Alpha-Synuclein in Skin Nerve Fibers as a Biomarker for Alpha-Synucleinopathies

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The common pathological features of synucleinopathies are abnormal aggregates of the synaptic protein alpha-synuclein (αSN) in the cytoplasm of neurons or glia. These abnormal aggregates appear several years before the onset of clinical manifestations, and so the early detection of αSN in body fluids or peripheral tissues (e.g., cerebrospinal fluid, colonic mucosa, salivary glands, and skin) is considered a potential tool for identifying synucleinopathies. Performing a skin biopsy is a practical option because it is a relatively noninvasive, safe, and reliable method to measure αSN deposition in the peripheral nervous system. Moreover, there is growing research interest in the use of cutaneous synuclein deposition as a biomarker for synucleinopathies. The aim of this study was to interpret the current data on cutaneous αSN deposition and present the current perspectives and future prospects.

Key Words alpha-synuclein, Parkinson's disease, Lewy bodies, multiple system atrophy, skin, biopsy.

INTRODUCTION

Alpha-synucleinopathies are a group of chronic, progressive neurodegenerative disorders that include idiopathic Parkinson's disease (IPD), dementia with Lewy bodies (DLB), multiple-system atrophy (MSA), and pure autonomic failure (PAF).[1] Similar to other neurodegenerative diseases, the prevalence and incidence of synucleinopathies increase with age. The key pathological findings of α-synucleinopathies are fibrillary aggregates of the protein alpha-synuclein (αSN) in the cytoplasm of neurons, neuritis, and glia, which have usually been found in neurons in IPD, DLB, and PAF, but in glia in MSA, thereby interfering with the axonal transport of synaptic proteins and promoting mitochondrial deficit and oxidative stress.[2] While synucleinopathies share the same pathological protein, they exhibit diverse clinical features.

Synucleinopathies are primarily diagnosed based on their respective clinical diagnostic criteria. The overlapping presentations and transitional phenotypes make an accurate diagnosis challenging. Some studies assessing the clinical diagnosis in early stages of the development of synucleinopathy found that the diagnosis accuracy was far lower than expected.[3-8] One clinicopathological study identified that the accuracy was 53% for a clinical diagnosis of PD in subjects with early PD responsive to medication (disease duration <5 years), and only 26% for PD in untreated or not-clearly-responsive subjects.[4] In another clinicopathological study of 100 cases, 76 subjects had neuropathologically confirmed PD, but many cases had a neuropathological diagnosis of progressive supranuclear palsy, MSA, or Alzheimer's disease despite the clinical diagnosis of PD.[5] The sensitivity of clinical diagnoses of MSA and DLB is very low, and more than half of patients with MSA or DLB remain either undiag-

nosed or misdiagnosed.[6-8] The clinical diagnosis of synucle-inopathies alone, especially during the early stages, is not particularly accurate, which indicates the need to identify a reliable biomarker.

Early detection in the prodromal stages is important for a prompt and effective intervention to treat and eventually slow the disease progression (as disease-modifying therapies are identified), ultimately leading to improving the patient's quality of life. Accordingly, the need for a more-accurate diagnostic tool for the early or prodromal stages is expanding. There is increasing evidence that the pathological process starts years before the onset of clinical manifestations. In PD, it is known that motor signs first appear when >50% of substantia nigra dopamine neurons are lost.[9]

