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RESEARCH ARTICLE

α-Synuclein inclusions in the skin of Parkinson's disease and parkinsonism

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Abstract

Objective: The presence in the brain of α-synuclein containing Lewy neurites, or bodies, is the histological hallmark of Parkinson's disease (PD). The discovery of α-synuclein aggregates in nerve endings of the heart, digestive tract, and skin has lent support to the concept of PD as a systemic disease. Our goals were, first, to demonstrate the presence of α-synuclein inclusions in the skin and, second, to detect quantitative differences between patients with PD and atypical parkinsonism (AP). **Methods**: Skin biopsies were taken from 67 patients and 20 controls. The biopsies underwent immunohistochemistry (IHC) and immunofluorescence (IF) testing for α-synuclein, whereupon its presence was quantified as the percentage of positive cells. Patients were divided into those with PD and those with AP. AP patients included AP with neurodegenerative disease (proteinopathies) and secondary AP. **Results**: Sixty-seven patients (34 with PD) and 20 controls were recruited. In the PD group, α-synuclein was detected in 58% of the cells in the spinous cell layer (SCL), 62% in the pilosebaceous unit (PSU), and 58% in the eccrine glands (EG). The AP-proteinopathies group showed 7%, 7%, and 0% expression of α-synuclein, respectively. No expression was found in the skin of the control group. **Conclusions**: The expression of α-synuclein in the skin was relatively high in the PD group, scarce in AP, and null for the individuals in the control group. While these findings require further confirmation, this minimally invasive technique may aid in the improvement of the accuracy of PD diagnoses.

Introduction

A major hallmark of neurodegenerative diseases is the abnormal deposition of aggregates of misfolded proteins. Although the cascade of molecular events that trigger and propagate protein aggregation is not completely understood, it has been firmly established that protein misfolding initiates a series of intracellular perturbations that lead to cell dysfunction and eventually cell death.[1] In nervous tissue, cellular alterations include endoplasmic reticulum stress, the disruption of calcium homeostasis, and intracellular signaling, which triggers synaptic dysfunction, morphological changes, and a deficit in energy supply leading to neuronal death.[2] Parkinson's disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA) all share the abnormal intracellular accumulation of α-synuclein and lead to posttranslational modifications, especially phosphorylation.[3,4] The anatomical distribution of protein aggregates determines the specific clinical manifestations of each of these diseases. In

PD, inclusions containing α-synuclein are found in the neurons of the substantia nigra, olfactory bulb, locus coeruleus, pontine tegmentum, hypothalamus, limbic system, and amygdala nuclei. Outside the central nervous system (CNS), sympathetic and parasympathetic pre- and postganglionic nerve endings in the heart,[5] the digestive tract,[6,7] and the skin[8,9] also present α-synuclein aggregates. It is still unclear whether these aggregates display similar toxicity in peripheral cells to that observed in the CNS, but multiple autonomic dysfunctions suggest a mechanism of widespread cell damage associated with α-synuclein aggregates.[10,11]

In DLB, intracytoplasmic neuronal inclusions are predominantly distributed in the neocortex, leading to dementia (visual hallucinations, fluctuations in cognitive status over the course of each day, and deficits in attention and executive function) and parkinsonism.[12] In MSA, the cytoplasmic inclusions are found in the oligodendroglial cells in the brainstem and cerebellum, resulting in parkinsonism with ataxia and dysautonomia.[13] Other proteinopathies show a set of distinctive clinical features and poor and transient responsiveness to levodopa. Classic non-α-synuclein parkinsonisms include tauopathies such as progressive supranuclear palsy (PSP), Alzheimer's disease (AD), fronto-temporal lobar dementia (FTLD), and corticobasal syndrome (CBS).[14]

