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REVIEW ARTICLE

Skin nerve α-synuclein deposits in Parkinson's disease and other synucleinopathies: a review

Vincenzo Donadio[1]

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Abstract

Purpose The in vivo diagnosis of synucleinopathies is an important research aim since clinical diagnostic criteria show low accuracy. The skin innervation, especially the autonomic subdivision, is a useful region to search for abnormal α-syn aggregates in synucleinopathies since the peripheral sympathetic nerves can be the earliest-affected neural region and autonomic symptoms may precede the classical symptoms of these disorders.

Methods The major advantages of skin biopsy as an in vivo diagnostic tool for synucleinopathies are that it is an inexpensive and easy-to-perform technique requiring only limited facilities, and that it is repeatable in long-term studies as it causes only minor discomfort to the patient.

Results This review analyzes current progress in this area of research that may facilitate the standardization of this method, potentially eliminating differences among laboratories in the implementation of the method.

Conclusions The most suitable and commonly used technique for identifying in vivo α-syn aggregates in skin nerves is indirect immunofluorescence, although several aspects of this approach need to be standardized, particularly when synucleinopathies without autonomic failure present a patchy distribution of abnormal α-syn aggregates in skin nerves. By contrast, synucleinopathies with autonomic failure may present widespread diffusion of abnormal aggregates in autonomic skin nerves.

Keywords Phosphorylated α-synuclein · Skin biopsy · Pure autonomic failure · Idiopathic Parkinson's disease · Dementia with Lewy bodies

Introduction

Synucleinopathies are neurodegenerative disorders characterized by the abnormal deposition of misfolded α-synuclein (α-syn), leading to neuron dysfunction and death [1]. They include acquired (i.e., idiopathic) and genetic disorders. The idiopathic variants are particularly frequent neurodegenerative disorders; indeed, Parkinson's disease (PD) is the second most common neurodegenerative disease—it is estimated to occur in approximately 1% of individuals > 60 years of age [2]. After Alzheimer's disease, dementia with Lewy bodies (DLB) is the second most common form of neurodegenerative dementia [3]. Other clinical variants of synucleinopathy are rare and include multiple system atrophy (MSA) and pure autonomic failure (PAF) [4]. In clinical practice, these disorders are mainly diagnosed based on clinical criteria. This frequently leads to misdiagnosis and thus delayed treatment or an increased risk of drug-related adverse events. The clinical accuracy of PD diagnosis is low; around 50% in the early stages of the disease [5]. For that reason, the in vivo diagnosis of these disorders is an important research aim, although reliable biomarkers are still lacking [6]. The skin innervation, especially the autonomic subdivision, is a useful region for searching for abnormal α-syn aggregates since the peripheral sympathetic nerves can be the earliest-affected neural region and autonomic symptoms may precede the classical symptoms of synucleinopathy, such as motor dysfunction in PD or dementia in DLB [7–9]. Furthermore, PAF is characterized by isolated peripheral autonomic nerve dysfunctions. These findings have driven research focusing on the analysis of the easily accessible skin innervation—which contains a large number of autonomic nerve fibers—to detect misfolded α-syn aggregates, such as autopsy studies of patients with synucleinopathies [10–13]. However,

✉ Vincenzo Donadio
vincenzo.donadio@unibo.it

[1] IRCCS Istituto Delle Scienze Neurologiche di Bologna (Italy), UOC Clinica Neurologica, Via Altura 3, 40139 Bologna, Italy

this approach is somewhat problematic due to the low rate of detection of abnormal α-syn aggregates (mainly in the abdomen; see Table 1) and other technical challenges [13]. The possibility of staining abnormal α-syn deposits in skin nerves in vivo has also been explored in numerous studies over the last few years [14–26]. Those independent studies demonstrated that abnormal α-syn deposits in skin nerves could be used to distinguish between patients with synucleinopathies from different neurodegenerative disorders (i.e., tauopathies) and healthy control subjects with high sensitivity and specificity (Table 2). In addition, Doppler et al. [16] reported that this approach was able to identify abnormal α-syn deposits in skin nerves and postmortem brain tissue of PD patients, supporting the idea that this approach could be applied as a diagnostic tool for synucleinopathies in vivo. The major advantages of skin biopsy as an in vivo diagnostic tool for synucleinopathies are that it is an inexpensive (a few tens of euros per patient for the reagents and a few hundreds of euros per patient for the laboratory work and all the necessary facilities) and easy-to-perform technique, although analyzing the skin sample is a technically challenging process that cannot be done in a routine laboratory setting. Also, this test can be repeated in long-term studies as it causes only minor discomfort to the patient. Taking these aspects into account, skin biopsy appears to be a promising diagnostic tool for synucleinopathies, and to be suitable for the in vivo study of pathogenetic mechanisms correlated with misfolded α-syn deposition in neurons.