αSN deposition can be detected in the early stage of disease in the nerve fibers in colonic submucosa and in neurons of the submucosal Meissner's plexus.[1,10-12] In addition, there is accumulating evidence of multiple organs being involved in synucleinopathies, since αSN pathology is detected in various peripheral tissues including the skin, salivary glands, sympathetic ganglia, vagus nerve, gastrointestinal tract, genitourinary tract, and heart.[13-18] Among the peripheral tissues that have been studied, the skin is one of the most-easily accessible organs, and is suitable for both single and repeated sampling. Although it is well established that αSN deposition can be seen in samples obtained from the gastrointestinal tract in PD, their acquisition procedure involves an invasive colonoscopy or endoscopy, requiring sedation and prior preparation. In addition, skin biopsies are already used for several indications, such as for determining the intraepidermal nerve fiber densities to detect and monitor small-nerve-fiber neuropathy.[19] Moreover, performing a skin biopsy is safe and minimally invasive. Thus, the identification and quantification of αSN deposition in cutaneous biopsy samples could become a valuable tool for α-synucleinopathies, as already suggested by several investigators.[20-31]

However, previous studies of cutaneous αSN deposition have produced inconsistent results. Systematic reviews found wide ranges for the sensitivity and specificity in detecting αSN pathology using skin biopsies, of 0−100% and 80−100%, respectively.[17,32] These marked differences in study results could be due to several factors such as the study design and other methodological differences. If the quantification of cutaneous αSN deposition using skin biopsy provides a reliable measure across studies, cutaneous αSN deposition could be a useful biomarker for α-synucleinopathies.

The purpose of this study was to evaluate and interpret the current data on cutaneous αSN deposition, to clarify its role as a potential biomarker in synucleinopathies, and to determine problems to be addressed before establishing αSN de-position as a biomarker and surrogate outcome.

THE ROLE AND DISTRIBUTION OF αSN

αSN is one of the key molecules in the pathogenesis of synucleinopathies. It is one of the normal proteins in soluble cytosolic brain fractions and is mainly localized at presynaptic terminals.[33] Its detailed physiological functions are still unclear, but recent studies suggest that it plays a key role in synaptic functions in cooperation with cysteine-string protein-α, which contains a typical domain for HSP40-type molecular cochaperones.[34] Physiologically, in its normal (or native) form, αSN modulates the stability of the neuronal membrane and influences presynaptic signaling and membrane trafficking via vesicular transport.[32] Environmental influences such as neurotoxins, low pH, and high temperature, as well as genetic mutations may result in misfolding of αSN that leads to its polymerizing into fibrils and accumulating throughout the nervous system, as represented by pathological hallmark inclusions such as Lewy bodies, Lewy neurites, and glial cytoplasmic inclusions.[32,35] There are increasing reports of αSN within Lewy bodies undergoing several posttranslational modifications, including phosphorylation, ubiquitination, cross-linking, truncations, and nitration, suggesting that these modifications play a key role in the regulation of αSN aggregation and toxicity *in vivo*.[36,37] In particular, the aberrant accumulation of phosphorylated αSN (p-αSN) at the residue serine 129 has been reported to be prominent in PD and enhance αSN toxicity both *in vivo* and *in vitro*, possibly by increasing the formation of αSN aggregates.[37] Recent studies have suggested that phosphorylation at serine 129 inhibits the interaction of αSN with membrane phospholipids or phospholipase D2 and promotes the formation of αSN filaments as well as oligomers, eventually causing neuronal death along with other alterations in the activities of various kinases and phosphatases.[37]

Early postmortem studies to detect αSN deposition used anti-αSN antibodies against amino-terminal and carboxyl-terminal sequences of the 140-amino-acid αSN protein by immunohistochemically staining brain tissues from PD or DLB patients.[38] Immunohistochemical studies measuring total αSN (both p-αSN and nonphosphorylated αSN) use a polyclonal antibody recognizing multiple binding sites from amino acids 111−131 of αSN, whereas those measuring p-αSN use a monoclonal antibody against p-αSN at serine 129. Some previous studies showed that total αSN is detectable in healthy individuals as well as patients with synucleinopathies,[23,26,27] which challenges the specificity of total αSN as a reliable biomarker for α-synucleinopathies. On the other hand, p-αSN is not usually found in healthy controls, and some authors have

suggested that p-αSN deposits would be a more-reliable biomarker than αSN for diagnosing PD and other synucleinopathies.[22,29,36] However, the exact role of p-αSN aggregation and toxicity *in vivo* remains controversial.