Since the diagnosis of PD is purely clinical, errors are common in the early stages even for specialists in the area.[15,16] Dopamine transporter (DAT) scans and magnetic resonance imaging (MRI) are helpful but cannot differentiate among PD, MSA, and PSP since all of them exhibit a similar dopamine deficit and characteristic signs are not always pathognomonic (the "hot cross bun" for MSA and the "hummingbird sign" for PSP).[17–20] There is not a strong biomarker to help differentiate between these diseases yet, but the presence of α-synuclein has been confirmed in PD[21] in a number of different tissues, including skin tissue.[22,23] While studies suggest that the sebaceous glands can express protein aggregates during neurodegenerative diseases,[22] there is little information available regarding the in vivo accumulation of α-synuclein,[24] since a low positive rate is reported in postmortem studies.[8,9] The aims of this study were to demonstrate the presence of α-synuclein inclusions in the skin of patients with PD and atypical parkinsonism (AP) and to detect quantitative differences between these groups compared with a control group. Researchers analyzed the epidermis, pilosebaceous unit (PSU), and eccrine glands (EG) of patients clinically diagnosed with PD and other forms of parkinsonism (AP) looking for immunopositive α-synuclein cellular inclusions.

Methods

Control group

The study included a control group of 20 age-matched neurologically asymptomatic subjects, comprising 10 males. These subjects were patients of the Dermatology Department, and had no neurological problems. They were invited to participate in the study and signed an informed consent document. The controls were recruited in order to make comparisons with the experimental subjects and confirm the absence of α-synuclein in unaffected individuals.

Patients

All patients were seen in the movement disorder clinic at the Central Hospital in San Luis Potosi, Mexico. The diagnosis of the different pathologies considered in the study was based on the following criteria or references: the diagnosis of PD used the United Kingdom PD Society Brain Bank[16]; the diagnosis of Lewy body dementia (LBD) used the report of the consortium on DLB international workshop[25]; the diagnosis of AD used the recommendations from the National Institute on Aging and Alzheimer's Association workgroups on diagnostic guidelines for AD[26]; the diagnosis of MSA used the *Second consensus statement on the diagnosis of multiple system atrophy*[27]; the diagnosis PSP used the report of the NINDS-SPSP International Workshop[28]; CBS[29] and we included a case with diagnosis of neurodegeneration with brain iron accumulation syndrome (NBIA).[30] Most of these criteria are summarized in the SIC Task Force appraisal of clinical diagnostic criteria for Parkinsonian disorders.[31] Patients were classified in one of the following groups: (1) PD – the patient initially presented asymmetric onset (with both rest and postural tremors with a frequency of between 5 and 7 Hz), rigidity, bradykinesia, postural instability, an acceptable and sustained response to levodopa, and previous and concomitant nonmotor characteristic semiology; (2) AP was divided into primary (neurodegenerative) and secondary (post encephalitic, vascular, drug induced, toxic) groups. All participants underwent a 4-mm punch biopsy in the retroauricular area. Informed written consent was obtained from each participant. The study was approved by the Research and Ethics Committee from our Institution and conducted at the Dermatology and Neurology Departments at Central Hospital in San Luis Potosi, Mexico. The protocol was registered at the U.S. National Institutes of Health Clinical Registry, NCT01380899.

Immunohistochemistry

Sections of 4 μm frozen tissue were fixed in acetone and processed for immunohistochemistry (IHC). A polyclonal antibody for α-synuclein (RB-9026-P; Thermo Fisher Scientific, Fremont, CA, USA) was used at a working dilution of 1:1500, as determined through sequential trials using PD midbrain, with abundant Lewy bodies as a positive control. Sections were blocked with horse serum and incubated with α-synuclein for 1 h and rinsed twice with a Tris-HCl buffer (pH 7.6) for 20 min. Sections were then incubated for 30 min with a biotinylated secondary antibody, rinsed, and treated with an Avidin–Biotin–Peroxidase complex (Vectostain Elite, Vector Laboratories, Burlingame, CA). The sections were developed with 3-amino-ethyl-carbazole for 3 min, rinsed with water and counterstained with Mayer's hematoxylin.