However, different methods of identifying abnormal α-syn deposits have been described, and the ideal approach and optimal biopsy site(s) need to be defined. In this paper, the technical and procedural aspects of the search for abnormal α-syn aggregates in skin nerves in vivo, as well as the different methods that have been employed to detect these aggregates will be investigated. This review summarizes current progress in this area of research that should facilitate the standardization of the method used to detect abnormal α-syn aggregates in skin nerves, thus potentially eliminating differences between the approaches used in different laboratories.

Immunohistochemistry procedures

The skin biopsy is generally performed in vivo using a 3-mm punch biopsy; exceptions include Doppler et al. [16, 21, 25], who used a 5-mm punch, and Miki et al., who used a 6-mm punch [14]. It is usually carried out under sterile conditions after local anesthesia, and usually without suture (Fig. 1).

Skin sections are usually cut frozen using a cryostat, but the thickness of the analyzed skin section varies widely, ranging from 3 to 50 µm (Table 2), which is mainly a reflection of differences in the underlying immunohistochemical technique used. The majority of the reported studies used an indirect immunofluorescence technique that utilizes primary antibodies against abnormal α-syn aggregates and skin nerves—usually the pan-neuronal marker protein gene product PGP 9.5. However, PGP 9.5 does not allow the identification of specific subtypes of skin fibers (i.e., sympathetic or somatic). This limitation is particularly important when skin annexes innervated by multiple neurotransmitters are evaluated in the presence of disorders that selectively affect specific subtypes of skin fibers (e.g., pure autonomic failure only affects autonomic fibers). In disorders involving all types of skin fibers, such as small-fiber neuropathy, this limitation is less important. Subsequently, primary antibodies are revealed by secondary antibodies associated with cyanine dye fluorophores that are visible using a confocal microscope with appropriate fluorescent filters. The use of distinct fluorophores allows different primary antibodies to be combined in a co-localization study, which is important for establishing the deposition of α-syn aggregates inside skin nerve fibers. By contrast, a few studies have used a bright-field immunohistochemistry technique in which the primary antibody against abnormal α-syn deposits is revealed by streptavidin/biotin and chromogen/

Table 1 Autopsy-based studies focusing on the detection of misfolded α-syn aggregates in skin nerves

Authors	Year	No. of patients	Disorder(s)	Technique	Skin section (µm)	Primary AB	Skin site	% of positive patients
Ilkemura et al.	2008	269	PD, DLB	BFI	6	P-syn 129	Arm/abdomen	20
Brach et al.	2010	92	PD, DLB, ILBD, ADLB	BFI	6	P-syn 129	Abdomen	0
Gelpi et al.	2014	15	PD, DLB	BFI	5	P-syn 129	Abdomen	0
Gibbons et al.	2017	11	PD	BFI	5	Native (binding sites from amino acids 111–131)	Scalp, abdomen	100

BFI bright-field immunohistochemistry, *PD* Parkinson's disease, *DLB* dementia with Lewy bodies, *ILBD* incidental Lewy body pathology, *ADLB* Alzheimer's disease with Lewy body pathology

Table 2 In vivo studies focusing on the detection of misfolded α-syn aggregates in skin nerves