PREVIOUS STUDIES OF αSN DEPOSITION IN CUTANEOUS NERVE FIBERS

The number of studies evaluating αSN deposition within cutaneous nerve fibers has increased in recent years. Most of these studies evaluating synuclein deposition in the dermal nerve fibers used a case–control design with clinically diagnosed cases, while only a few studies used tissue from cases verified by autopsy.[13,14,20-31,39,40] Table 1 summarizes the studies that used autopsy samples, while Table 2 summarizes the studies that used *in vivo* samples from clinically diagnosed subjects.

Studies of autopsy cases

The methods used in the four postmortem studies were comparable in regard to tissue preparation, with the autopsy samples being fixed in formalin or formaldehyde solution (in 3.75% or 10% solution for more than 24 hours), embedded in paraffin, sectioned (at 5−6 µm), and stained.[13,14,39,40] One major difference was the antibodies used: two studies selected antibodies against p-αSN, while the other two studies used antibodies against total αSN. Another significant methodological difference was the method of image analysis, with two studies evaluating αSN deposition using light microscopy[14,39] and the others using fluorescence microscopy.[13,40] The two postmortem studies that used light microscopy identified multiorgan involvement of αSN pathology, but no cutaneous αSN deposition in either the patient or control groups.[14,39] The two postmortem studies using fluorescence microscopy found αSN deposition in the dermal fibers of patients, each using a different antibody; the study of Ikemura et al.[13] showed a sensitivity of p-αSN immunoreactivity of 70% in PD and PD with dementia and 40% in Lewy-body disease, and a specificity of 100%. This suggests that Lewy-body-related pathology also involves cutaneous nerves in Lewy-body disease, and that skin biopsies may be of value in PD or Lewy-body disease presenting with advanced autonomic failure. The more-recent study of Gibbons et al.[40] assessed total αSN with immunostaining and confocal microscopy in autopsy samples obtained from abdominal skin and the scalp of 11 individuals with PD, and compared this to 5 controls with nonsynucleinopathy. That study found that the amount of αSN deposited in cutaneous autonomic nerves was significantly greater in subjects with a postmortem confirmation of a diagnosis of PD than in

Table 1. Summary of studies measuring cutaneous synuclein deposition in autopsied cases

Reference	Cases	HC	Sites	Section thickness (µm)	Fixative / Microscope type	Antibody αSN	Antibody p-αSN	Results
Ikemura et al.[13]	279 prospective cases 142 retrospective cases with CNS LB stage ≥II	194 HC without CNS LB pathology	Arm Abdomen	6	4% paraformaldehyde or 10% formalin FM		+	p-αSN (–) in 194 HC without CNS LB pathology SPE: 100% SEN: 70% in PD & PDD; 40% in DLB
Beach et al.[14]	17 PD 9 DLB 7 ILBD 19 ADLB	23 HC 17 ADNLB	Abdomen Scalp	6	3.75% formaldehyde LM		+	(–) Abdominal skin & scalp in patient & HC groups SEN: 0%; SPE: 100%
Gelpi et al.[39]	10 PD 5 DLB	13 non-LBD HC	Abdomen	5	10% formaldehyde LM	+		(–) Abdominal skin in patient & HC groups SEN: 0%, SPE: 100%
Gibbons et al.[40]	11 PD	5 HC	Abdomen Scalp	5	10% formalin FM	+		Greater deposition of αSN within pilomotor, sudomotor, & vasomotor nerve fibers in PD SEN: 100%

αSN: alpha-synuclein, ADLB: Alzheimer's disease with Lewy bodies, ADNLB: Alzheimer's disease but no Lewy body pathology, DLB: dementia with Lewy bodies, FM: fluorescence microscope, HC: healthy controls, ILBD: incidental Lewy-body disease, LB: Lewy bodies, LBD: Lewy bodies disease, LM: light microscope, p-αSN: phosphorylated alpha-synuclein, PD: Parkinson's disease, PDD: Parkinson's disease dementia, SEN: sensitivity, SPE: specificity.

