., MD: Recruitment and diagnosis of patients. V. M.-M., MD: Biopsy procedure and obtention of informed consent. M. G. M.-G., PhD: Rat and brain tissue processing, Western blot. S. Z.,

PhD: Western blot and critical revision of manuscript. L. E.-M., MSc MSci²: Tissue processing for immunohistochemistry and design of figures. A. C., MSc: Western blot. A. L. C.-G., MD, PhD: Analysis of immunostained sections in order to establish quantification criteria; critical revision of manuscript. M. E. J.-C., ME, PhD: Study design, obtention of microphotographs of light microscopy, confocal analysis, statistical analysis, manuscript redaction, team leader, and obtention of financial support.

Conflict of Interest

None declared.

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