Authors	Year	No. of patients	Disorder(s)	Fixative		Skin sections		Primary antibody	Technique	Skin site	Syn positivity rate	
				Type	Time	Thickness (μm)	Number				(%)	Site(s) with the Higest rate
Miki et al.	2012	20	PD	Paraffin	NS	6	3	P-syn 129 (monoclonal)	BFI	Chest wall and leg	10	Proximal
Donadio et al.	2013	9	PAF	Zamboni	12 h	10	4	P-syn 129 (M monoclonal)	IF	Two samples in C7, thigh, and leg	100	All
Wang et al.	2013	20	PD	Zamboni	18 h	50	20	Native* (polyclonal)	IF	Proximal/distal thigh and leg	100	All
Doppler et al.	2014	31	PD	4% PFA	NS	20	3	P-syn 129 (NS)	IF	Th 12, proximal/distal leg, and finger	52	Proximal
Donadio et al.	2014	21	PD	Zamboni	12 h	10	4	P-syn 129 (M monoclonal)	IF	Two samples in C7, thigh, and Leg	100	Proximal
Navarro-Otano et al.	2014	6	PD	Paraffin	NS	5	NS	P-syn 129 (NS)	BFI+	Distal leg	0	–
Zange et al.	2015	10, 10	PD, MSA	Paraffin	24 h to 2 weeks	3	NS	P-syn 129 (M monoclonal)	BFI	Forearm	100, 0	–
Doppler et al.	2015	30, 12	PD, MSA	4% PFA	30 min	50	5	P-syn 129 (NS)	IF	Th12, thigh, and leg	67 for both	Proximal
Donadio et al.	2016	14, 16	PAF, PD	Zamboni	12 h	10	4	P-syn 129 (monoclonal)	IF	Two samples in C7, thigh, and leg	100 for both	PD: proximal PAF: all sites
Gibbons et al.	2016	28	PD	Zamboni	18 h	50	20	Native* (polyclonal)	IF	Proximal/distal thigh, distal leg, and forearm	100	All
Donadio et al.	2017	18	DLB	Zamboni	12 h	10	4	P-syn 129 (R monoclonal)	IF	Two samples in C7, thigh, and leg	100	Proximal
Doppler et al.	2017	25, 18	PD, RBD	4% PFA	30 min	20	5	P-syn 129 (NS)	IF	C7, Th10, thigh, and leg	80, 56	PD: no difference iRBD: proximal
Antelmi et al.	2017	12	RBD	Zamboni	12 h	10	4	P-syn 129 (R monoclonal)	IF	Two samples in C7 and leg bilaterally	75	Proximal

BFI bright-field immunohistochemistry, *M monoclonal* mouse monoclonal, *R monoclonal* rabbit monoclonal, *PD* Parkinson's disease, *DLB* dementia with Lewy bodies, *MSA* multiple system atrophy, *PAF* pure autonomic failure, *iRBD* idiopathic REM behavior disorder, *PFA* paraformaldehyde, *NS* not specified, * binding sites at amino acids 111–131, + citrate buffer was used for antigen retrieval in this work (antigen retrieval was not performed in the other works)

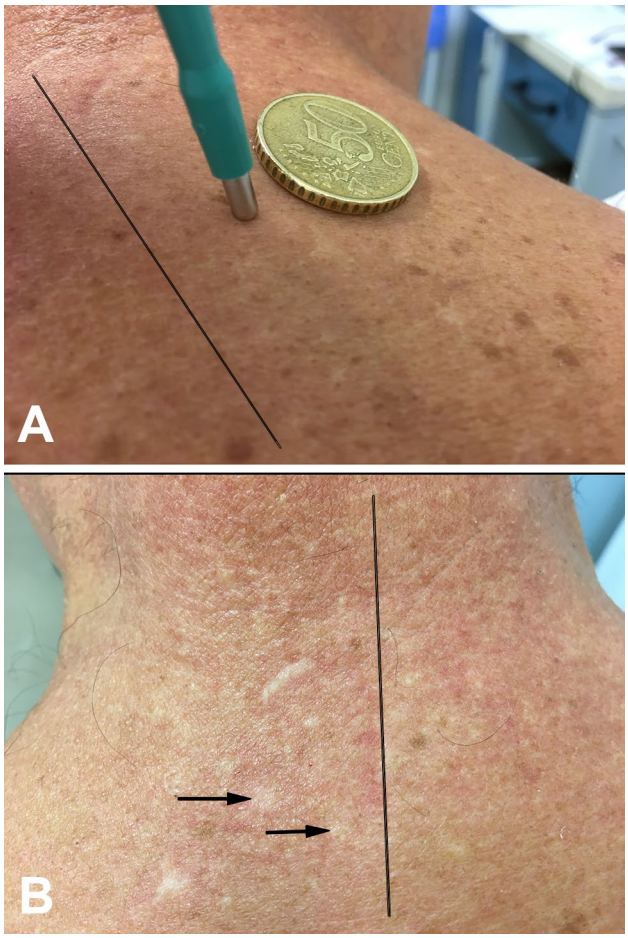

Fig. 1a–b Punch skin biopsy from the C7 paravertebral skin area. **a** The skin biopsy can be taken using a 3-mm punch with a diameter of less than that of a small coin. A small-diameter punch biopsy imparts only minor discomfort to the patient. **b** The biopsy does not require suture and leaves a small, scarcely visible scar (*arrows*). The *continuous black line* corresponds to the midline spine and connects spinous processes of the cervical vertebrae

