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The aggregation state of α-synuclein deposits in dermal nerve fibers of patients with Parkinson's disease resembles that in the brain

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ABSTRACT

Introduction: Phosphorylated α-synuclein (p-α-syn) can be detected in dermal nerve fibers of patients with Parkinson's disease (PD) and multiple system atrophy (MSA). Here we investigated whether p-α-syn in the cutaneous nerve fibers represents misfolded aggregated protein.

Methods: Using immunofluorescence with conformation specific antibodies and digestion with proteinase K (PK), we studied skin biopsies from a cohort of patients with early stage PD (Hoehn and Yahr I/II, n = 27), MSA with predominant parkinsonism (MSA-P, n = 8) and normal controls (n = 21).

Results: We could show that α-synuclein (α-syn) found in the dermal nerve fibers in PD and MSA-P is not only phosphorylated but represents PK resistant and truncated aggregated protein. Comparison with a post mortem midbrain sample revealed a similar staining pattern of pathologic α-syn lesions in the PD brain.

Conclusion: Immunostaining of nerve fibers with different conformation specific antibodies and digestion with PK gave comparable results between midbrain and skin sections, showing that cutaneous nerve deposits of α-syn are structurally similar to Lewy pathology in the brain and are highly specific for synucleinopathy.

1. Introduction

Phosphorylated α-synuclein (p-α-syn) can be detected within dermal nerve fibers of patients with Parkinson's disease (PD) [1–4]. In contrast to native α-synuclein (α-syn) found in dermal nerve fibers of healthy controls, p-α-syn is highly specific for synucleinopathies and is a potential biomarker for PD. In recent studies, dermal p-α-syn was detected even in early stages of PD and in patients with REM sleep behavior disorder, who bear a high risk of converting to PD and therefore presumably represent a premotor stage of PD [5,6]. With regard to disease-modifying therapies that target α-syn deposition, quantification of the dermal deposits may serve as a feasible outcome measure in clinical trials. Furthermore, skin is an easily accessible tissue to study the pathogenicity of α-syn deposition in PD. Any conclusions from such research would require that the dermal p-α-syn deposits correspond to the central nervous system (CNS) deposits regarding their composition and aggregation state. Since aggregated α-syn is found to be mostly phosphorylated in the brain [7] and p-α-syn-positive dermal nerve fibers morphologically resemble Lewy neurites [1,3], p-α-syn in the skin is supposed to represent aggregates. The pioneer study of cutaneous nerves in PD made use of immunoelectron microscopy to show that p-α-syn-immunoreactive lesions are composed of granulofilamentous profiles seemingly similar to those found in the CNS [1,3]. However, this has not been since definitively characterized in a larger number of patients by more widely accessible and potentially diagnostic immunohistochemistry.

Specific antibodies are available to detect α-syn conformations: AsyO5 was proposed as marker of oligomers and 5G4 - for aggregates. Antibodies to C-terminal truncated α-syn, a posttranslational modification that is also increased in Lewy bodies in the brain, are also available [8]. Proteinase K (PK) resistance is an established marker of aggregated α-syn [9]. In this study, we aimed to characterize the aggregation state of dermal p-α-syn deposits by immunohistochemistry (IHC) with the above mentioned antibodies and using PK digestion and to compare our skin biopsy findings with the α-syn found in the brain.

2. Materials and methods

2.1. Patients and controls

We used 20-μm skin biopsy serial cryosections from patients diagnosed with idiopathic PD (n = 27) and controls (n = 5) who were prospectively recruited between November 2014 and June 2016 for another study [5]. All patients were diagnosed with PD according to the UK Parkinson's Disease Society Brain Bank Criteria and were staged as Hoehn and Yahr (HY) stage I (n = 13) or II (n = 14), see Table 1 [10].