Confocal immunofluorescence

This study employed a triple labeling technique in either single or combined form (α-synuclein, cytokeratins, and DAPI). (4',6-Diamidino-2-Phenylindole, Dihydrochloride) The identification of α-synuclein and cytoskeletal cytokeratins was performed on frozen sections. The samples were incubated for 1 h with rabbit anti-α-synuclein antibodies (1:750; Thermo Scientific Fremont, CA, USA) and mouse anti-cytokeratin AE1/AE3 (1:50, DAKO, Carpinteria, CA). For detection, we used goat anti-rabbit Alexa Fluor 568 (1:500; Invitrogen, Carlsbad, CA) and goat anti-mouse Alexa Fluor 488 (Invitrogen). Nuclei were contrasted with DAPI Vectashield® Mounting Medium (Vector Laboratories, Burlingame, CA). The samples were analyzed with a Zeiss LSM 700 Carl Zeiss GmbH, Jena, Germany. Confocal microscope with 49 DAPI shift-free filters, 38 Endow GFP GFP (Green Fluorescent Protein) shift-free y 45 HQ Texas Red, $d = 25$ shift free. Objectives: Plan-Apochromat 40×/1.3 Oil DIC M27, Plan Q Apochromat 63×/1.40 Oil DIC M27, and Plan-Apochromat 100×/1.40 Oil DIC M27. The fluorochromes were excited with a 405-nm laser line (5 mW fiber output), 488-nm laser line (5 mW fiber output), and 561-nm laser line (5 mW output fiber) for LSM 700. For the three-dimensional reconstruction, optical cuts of 0.198 μm were made with Zeiss Zen 2011® software.

Semiquantitative analysis of α-synuclein

Every 4 μm processed section was digitally captured with a 40× magnification using a 6-megapixel Olympus SP-320 camera, mounted on an Olympus CX31 microscope that was connected to a PC. Images were processed using the software Image J v1.44 (National Institutes of Health, Bethesda, MD). We determined the presence (+) or absence (−) of α-synuclein in the cells of the spinous cell layer (SCL) of the epidermis, the PSU, and the EG. Both a dermatopathologist and a neuropathologist independently counted IHC α-synuclein positive cells blinded to the clinical group. The intra- and interobserver researchers' agreements were evaluated using positive slides of α-synuclein in mesencephalon. The intraclass correlation coefficient indicated a value of 0.94 ($P \leq 0.001$) and 0.89 ($P \leq 0.001$) for an intra- and interobserver reliability, respectively.

Using light microscopy, positive staining was considered when two or more reddish nodular intracellular structures 1–2 microns wide, perfectly delimited and separated from each other, were observed (Figs. 1, 2). Cells were viewed under a 40× objective. A 10× ocular was used. The number of positive cells per 100 in at least five different zones (total count of 500 cells) was determined. The result was expressed as the percentage of positive cells in each of the observed structures (epidermis, the PSU, and EG).

Statistical analysis

The patients were stratified according to the clinical diagnosis made on the basis of the clinical findings, the MRI, and the progression of the disease. Both the clinical diagnosis and the semiquantitative histological analysis where carried out independently and blind to each other. The presence of aggregates of abnormal α-synuclein, expressed as the percentage of cells with positive inclusions in each experimental group, was tested for normality with the Shapiro–Wilk test. Next, the groups were analyzed with the nonparametric Kruskal–Wallis followed by Mann–Whitney U tests. One independent analysis was performed for each structure, namely the epidermis, PSU, and EG. Assessments were performed using the software Statistica 7.0 (Tulsa, OK) at 95% confidence.