peroxidase reactions (Tables 2). Bright-field immunohistochemistry is a simpler technique than indirect immunofluorescence but it does not allow the combination of different individually identified signals, which means that a co-localization study for establishing the intraneural deposition of α-syn aggregates cannot be performed.

In any case, reported immunohistochemical procedures for detecting α-syn aggregates in skin nerves differ widely among laboratories in terms of the type of fixation used, the primary antibody applied, and the signal development method employed. Standardization of these procedures is desirable to facilitate the large-scale automation of this technique.

Fixative

Different fixative solutions are used depending on the technique adopted. In the indirect immunofluorescence technique, skin samples are usually fixed in cold 4% paraformaldehyde or Zamboni's (4% paraformaldehyde plus picric acid) fixative for a period ranging from 30 min at room temperature [16, 21, 25] to 4 °C overnight (approximately 18 h) [15, 17–20, 22, 26, 27]. Although a direct comparison between these two different fixative methods is lacking, a recent study involving two European laboratories (Würzburg, Germany and Bologna, Italy) underlined that these two fixative procedures showed similar findings, no difference in intraneural p-syn staining, and are both suitable for indirect immunofluoresce analysis of p-syn [27]. Bright-field immunohistochemistry requires paraffin-embedded samples fixed in 10% formalin for 24–72 h. Although a study comparing the fixative procedures used for indirect immunofluorescence and bright-field immunohistochemistry is not available, it should be noted that paraffin-embedded samples are fixed in a more highly concentrated solution (10% vs 4% of paraformaldehyde) and for a longer period (24–72 vs ≤ 18 h for paraformaldehyde and Zamboni), which tends to result in the overfixation of skin tissue, probably reducing peripheral nerve immunostaining [13]. This overfixation likely decreases the co-localization of the two different primary antibodies (i.e., those against PGP 9.5 and α-syn deposits), and it is the co-localization of different antibodies that allows the intraneural deposition of abnormal α-syn to be reliably established [13–27]. This is an important point, as nonspecific staining was reported to occur with both techniques when co-localization was not employed [17, 18, 28, 29]. Nonspecific signals are seen as a distinct staining of the primary antibody against abnormal α-syn in patients and healthy controls, when native (normal) α-syn is mistakenly stained in presynaptic terminals because of low epitope specificity of the primary antibody or because of precipitation of the fluorophore as dot-like background staining, particularly inside sweat gland tubules or vessel endothelium (Fig. 2A) [17–19, 27]. A possible strategy for improving the specificity of abnormal α-syn detection is to eliminate the native α-syn through pretreatment with proteinase K [28]. However, because of the technical challenges involved in studying paraffin-embedded tissue, it is thought that the utility of this technique for detecting cutaneous α-syn deposition is limited [13]. By contrast, the indirect immunofluorescence approach seems to be more appropriate for highlighting abnormal α-syn deposits, although it should be noted that this is a more complex technique that is difficult to standardize on a large scale.