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Abbreviations

α-syn	α-synuclein
CNS	central nervous system
HY	Hoehn and Yahr
IF	Immunofluorescence
IHC	immunohistochemistry/immunohistochemical
MAP	musculus arrector pili
MSA	multiple system atrophy
NC	normal control
p-α-syn	phosphorylated α-synuclein
PD	Parkinson's disease
PGP 9.5	protein gene product 9.5
PK	proteinase K
SN	substantia nigra

All patients gave informed consent to participate in the study, and the study was approved by the Ethics committee of the University of Würzburg. Skin punch biopsies had a diameter of 5 mm, and four different biopsy sites were analyzed per patient: distal leg, proximal leg, paravertebral Th10 and C7. Skin biopsy procedure, fixation and serial sections were performed as previously described [5], namely 100 serial sections of 20 μm thickness were cut from each of the four biopsies and stored at −20 °C until use. One slide (carrying every tenth section – in total 5 sections, s. Suppl. Table 5) for each of the four biopsy sites was analyzed per staining.

To confirm the specificity of the used IHC methods, additional skin biopsies of controls (n = 16, 2–4 sections from at least two biopsy sites: lower leg and back) and multiple system atrophy with predominant parkinsonism (MSA-P) (n = 8; see Table 1 and Suppl. Table 6; three biopsy locations (distal and proximal leg and Th10) in six patients, and additionally C7 in two patients) that were stored in our department from former studies [1,11] were used. Biopsies were sectioned within a month prior to the staining.

Post-mortem brain tissue of the substantia nigra (SN) was obtained from a patient with clinical and neuropathological diagnosis of PD (Braak stage 6) and a normal control (NC) and was cryoconserved at −80 °C immediately after dissection. Both midbrain samples were obtained from the Brain Bank Center Würzburg, member of the BrainNet Europe Brain Bank Consortium Network (https://www.brainnet-europe.org). 20-μm sections were cut with a cryostat and post-fixed with 4% paraformaldehyde for 10 min before staining.

2.2. Double-immunofluorescence staining

Double-immunofluorescence (IF) staining with anti-p-α-syn antibody (P-α-syn/81A or D1R1R), syn105 for truncated α-syn, anti-oligomeric α-syn (ASyO5) or anti-aggregated α-syn (5G4) and anti-protein gene product (PGP) 9.5 (pan neuronal marker) and appropriate secondary antibodies was performed as previously described [1] (see suppl. Table 1 for the detailed antibody list). Prior to 5G4 staining sections were subjected to an extra post-fixation with 4% PFA for 10 min followed by antigen retrieval with 70% formic acid for 5 min. Treatment with formic acid (or PK) without additional fixation led to signal loss and destruction of tissue (the short initial fixation of 30 min was likely insufficient for these methods). Colocalization of new markers with p-α-syn was assessed as percentage of deposits positive for both markers (in case of double-IF) or biopsies positive for both markers in the adjacent sections in relation to deposits/biopsies positive solely for p-α-syn.

2.3. Proteinase K digestion

The air-dried sections were additionally post-fixed with 4% paraformaldehyde for 10 min, washed twice in PBS for 5 min and incubated in 10 μg/ml PK in Tris-buffered saline TBS-B (10 mM Tris–HCl, pH 7.8, 100 mM NaCl, 0,1% Brij 35) for 10 min. After washing the sections were stained with syn211 and anti-PGP9.5. The initially used 20 min incubation time (determined in freshly sectioned midbrain) led to almost complete disappearance of signal in stored tissue sections and distortion of tissue morphology. The reduced incubation time of 10 min preserved PGP 9.5 signal, but did not lead to false positive results in NC.

2.4. Immunofluorescence microscopy

All sections were analyzed immediately after staining using a fluorescence microscope (Ax10, Zeiss, Oberkochen, Germany) with CARVII system and Visiview software (Visitron GmbH, Puchheim, Germany). Slides were scanned for immunoreactive nerve fibers and were considered positive if at least one dermal nerve fiber was immunoreactive for both α-syn and PGP9.5. In case of a double staining of anti-p-α-syn with syn105 or 5G4 nerve fibers were identified by morphology. To quantify the extent of α-syn deposits we counted the number of distinct dermal structures containing at least one nerve fiber positive for a chosen α-syn marker.

Colocalization was measured by Colocalization Analysis plugin over five representative region scans of SN [12] and the extent of lesions was quantified using Object finder 3D [13] in ImageJ. The average “lesion burden” was calculated as volume times number of objects; two sided paired Student test was used to determine statistical significance in R Version 3.3.2. A significance level of 5% was applied.