Results

General characteristics of the population and comparison of immunopositive cells (percentage) found in each group (Control, PD, and AP) are presented in Table 1. We enrolled 67 patients, of whom 34 had PD and 33 had AP, 26 were found to have a neurodegenerative disease clinically (18 had synucleinopathy-related diagnosis: MSA, LBD and 8 had tauopathies: PSP, AD). We also included seven patients with secondary parkinsonism. A control group ($n = 20$) of similar age and sex distribution to the patient population was included. There was no significant difference in either age or evolution of disease between the two groups with parkinsonism (Table 1). IHC analyses revealed intense α-synuclein positive immunostaining in PD patients, showing small nodular deposits from 1 to

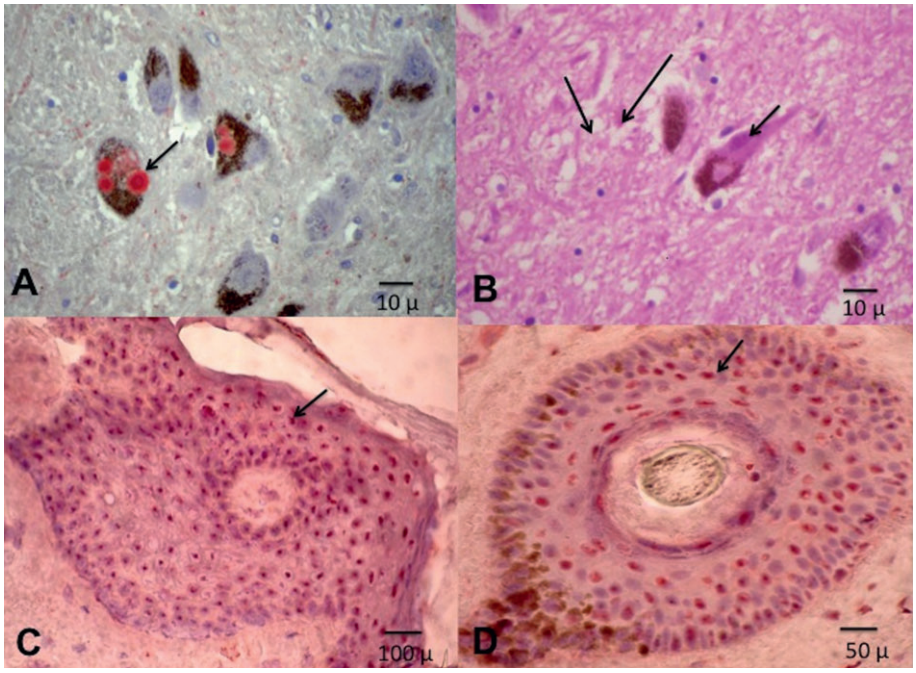

Figure 1. Immunohistochemistry (IHC), α-synuclein. Upper view. (A, B) Mesencephalon of a Parkinson's disease (PD) subject. (A) Positive IHC-control specimen showing Lewy bodies (in red). (B) H&E. Lewy bodies (arrow). (C, D) Skin biopsy, IHC, PD patient. (C) Positive α-syn aggregates in spinous cells (arrows). (D) Hair follicle emerging through epidermis. Red, juxtanuclear nodular aggregates in keratinocytes (arrows).