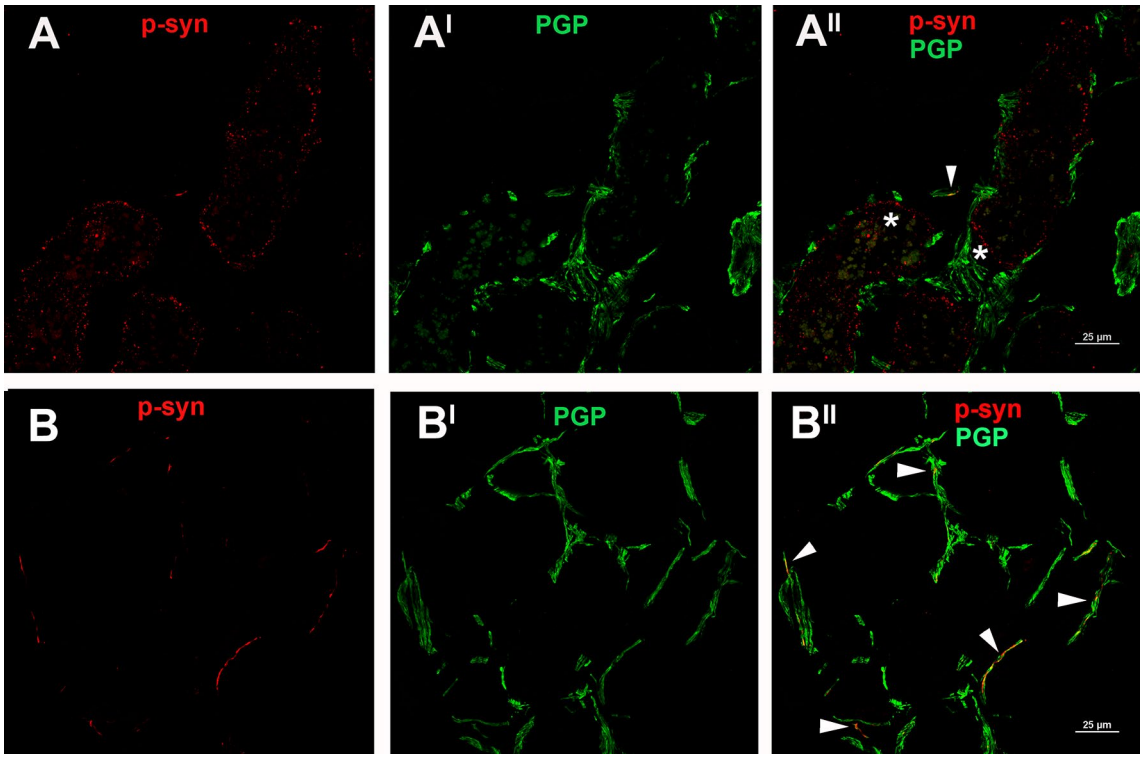

Fig. 2A–B False and true intraneural p-syn staining around sweat glands. **A** The primary antibody against p-syn produced a series of staining around sweat tubules. $\mathbf{A^I}$ The pan-neuronal marker PGP 9.5 is crucial for detecting the true intraneural deposition of abnormal α-syn through co-localization with the p-syn signal. $\mathbf{A^{II}}$ Using a combination of antibodies against p-syn and PGP 9.5 allows us to detect the true intraneural deposition of abnormal α-syn. In the true intraneural deposition, the p-syn and PGP 9.5 signals are co-localized (*arrowhead*); p-syn signal without co-localized PGP 9.5 staining (*asterisks*) may occasionally arise from the background (sweat gland tubules) as a nonspecific signal due to fluorophore precipitates. The nonspecific signal often presents a dot-like staining distribution that is different from true intraneural deposition, which exhibits a more continuous signal along a nerve fiber. **B** A distinct p-syn signal was observed upon using the indirect immunofluorescence technique. $\mathbf{B^I}$. The pan-neuronal marker PGP 9.5 can allow the detection of all nerve fibers around sweat tubules. $\mathbf{B^{II}}$ Using the combination of antibodies against p-syn and PGP 9.5, it was possible to establish the true intraneural deposition of abnormal α-syn by looking for co-localized signals (*arrowhead*)

Antibodies

Two types of primary antibodies have been used to detect abnormal α-syn deposits in skin nerves: (1) those against native α-syn (binding sites at amino acids 111–131) [13, 15, 22] and (2) those against α-syn phosphorylated at Ser129 (p-syn) [10–12, 14, 16–21, 23–27].