3. Results

3.1. Staining results in the skin of NC

No dermal nerve fibers positive for aggregated (syn211 after PK digestion or 5G4), oligomeric (ASyO4), truncated (syn105) α-syn or p-α-syn were detected in the controls, corresponding to a 100% specificity of all antibodies to detect pathological α-syn deposits (n = 16 (single sections) + 5 (serial sections); n = 5 (serial sections) in case of syn105).

3.2. A-syn aggregation in dermal nerve fibers of PD patients is revealed by different IHC methods

P-α-syn could be detected in 82% of patients using freshly cut serial sections in agreement with our previous studies [5,11], in the subsequent stains done simultaneously with stains for truncated and aggregated forms to allow a better comparison, p-α-syn detection rate fell with increased storage time (Table 1, Suppl. Table 2).

Truncated and PK resistant α-syn were detected in more than half of patients and in a similar number of patients as p-α-syn that was stained for simultaneously; aggregated and oligomeric protein as detected by 5G4 and AsyO5 was found in a lower proportion of patients (Table 1).

Among patients positive for p-α-syn, 94% were also positive for truncated α-syn, 90% and 69% for aggregated α-syn as detected by 5G4 and PK digestion, respectively, and 89% for oligomeric α-syn as detected by ASyO5 (Suppl. Table 2). To allow a better comparison of prevalence of the aggregated and oligomeric forms in relation to p-α-syn, percentage of sections positive for both markers in relation to p-α-syn positive sections was calculated for PK digestion and ASyO5 stain as seen in the adjacent sections (20–40 μm apart) or, in case of truncated α-syn and in a subcohort for the 5G4 stain, single deposits positive for both markers in relation to p-α-syn positive deposits were quantified in a double staining (Table 1).

Table 1
Summary of results.

	PD			MSA	Controls	
Subjects, n	27			8	5 with serial sections +16 with single sections	
Male to female ratio, M:F	1.25			1.0	1.33	
Mean age, yrs. (+/− SD)	63 (± 8.1)			65 (± 8)	58 (± 12)	
Mean UPDRS3, points (+/− SD)	15.3 (± 7.8)			56 (± 15.6)	–	
Mean duration of disease, yrs. (+/− SD)	3.6 (± 3.7)			4.6 (± 3.1)	–	
Mean HY stage, (+/-SD)	1.52 (± 0.5) HY I: n = 13, HY II: n = 14			4.2 (± 0.75)	–	
Subjects positive for the specified marker, n (%)	fresh sections (stain #1)	sections stored for 2 months (stain #2)	sections stored for 6 months (stain #3)	fresh sections	stored sections, n = 5	fresh sections, n = 16
P-α-syn	**22 (82%)**	**17 (63%)**	**13 (48%)**	**6 (75%)**	**0**	**0**
Truncated α-syn		**16 (59%)**		**n/a**	**0**	**0**
	Colocalization of p-α-syn-positive *and truncated α-syn deposits in double IF (n = 27)*	*120 of 256 total (47%)*		–		
PK resistant α-syn			**13 (48%)**	**7 (87.5%)**	**0**	**0**
	Number of p-α-syn -positive *biopsies also containing PK resistant deposits (%) based on analysis of consecutive sections*		*13 of 22 (59%)*	–	-	
Aggregated α-syn (5G4+)			**10 (37%)**	**3 (37.5%)**	**0**	**0**
	Colocalization of p-α-syn-posivtive *and aggregated α-syn deposits in double IF in a subcohort of patients (n = 18)*		*9 of 20 (31%)*	–	–	
Oligomeric α-syn (ASyO5+)			**9 (33%)**	**4 (50%)**	**0**	**0**
	Number of p-α-syn -positive *biopsies also containing ASyO5*-immunoreactive *deposits (%) based on analysis of consecutive sections*		*10 of 22 (45%)*	–	–	

α-syn – α-synuclein; IF – immunofluorescence; HY – Hoehn and Yahr scale; p-α-syn - phosphorylated α-synuclein; MSA – multiple system atrophy; PD – Parkinson's disease, SD – standard deviation; UPDRS –Unified Parkinson's Disease Rating Scale.