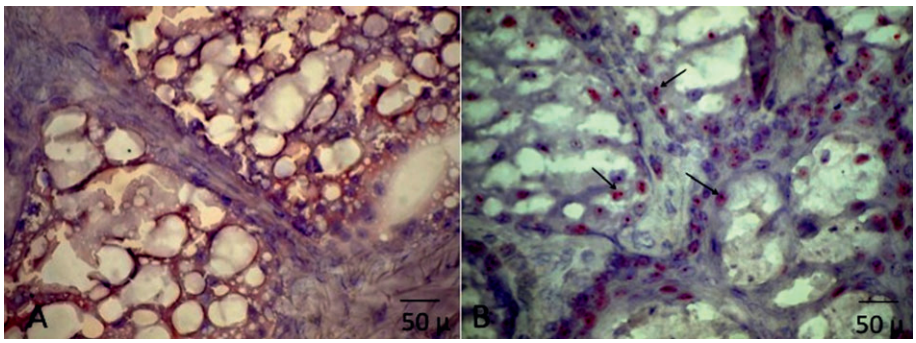

Figure 2. Immunohistochemistry (IHC), α-synuclein. Pilosebaceous unit. Left view. Atypical parkinsonism subject, negative staining. Right view. Parkinson's disease subject with numerous α-synuclein, juxtanuclear, red aggregates in spinous and sebaceous cells.

2 μm in diameter in the SCL of the epidermis including the epidermal component adjacent to hair follicles, and in cells from PSUs (Figs. 1, 2), while control subjects presented null immunoreaction. The α-synuclein inclusions in the two groups of patients were morphologically similar but quantitatively different. Using light microscopy, these inclusions appeared to be juxtanuclear (Figs. 1, 2). Table 2 compares the percentages of α-synuclein positivity in patients, divided into PD and AP. AP was classified as neurodegenerative (synucleinopathies other than PD, and tauopathies) and secondary (post encephalitic, vascular, drug induced, toxic). Close to 60% of the cells from the epidermis and PSUs of the PD patients presented α-synuclein inclusions. In contrast, in AP patients, α-synuclein immunopositive inclusions were scarcely observed. In general, as a group, AP patients with a neurodegenerative disorder showed a median of 6.9%, 7.7%, and 0% of immunopositive cells in the epidermis, PSUs and EG, respectively. The EG showed deposits mainly at the acini component in 58% of cells in the PD group, while in the AP group, α-synuclein positive cells were almost inexistent in these structures.

Further analysis made using confocal microscopy and the three-dimensional image reconstruction of optical

Table 1. General characteristics and classification of patients according to the clinical diagnosis.

	PD	Neurodegenerative AP				Secondary AP		Statistical analysis
		Related to other α-synucleinopathies		Tauopathies and other proteinopathies		Not degenerative		ANOVA (*F* value) *P*
Males	24	LBD	6	PSP	3	PID	1	
		MSA	2	CBS	1			
Females	10	LBD	6	PSP	3	VaP	2	
		MSA	3			PEP	2	
		NBIA	1	AD	1	PID	2	
Total	34	26				7		
Age (mean ± SD)	66.82 (11.4)	68.2 (13.5)				74 (7.9)		NS
Duration (years ± SD)	5.02 (4.1)	5.84 (3.7)				7.7 (5.2)		NS
Hoehn and Yahr scale	2.1 (0.8)	2.92 (0.8)				2.7 (0.75)		(7.4) 0.001

ANOVA *F* and *P* values arise from the comparison of the three groups: PD ($n = 34$), neurodegenerative AP ($n = 26$), and secondary AP ($n = 7$). Only Hoehn and Yahr scale values showed a significant difference. PD, Parkinson's disease; AP, atypical parkinsonism; ANOVA, analysis of variance; LBD, Lewy body dementia; MSA, multiple system atrophy; NBIA, neurodegeneration with brain iron accumulation; PSP, progressive supranuclear palsy; PID, parkinsonism induced by drugs; CBS, corticobasal syndrome; AD, Alzheimer's disease; VaP, vascular parkinsonism; PEP, postencephalitic parkinsonism.

Table 2. Comparison of positive α-synuclein expression in skin cells (percentage) by skin structures in PD and AP (neurodegenerative and secondary).