Staining native α-syn is useful for identifying large misfolded aggregates of this protein, i.e., Lewy bodies, in cerebral cell bodies during the autopsy analysis [30]. By contrast, in skin nerves, abnormal deposits are mainly found in autonomic nerve terminals, and the cell bodies of these neurons are located in the spinal ganglia. Accordingly, no Lewy bodies are detected in the skin innervation, only Lewy neuritis. The open question is whether an antibody against native α-syn will recognize Lewy neuritis. It has been proposed that the antibody that recognizes amino acids 111–131 of native α-syn is also able to detect misfolded aggregates of phosphorylated α-syn [13, 15]. However, the corresponding staining was also observed in healthy controls [15]. In addition, a direct correlation between antibodies against native α-syn (binding to sites in amino acids 111–131) and p-syn highlighted that misfolded aggregates of α-syn identified by antibodies against p-syn were only seldom stained by the antibodies against native α-syn, supporting the conclusion that the latter antibodies are not suitable for detecting α-syn aggregates in skin nerves [19], although staining with these antibodies may reflect the stage of disease and the degree of autonomic dysfunction, making such staining a biomarker of PD severity [15, 31]. By contrast, antibodies against p-syn were not correlated with the clinical severity of the disease [18, 19, 31], and they were never found in skin nerves of healthy controls, only in patients with supposed synucleinopathies, suggesting that staining with these antibodies could be a promising disease biomarker [31]. In a recent study, we tested additional antibodies against C-terminal α-syn epitopes involved in the post-translational modification of α-syn, such as Tyr125 or nitration at Tyr125–133.

However, these antibodies did not stain abnormal α-syn aggregates in skin nerves, so they are probably not useful as a disease biomarker [32]. Several working antibodies against p-syn are commercially available. However, because some of them may present nonspecific staining, a reliable approach is to refer to the working protocols of laboratories with considerable experience in skin p-syn staining using an indirect immunofluorescence method [16–21, 25, 26]. In this regard, the abovementioned comparative study between skin laboratories in Würzburg (Germany) and Bologna (Italy) found that monoclonal p-syn antibodies of different species produced by different companies (mouse p-syn, BioLegend, cat. no. 825701; rabbit p-syn, Abcam, cat. no. ab-51253) yielded similar results in terms of detecting abnormal α-syn aggregates in skin nerves [27].

Skin section thickness

Different skin section thicknesses have been used to search for p-syn in skin nerves (Table 2). Differences in thickness have mainly related to the technique used, as a thickness of less than 10 µm was adopted for paraffin-embedded tissue (i.e., bright-field immunohistochemistry), whereas frozen sections of thickness ≥ 10 µm were analyzed when using indirect immunofluorescence. Frozen sections present several advantages compared to paraffin-embedded sections, as thick skin sections provide a larger fraction of the skin volume, including a higher number of dermal autonomic annexes (such as sweat glands, blood vessels, and arrector pilomotor muscles) randomly scattered throughout the skin. Autonomic annexes are important when searching for p-syn, as abnormal α-syn deposits are mainly localized in skin autonomic nerves. Therefore, decreasing the sampling volume decreases the probability of appropriate specimen acquisition [13]. Furthermore, in the thinner paraffin-embedded tissue sections, nerve fibers appear as small dots rather than traceable nerve fibers, thus reducing the ability to co-localize p-syn within nerve fibers, which is important for differentiating true p-syn deposits from artefacts that are often visible in healthy subjects [17–21]. By contrast, bright-field immunohistochemistry has the advantage compared to indirect immunofluorescence of a more stable signal. Also, the fluorescent signal may dissipate with time, preventing the re-evaluation of stained skin sections over time, although in our experience the two signals that are needed to detect abnormal α-syn deposits (i.e., antibodies against PGP 9.5 and p-syn) are reasonably stable over several months if the slides are stored away from light. Bright-field immunohistochemistry, on the contrary, presents a more stable signal with a negligible decline over time. In addition, paraffin-embedded tissue (i.e., that used with bright-field immunohistochemistry) is the method commonly used in neuropathology laboratories, so it is a better candidate for the widespread automation of a new method. Despite these advantages, the technical challenges of bright-field immunohistochemistry reported above and the experience gained so far with indirect immunofluorescence, which suggests that it yields a higher positive rate for α-syn deposits (Table 2), point to the latter technique being more suitable for detecting p-syn in skin nerves, whereas the utility of bright-field immunohistochemistry appears limited [13]. Nevertheless, the optimal thickness of skin sections analyzed by indirect immunofluorescence has not been established. We have reported that p-syn staining was visible at thicknesses of both 10 and 50 µm [19]. Preliminary data showed that a thickness of 10 µm led to more intense staining, but a thick section (i.e., 50 µm) allows a larger skin volume to be analyzed, which could be a real advantage, particularly in disorders with patchy α-syn deposition, such as PD or DLB without orthostatic hypotension (OH; see below). Thus, a focused indirect immunofluorescence study comparing thin (10 µm) and thick (50 µm) skin sections is needed to establish the optimal section thickness in synucleinopathy, particularly in disorders leading to patchy α-syn aggregates in skin nerves.