Analysis of consecutive sections revealed that the fibers immunoreactive for p-α-syn, 5G4, syn211 (post PK treatment) and AsyO5 were commonly located in the same area within each consecutive section suggesting colocalization (Fig. 1). Deposition of truncated α-syn in the dermal nerve fibers always colocalized with p-α-syn, but some p-α-syn deposits were found to be negative for truncated α-syn (see Fig. 3A–C and Suppl. Tables 2 and 3 for exact numbers). The highest number of deposits were found in the nerve fibers surrounding blood vessels followed by dermal nerve bundles. No difference in deposit distribution across different structures was seen among the stains (Suppl. Table 3). Also no proximal gradient was seen in the distribution, as more deposits were found in the biopsies of distal leg (see Suppl. Table 3). There was no significant difference in the number of deposits between HY stages I and II and no correlation with disease duration.

In the biopsies of seven out of eight MSA-P patients deposits of aggregated α-syn could be revealed by at least one of the methods, PK digestion being most sensitive (7 out of 8 patients positive, followed by p-α-syn (6/8), 5G4 (3/8) and ASyO5 (4/8), Table 1 and Suppl. Table 6). In five patients the deposits were limited to somatosensory subepidermal and dermal plexuses. In two patients pathological deposits were found in musculi arrector pili (MAP) and near blood vessels. The two aforementioned patients were clinically diagnosed with possible MSA [25] due to a relatively good L-DOPA response. One of these patients was additionally found to have typical for iPD cardiac denervation on MIBG scintigraphy (Suppl. Table 6).

3.3. A-syn aggregation state in the substantia nigra (SN) in PD as assessed by IHC

To compare our skin biopsy findings with the aggregation state in the brain, SN samples of a PD patient along with a NC were immunostained with all of the above mentioned methods. We found numerous Lewy body and neurite like accumulations of α-syn along with a diffuse neuropil signal when staining for total α-syn (syn211) in the brain sections of a PD patient (Suppl. Fig. 1C). The syn211 staining of Lewy bodies was intensified by PK pretreatment, while the diffuse neuropil staining disappeared (Suppl. Fig. 1D). In the normal brain, only a diffuse α-syn staining was visible when staining with syn211 (Suppl. Fig. 1A), but was not found when sections were pretreated with PK (Suppl. Fig. 1B).

AsyO5 at the 1:10,000 dilution revealed characteristic Lewy lesions in the PD brain (Suppl. Fig. 2B) that were paler compared to a staining with 1:100 dilution (Suppl. Fig. 2A) and did not show any staining in the NC at 1:10,000. ASyO5 signal persisted after a 20 min pretreatment in 10 μg/ml (Suppl. Fig. 2C) or 50 μg/ml PK (Suppl. Fig. 2D) and was intensified by it.

5G4 only stained the PD but not the control brain (Fig. 2D–F, Suppl. Fig. 3), resulting in a characteristic pattern of neural cytoplasmic and neurite inclusions. In a double-IF with p-α-syn some of the neurites were exclusively positive for p-α-syn (Fig. 2F, marked with arrowheads), few small lesions were positive only for 5G4 (marked with white *, Fig. 2F), while Lewy bodies and the majority of larger size neurites were positive for both (Fig. 2F, Suppl. Fig. 3F, arrows). A high degree of colocalization was supported by a Mander's coefficient of 0.89. The average “lesion burden” was lower in the 5G4 stain compared to anti-p-α-syn stain ($p < 0.05$, two-sided paired Student t-test), on average comprising 55.5% of that of the anti-p-α-syn scans (Suppl. Table 4).

Truncated α-syn (revealed by syn105) was also found to be highly colocalizing with p-α-syn (Mander's coefficient = 0.86). Here, similar to the skin biopsy findings, the majority of lesions were also positive for both markers (Fig. 3F, arrows), while some lesions were staining positive only for p-α-syn (Fig. 3F, arrowheads). The overall “lesion burden” was higher in the anti-p-α-syn staining ($p < 0.05$ two-sided paired Student t-test), the “lesion burden” for truncated α-syn made up 59% of that of the anti-p-α-syn scans (Suppl. Table 4).