	Epidermis		Pilosebaceous unit		Eccrine gland	
Condition	*n*	Median (range)	*n*	Median (range)	*n*	Median (range)
PD	34	57.9* (44.9–62.6)	31	62.1* (48.5–71.2)	16	58.4* (47.4–69.3)
AP-neurodegenerative (18 synucleopathies eight with tauopathies)	26	6.9 (0–18.3)	23	7.7 (0–20.7)	9	0 (0–0)
AP (secondary)	7	0 (0–1.7)	7	0 (0–8.7)	7	0 (0–0)

PD, Parkinson's disease; AP, atypical parkinsonism, further divided into neurodegenerative AP (related to synucleinopathies, tauopathies, and other proteinopathies) and secondary AP (related to non-neurodegenerative conditions). Comparisons among groups were performed by Kruskal–Wallis followed by Mann–Whitney *U* test.

*$P < 0.001$ PD versus both neurodegenerative and secondary AP.

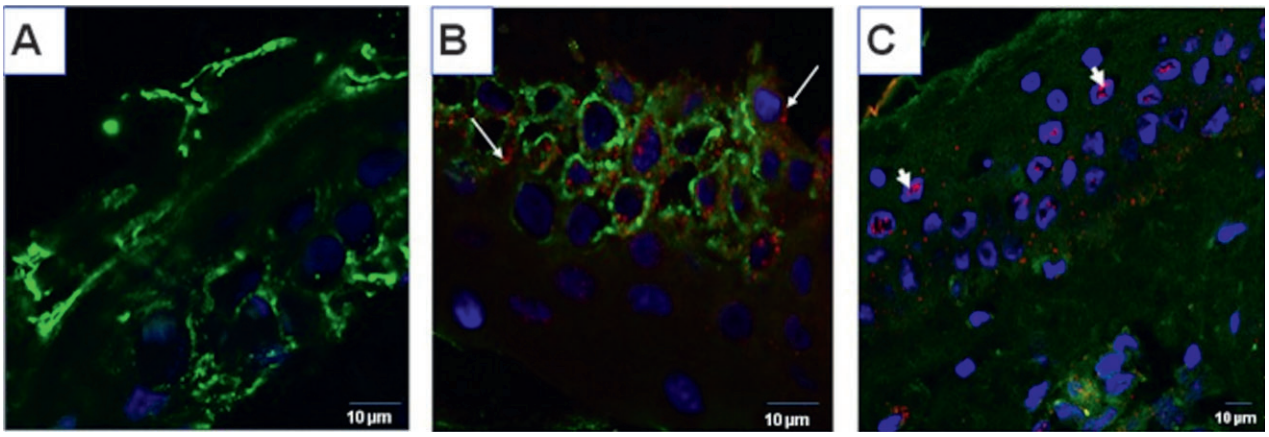

Figure 3. Confocal microscopy. Skin biopsy, epidermal cells (keratinocytes). (A) Negative control. (B, C) Parkinson's disease patient with positive juxtanuclear, α-syn inclusions (arrows). α-Synuclein is in red (Alexa 568); nuclei are in blue (DAPI); and cytokeratins AE1/AE3 are in green.

sections confirmed the findings reported in the light microscopy study. In PD patients, immunoreactivity to α-synuclein was observed in the epidermal cells, PSU, and EG. The confocal study determined that the α-synuclein inclusions were predominantly located adjacent to the nucleus (nuclear juxtaposed, Fig. 3).

Discussion

Although the sample is small, the data observed in this study is encouraging. We found and described deposits of α-synuclein, with intracytoplasmic and juxtanuclear location, in the epidermis and its appendages that occurred with a very strong expression in PD when compared to AP. Controls did not have any α-synuclein positive inclusions. To our knowledge this is the first study to detect α-synuclein expression in the epidermis and its appendages and to describe its potential as a biomarker for the differentiation between PD and AP.[32]

Given the complexity and heterogeneity of the genetics, the underlying molecular mechanisms, and the environmental risk factors in PD and other neurodegenerative diseases, there is an increasing need for a reliable