In vivo skin sites and number of samples

Available data indicate that a patchy expression of p-syn throughout skin sites (i.e., proximal vs distal) is a biomarker of the most frequent variants of synucleinopathy, i.e., DLB and PD [16, 18–20], whereas synucleinopathies leading to autonomic failure such as OH (i.e., PAF, PD with OH and DLB with OH) show widespread deposits of p-syn in nearly all analyzed proximal and distal skin samples [17, 20, 32, 33]. Although the relevant data have mainly originated from a few skin biopsy laboratories and therefore require confirmation on a larger scale, the diffuse p-syn depositions seen in autonomic skin nerves in synucleinopathies with OH have important pathogenetic and practical implications. The widespread p-syn involvement of autonomic skin nerves in patients with OH may suggest that autonomic failure associated with PAF, PD, and DLB is characterized by diffuse damage to the peripheral autonomic innervation, which significantly contributes to the underlying pathogenesis of these disorders. From a practical point of view, these data may indicate that the choice of skin site may be irrelevant when searching for abnormal α-syn aggregates in synucleinopathies associated with OH because of the likely diffuse p-syn deposition. By contrast, synucleinopathies without OH may be associated with a patchy distribution of p-syn in skin nerves, meaning that appropriate site selection is crucial to increasing the probability of detecting misfolded p-syn. At the same time, the number of skin samples and sections analyzed may also be relevant when the distribution of α-syn aggregates in skin nerves is likely to be irregular.

Skin site

According to the available data, in PD as well as DLB without OH, the incidence of p-syn in autonomic nerves differs between proximal and distal skin sites, with the highest incidence observed in proximal sites [16, 18–20], although a nonsignificantly higher incidence at the distal skin site in PD was reported in a more recent paper [25]. Interestingly, the same distribution with the highest incidence of p-syn at proximal sites has been described in idiopathic REM sleep behaviour disorder (iRBD), a clinical condition that often precedes PD and DLB [25, 26]. As previously specified, these data originate from only a few skin laboratories and require confirmation on a larger scale. However, proximal sites included the skin around the spine, i.e., close to autonomic ganglia, and the detection of p-syn with a higher incidence at this site may suggest that misfolded α-syn comes from ganglia and spreads to the closest autonomic skin terminals first. According to this hypothesis, abnormal α-syn deposits may reach the longest autonomic terminals in the distal leg later and to a lesser extent. By contrast, it is possible to speculate that the widespread p-syn distribution in skin autonomic nerves in disorders associated with OH support a different pathogenetic mechanism, with misfolded α-syn arising in autonomic nerve terminals, or the increased α-syn aggregates observed in these disorders could be due to more favorable predisposing conditions.

Data from our laboratory indicated a higher incidence of p-syn in the skin of the paravertebral cervical C7 area than in the skin of the thoracic Th12 area, implying a spine gradient [34], although a different study showed a slightly higher incidence of p-syn in the thoracic area as compared to the cervical skin region [25]. Importantly, analysis of the distribution of p-syn in the skin at C7 in patients with unilateral PD showed that abnormal deposits were evenly distributed between the affected and unaffected sides, suggesting that the distribution of p-syn in skin nerves may be independent of motor and nigrostriatal damage, whereas PD patients with bilateral motor symptoms presented widespread diffusion of p-syn along the spine. These findings point to the need to perform skin biopsies on both cervical sides when searching for p-syn in PD patients with prevalent unilateral motor dysfunction [34].

The p-syn in skin nerves in MSA presented a peculiar distribution, with rather selective involvement of somatosensory fibers [21, 32], although the pathogenetic role of these abnormal deposits remains to be established. In particular, it is necessary to define whether these abnormal deposits are associated with decreased epidermal nociceptors and a possible sensory deficit. A slight prevalence in the spine region was also reported in MSA [21], but a different pattern of distribution with higher prevalence in the leg was subsequently reported [32]. In addition, no p-syn staining in skin nerves of MSA patients was observed in a different study [24]. These contrasting findings suggest that more studies involving a larger cohort of patients are needed to clarify the distribution pattern of p-syn in skin nerves in MSA. Taken together, available data support the hypothesis that the skin around the spine is the optimal site to search for p-syn in skin nerves for diagnostic purposes in synucleinopathies without autonomic failure except MSA, for which more data are needed to identify the optimal site. Among spine sites, the cervical area is preferable because a thoracic site may cause discomfort to the patient, particularly when sitting.