4. Discussion

Using a set of IHC methods, we could show that α-syn deposited in dermal nerve fibers in PD is not only phosphorylated at S129, but is PK resistant and stains positive with antibodies specific for aggregated (5G4), oligomeric (AsyO5) and truncated (syn105) forms similar to synucleinopathy in the brain. Dermal α-syn deposits can therefore be classified as protein aggregates.

Stains for oligomeric, aggregated and truncated forms of α-syn did not reveal any positive signal in the midbrain or the skin biopsies of normal controls indicating that these methods specifically stained α-syn

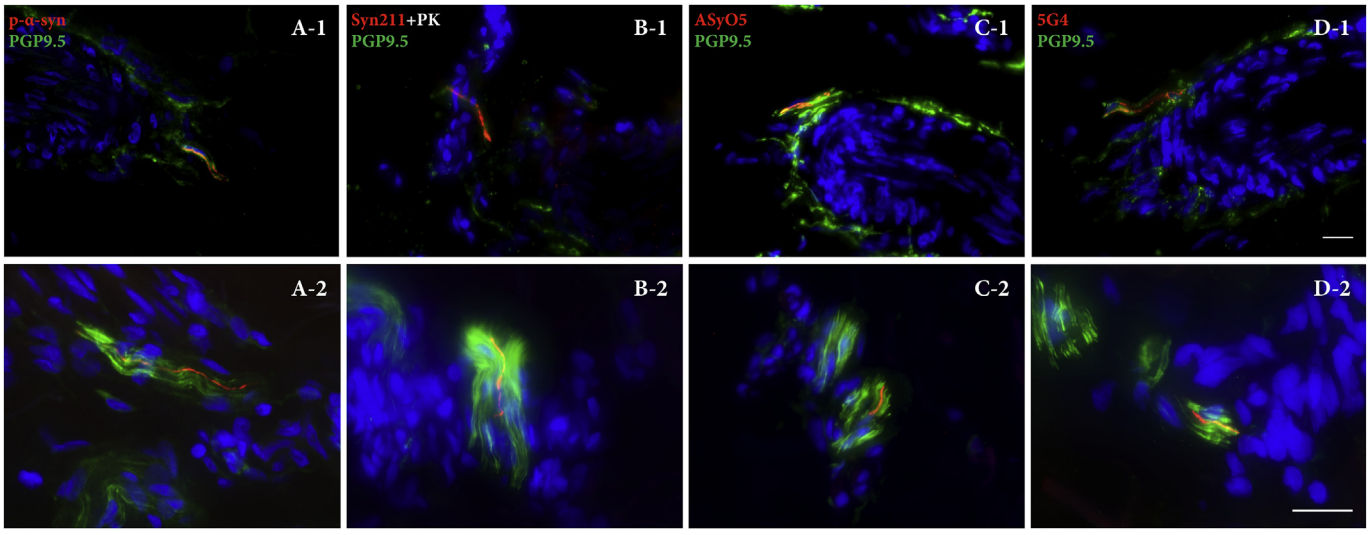

Fig. 1. α-syn aggregates in dermal nerve fibers. Double-immunofluorescence of a skin biopsy section of a PD patient with anti-p-α-syn (red) and anti-PGP9.5 (green) (A), syn211 (red) and anti-PGP9.5 (green) after PK digestion (B), AsyO5 (red) and anti-PGP9.5 (green) (C) and 5G4 (red) and anti-PGP9.5 (green) (D). All images in the upper panel show the same vessel (identified by nuclear staining with Hoechst 33342), images in the lower panel - the same dermal nerve bundle in subsequent sections of the biopsy, made possible due to serial sectioning. The single nerve fiber immunoreactive for p-α-syn, syn211 after PK digestion, AsyO5, and 5G4 is found in all subsequent sections, indicating colocalization of these markers. PK digestion in B-1 was done for 20 min, accordingly PGP 9.5 signal is weaker than in B-2 (10 min). Scale bar = 20 μm. PK = proteinase K, p-α-syn = phosphorylated α-synuclein.