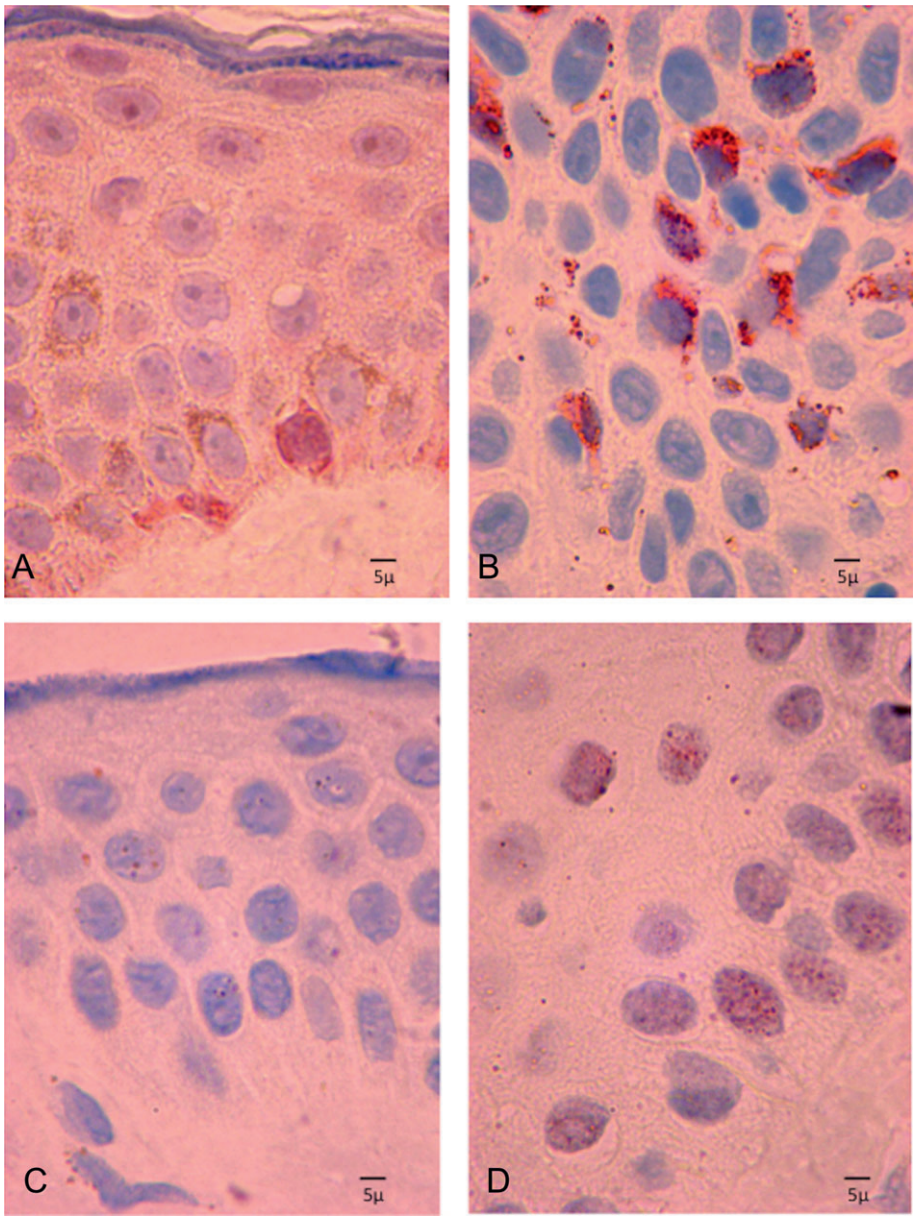

Figure 4. Skin biopsies embedded in paraffin. Immunohistochemistry with antibody to nonphosphorylated (A, B) and phosphorylated α-synuclein (C, D). Control samples shows melanin in basal cells and scarce red granules in melanocytes (A) and scarce perinuclear red granules in squamous cells (C). Parkinson's disease patient shows red granular inclusion in perinuclear (juxtanuclear) region (B and D) and cytoplasm (B) with both antibodies