Number of skin samples and sections

The search for p-syn in skin nerves was performed in vivo using one skin sample 3, 5, or 6 mm in diameter [14, 16, 21, 25–27] or two samples (3–4 cm away) 3 mm in diameter taken close together [17–20, 26, 31–33] at proximal and distal sites (Table 2). The number of skin sections per sample that were used to search for p-syn ranged from 3 to 5; these were obtained at regular intervals throughout the sample (Table 2). When two skin samples were taken, the number of skin sections analyzed was obviously double the number analyzed with one skin sample.

Taking two close skin samples led to a higher rate of p-syn positivity than taking one skin sample of the same diameter (i.e., 3 mm) in synucleinopathy without autonomic failure [18–20, 26, 33]. This is probably due to the higher number of skin sections analyzed, which increased the possibility of detecting patchy p-syn such as that found in PD and DLB. In line with this explanation, the p-syn positivity rate identified via two skin samples was often higher than that detected with one sample when analyzing skin sections of the same thickness (Table 2) [16, 18, 19, 25, 26, 34]. In fact, in cervical skin at C7, PD patients showed a p-syn positivity rate of 100% with two close skin samples [18, 19] but 40% when only one sample was taken [25]; these values were 62% [34] vs 35% [16] in skin at Th12, and 24–31% [18, 19] vs 13–60% [16, 25] for the distal leg. In iRBD, skin at C7 presented a p-syn positivity rate of 67% when two samples were analyzed [26], but 50% with one sample [25]. The corresponding values were 58% [26] vs 50% [25] for the distal leg. This correlation obtained by extrapolating data from published papers is, however, rather sketchy as the number of recruited patients and the selection criteria adopted differed among the various studies. However, it may suggest that the number of skin sections analyzed is strongly correlated with the probability of detecting abnormal α-syn aggregates in skin nerves. Data from Doppler et al. also support this conclusion, since they reported that the number of PD, MSA, and iRBD patients found to be positive for p-syn in skin nerves rose upon increasing the number of skin sections analyzed [16, 21].

Anyway, the optimal number of skin samples and sections needed to detect p-syn in skin nerves remains to be defined in synucleinopathies without autonomic failure. A focused study with this specific aim is desirable to affirm skin biopsy as a diagnostic tool for synucleinopathy.

Conclusions

Skin biopsy is a promising diagnostic tool for synucleinopathies because of its high specificity, its low cost, and because it causes only minor discomfort to the patient. The most suitable and commonly used technique for identifying α-syn aggregates in skin nerves is indirect immunofluorescence. Rather than presenting some advantages over indirect immunofluorescence, bright-field immunohistochemistry has technical challenges and limitations preventing its use in this field. Nevertheless, experience with the indirect immunofluorescence technique is restricted to a few skin biopsy laboratories, and several aspects of it need to be standardized. According to the available data, the distribution of abnormal α-syn aggregates in skin nerves depends on whether autonomic failure is present. In synucleinopathies associated with autonomic failure (except for MSA), α-syn aggregates show widespread diffusion in autonomic skin nerves. By contrast, disorders without autonomic failure present a patchy distribution of abnormal aggregates in the skin innervation. In the latter disorders, the ideal skin site(s), the appropriate number of skin samples and sections, and the optimal section thickness to analyze in order to obtain the highest sensitivity and specificity remain to be defined. Similarly, more studies involving larger cohorts of patients are needed to clarify the distribution pattern of p-syn in mainly somatic skin nerves in MSA patients. The future of this field is the large-scale standardization of skin biopsies when used as a tool for the diagnosis of synucleinopathies. This standardization concerns the technical procedures (i.e., indirect immunofluorescence) used to detect misfolded α-syn aggregates as well as the skin sites to be analyzed. Another important future aim is the automation and quantification of skin biopsy findings, which may lead to a more objective measure of p-syn load in skin nerves.

Compliance with ethical standards

Conflict of interest There is no conflict of interest to report for this manuscript.

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