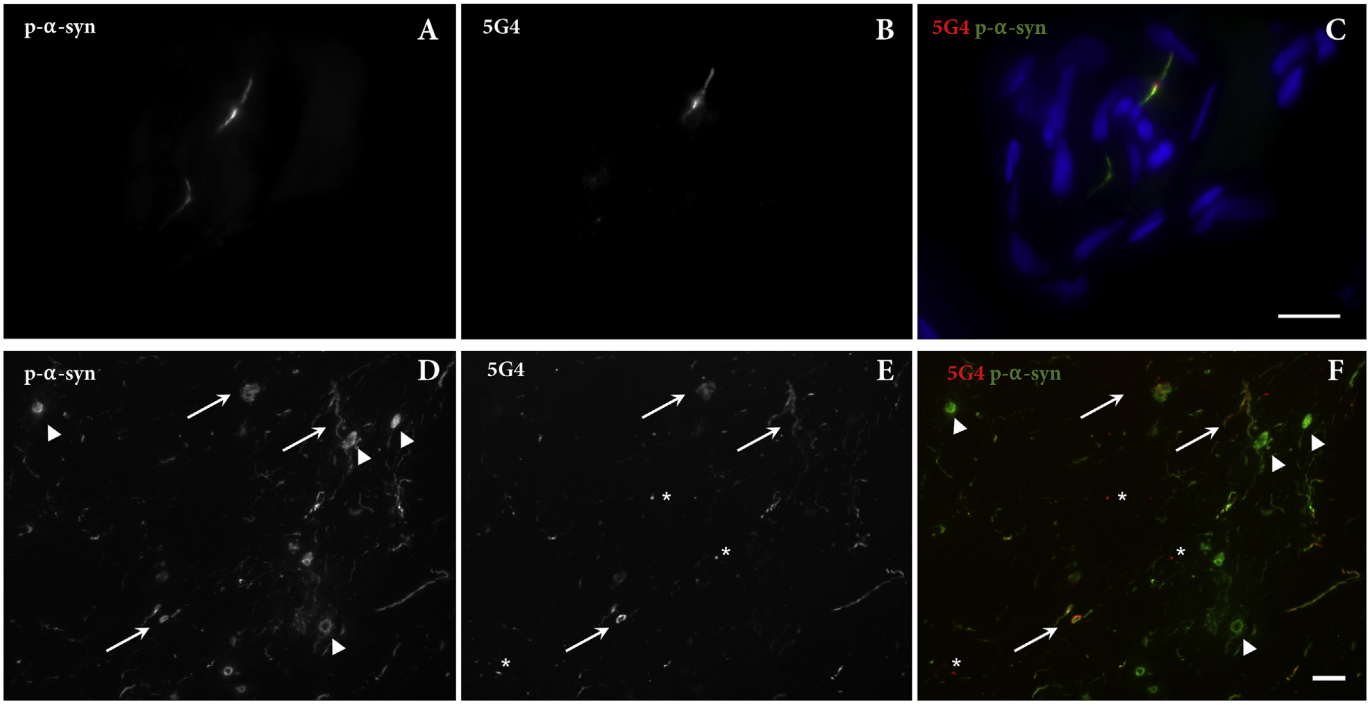

Fig. 2. Immunostaining of p-α-syn and aggregated α-syn (5G4) in the skin and brain in PD. Double-immunofluorescence of a skin biopsy of a PD patient (top panel, A-C) and of a midbrain section from an autopsy of a patient with neuropathologically confirmed PD (lower panel, D-F) showing colocalization of anti-p-α-syn (green) and 5G4 signals (red) in a dermal nerve fiber (C) and Lewy bodies and neurites (F). Many lesions colocalize completely (arrows), some are positive only for p-α-syn (arrowheads), and few small lesions are only positive for 5G4 (*). Scale bar = 20 μm. P-α-syn = phosphorylated α-synuclein, PD = Parkinson's disease, PK = proteinase K.