biomarker in living patients that correlates with the histopathological changes in the brain derived from the proteinopathy.[32] Besides the motor characteristics of PD (bradykinesia, rigidity, tremor, and postural instability), its nonmotor symptoms and signs are common (sensory, autonomic, cognitive, and behavioral), and at least 60% of PD patients have more than one nonmotor symptom or sign.[33] These manifestations, however, are also common in AP and, although neurologists specializing in movement disorders achieve a high degree of diagnostic accuracy, more than 60% of cases with a final diagnosis of AP had their diagnosis changed during the course of the illness.[34]

Previous studies of the occurrence of aggregated α-synuclein outside the nervous system have demonstrated that PD is a multiorgan disease.[10,22] While α-synuclein deposits have been evidenced by studies describing IHC in paraffin sections of cutaneous nerve endings,[8,9,16] including a recent report on cutaneous autonomic nerves,[24] the authors did not mention its expression in other skin appendages or in the epidermis. After Ikemura et al. demonstrated in 20 of the 85 autopsies α-synuclein-positive unmyelinated fibers in the skin,[8] Miki et al. found immunoreactivity to α-synuclein in unmyelinated fibers near the blood vessels and sweat glands in skin biopsies of the chest wall for 2 of the 20 PD patients.[9] Subsequently, Shishido et al. showed the clear expression of α-synuclein aggregates in the autonomic nerves in the skin of one 73-year-old patient.[23] Concerning the analysis of the skin, Beach et al. reported the absence of α-synuclein in the abdominal skin of 14 subjects; however, those samples were autopsies,[35] not biopsies as is the case in this study, whose research process was also different. The main differences between those studies and the study presented here are that they employed antibodies for phosphorylated α-synuclein and paraffin-embedded tissue sections, whereas in this study, frozen sections "ex vivo" were used with an anti-α-synuclein antibody (nonphosphorylated). Although this study used fresh tissue and a polyclonal antibody for nonphosphorylated α-synuclein, we are now conducting a study with formalin-fixed material (I. Rodriguez-Leyva et. al., unpublished results) (Fig. 4). The same antibody plus an antibody for phosphorylated α-synuclein are used, and with which the preliminary results obtained are very similar. Although we recognize that our patients are not autopsy-confirmed diagnosis, all the included patients had clear clinical manifestations.

The exclusive occurrence of immunoreactivity in mesencephalic neurons presenting Lewy bodies, its absence in the skin of control subjects, and scarce occurrence in patients with AP confirms that the well-defined, red nodular structures correspond to α-synuclein insoluble aggregates. The intracellular distribution of α-synuclein was thought to be only cytoplasmic, but recent studies indicate α-synuclein translocation to the nucleus.[36,37] The findings achieved using confocal immunofluorescence that are reported here, are in agreement with a predominant cytoplasmic localization of the protein; moreover, its juxtanuclear and nuclear presence in keratinocytes was also observed. Although evidence of this translocation has been generated in vitro in dopaminergic cell lines, the multiorgan expression of α-synuclein aggregates suggest the possibility of similar protein trafficking in other cell types. Finally, although the number of cases is small, the sample is representative of the patient population with PD who are treated in our hospital, and from which it was possible to observe, as previously reported by other authors, the predominance of males, something which was not observed in the case of AP.[38,39] This finding is also an area of opportunity for future research.

In conclusion, our findings suggest an important quantitative difference between the expression of α-synuclein in the epidermis and its appendages when comparing patients with PD and AP. Pending further confirmation in larger cohorts, we propose α-synuclein screening in skin biopsies as a minimally invasive, affordable, and safe technique that may distinguish PD from AP.

Conflict of Interest

Ildefonso Rodriguez-Leyva is the speaker for Novartis, Boehringer Ingelheim, UCB and Genzyme (Sanofi Company) but they are not related with the research that he is making and pretending to publish.

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