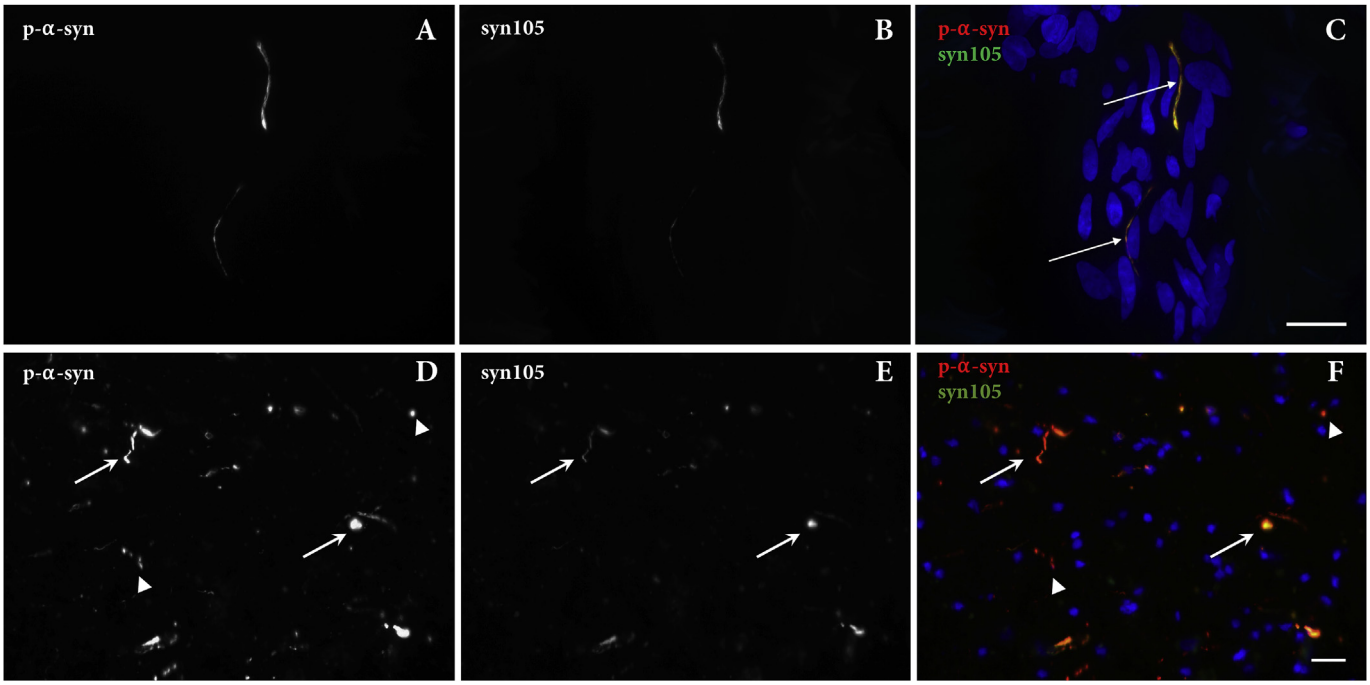

Fig. 3. Immunostaining of p-α-syn and truncated α-syn in the skin and brain in PD. Double-immunofluorescence staining of a skin biopsy of a PD patient (top panel, A-C) and of a midbrain section from an autopsy of a patient with neuropathologically confirmed PD (lower panel, D-F) showing colocalization of truncated α-syn detected by syn105 (green) and p-α-syn (red) markers in a nerve fiber in the skin (identified by morphology, arrows, C) and in Lewy bodies and neurites in the midbrain (F, arrows). Some lesions are solely positive for p-α-syn (arrowheads). Scale bar = 20 μm. P-α-syn = phosphorylated α-synuclein, PD = Parkinson's disease.

pathology in peripheral nervous system (PNS) as well as in the CNS and may be potentially used for diagnostics. In the present study sections of a single normal and diseased brain were stained. Although 5G4, ASyO5 and PK digestion have already been previously well characterized in human brain samples [9,14,15], human histological data for the specificity of syn105 (anti-truncated) is not available to the best of our knowledge, and use of a single NC and PD samples might represent a limitation. P-α-syn has also been shown to be highly specific for synucleinopathy in agreement with earlier skin biopsy studies [2,5,6].

Truncated α-syn was detected in dermal nerves of more than half of PD patients and none of the controls, and always colocalized with p-α-syn. To the best of our knowledge, the current study is the first to demonstrate the presence of truncated α-syn in PNS in PD. Based on this data it does not physiologically occur in dermal nerve fibers and represents another marker for pathological α-syn deposition.

Further we investigated whether aggregated α-syn is detectable in the dermal nerves in early stage PD using three different approaches: exploiting resistance of α-syn aggregates to PK [9], immunofluorescence with a conformation specific antibody 5G4 [14] and a highly diluted antibody (ASyO5), leading to preferential staining of oligomeric protein [15].

Pathological α-syn could be detected using all of the three approaches though with a varying sensitivity. By analysis of consecutive sections, it could be inferred that aggregated and oligomeric α-syn forms in the dermal nerves often colocalize with p-α-syn as well as with each other suggesting that the very same structure is being stained.

PK resistant α-syn was detected in about half of the patients - similar to p-α-syn when stained simultaneously. Stains with 5G4 and ASyO5 antibodies were less sensitive (37% and 33% of patients, accordingly). The superiority of the PK method may be explained by an antigen-retrieval effect of PK that makes more epitopes available [16]. The overall low detection rates of pathological α-syn in this study are explained by the use of stored sections. The relatively high occurrence of PK resistant aggregates suggests that all dermal p-α-syn deposits may represent aggregated protein, however, a direct comparison of PK resistant and p-α-syn is methodologically not possible. On the other hand, detection of lesions positive for p-α-syn and negative for 5G4 in the double-IF in the skin and midbrain could either mean that indeed some p-α-syn deposits are not aggregated or that 5G4 doesn't reveal a part of the aggregates or could even be a methodological issue, as in a very recent study pathological aggregates of α-syn in the skin were detected using the very same 5G4 antibody with a diagnostic accuracy similar to that of the anti-p-α-syn staining; main methodological difference being use of freshly cut sections and different fixation procedure [17]. In another recent study [18] however aggregated α-syn (as detected by antibody F1) was found less frequently than p-α-syn. As detection of one or other α-syn form is always dependent on the characteristics of the chosen antibody the true relationship of phosphorylation and aggregation cannot be definitively stated. Interestingly, in the aforementioned study [17] 5G4 positive lesions were found in some of the normal controls. This could either be another methodological issue (supported by the fact that no positive samples among the controls in our study were found) or a PNS manifestation of incidental Lewy body disease, as p-α-syn [19,20] and PK resistant aggregates [21] have previously been shown in the enteric nervous system of NC, their incidence increasing with age.

Due to limitations of IHC it is not possible to clearly classify the stained higher molecular weight protein into oligomeric or aggregated. 5G4 is targeted against a disease associated epitope available both on the β-sheet rich oligomers as well as on aggregates [14]. The presence of ASyO5-positive deposits detectable even after an intensive digestion with PK strongly suggests that ASyO5 also stains aggregates, as oligomers were shown to have an intermediate degree of PK resistance [22]. The preferential binding of ASyO5 to oligomers/aggregates is achieved due to a higher antibody affinity to a ligand with more epitopes at higher dilution and might also explain the recently reported increase in total α-syn in PD when analogously stained with a highly diluted anti-total-α-syn antibody [23]. If the same antibody was used at a moderate dilution, no difference between patients and controls was seen [24].

Similarly to PD, aggregated α-syn was found in the majority of the MSA-P cases. Deposits were mostly limited to somatosensory subepidermal and dermal plexuses, as has been shown for p-α-syn in our previous work [11] and confirmed most recently [23].

In conclusion, using a set of immunohistochemical methods we could demonstrate that α-syn deposits in the dermal nerve fibers in PD are similar in regards to their aggregation state to the α-syn lesions in the brain. This is yet another striking similarity with prion diseases, where involvement of dermal nerves has also been shown recently [26]. However, the intriguing question of *when* the skin pathology begins in the course of disease is yet to be determined for either condition. This finding supports the use of skin biopsy IHC as a specific tool in the diagnostic work-up of synucleinopathies and as an easily accessible tissue for research on α-syn pathology in general. For diagnostic purposes, staining of aggregated or truncated α-syn using the studied antibodies was not superior to the comparatively better established anti-p-α-syn stain.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parkreldis.2019.03.003.

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Author contributions

Conceptualization C.S., K.D.; Methodology K.D., A.K., Investigation A.K., L.S., Resources C.M., Writing – Original Draft K.D., A.K., Writing – Review & Editing A.K., K.D., C.S., J.V., C.M., L.S., Supervision K.D., C.S.; Project Administration C.S., J.V.; Funding Acquisition C.S., K.D.

Declarations of interest

None.

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