Table 2. Summary of studies measuring cutaneous synuclein deposition in samples from clinically diagnosed subjects

Reference	Cases	HC	Sites	Biopsy punch diameter / Section thickness	Fixative	Antibody αSN	Antibody p-αSN	Results
Miki et al.[20]	20 PD	0 HC	Distal leg, Chest wall	6 mm, 6 μm	10% formalin		+	αSN (+) in only 2 cases, SEN: 10%
Doppler et al.[21]	31 PD	35 HC	Distal leg, Prox. leg, Back (Th12), Index finger	5 mm (leg, back), 3 mm (finger), 20 μm	4% paraformaldehyde		+	p-αSN (+) in 16/31 cases, in 0/35 HC, SEN: 52%, SPE: 100%
Donadio et al.[22]	21 PD	20 PAR, 30 HC	Distal leg, Distal thigh, Cervical (C8)	3 mm, 10 μm	Zamboni		+	p-αSN (–) in HC & PAR SPE: 100%, SEN of p-αSN (+) in PD: 100% at cervical, 52% at thigh, 24% at leg
Doppler et al.[23]	30 PD, 12 MSA	15 taupathy, 39 HC	Distal leg, Prox. leg, Back (Th12)	5 mm, 20 μm	4% paraformaldehyde		+	SEN: 75% in MSA & 73% in PD, SPE: 100%
Zange et al.[24]	10 PD, 10 MSA	6 ET	Volar forearm	3 mm, 3 μm	4% formaldehyde		+	p-αSN (+) in all PD, p-αSN (–) in MSA & ET, SPE: 100%
Haga et al.[25]	38 PD	13 MSA	Chest wall, Distal leg	6 mm, 60 μm	Zamboni		+	p-αSN (+) in 5.3% PD; p-αSN (–) in MSA
Wang et al.[26]	20 PD	14 HC	Distal leg, Distal thigh, Prox. thigh	3 mm, 50 μm	Zamboni	+		αSN (+) associated with Hoehn & Yahr score, autonomic dysfunction, SEN: 100%, SPE: 0%
Gibbons et al.[27]	28 PD	23 HC	Distal leg, Distal thigh, Prox. thigh, Mid-volar forearm	3 mm, 50 μm	Zamboni	+		SEN: >90%, SPE: >90%
Rodríguez-Leyva et al.[28]	17 PD	10 PSP, 17 HC	Occipital area	4 mm, 5 μm	4% paraformaldehyde	+		Higher αSN immunopositivity in PD
Donadio et al.[29]	16 PD, 14 PAF	15 HC	Distal leg, Distal thigh, Cervical (C8)	3 mm, 50 μm (αSN), 10 μm (p-αSN)	Zamboni	+	+	αSN (+) in PD, PAF & HC; p-αSN (+) in only PD & PAF, SEN of p-αSN (+) in PD: 100% at cervical, 75% at thigh, 31% at leg
Donadio et al.[30]	9 PAF	12 AAN, 15 HC	Distal leg, Distal thigh, Cervical (C8)	3 mm, 10 m	Zamboni		+	p-αSN (+) in all PAF, p-αSN (–) in HC & AAN, SEN: 100%, SPE: 100%
Donadio et al.[31]	18 DLB	13 AD, 6 FTD, 4 VD, 25 HC	Distal leg, Distal thigh, Cervical (C8)	3 mm, 10 μm	Zamboni		+	p-αSN (+) in all DLB, p-αSN (–) in HC & other dementia cases, SEN: 100%, SPE: 100%

αSN: alpha-synuclein, AAN: acquired autonomic neuropathy, AD: Alzheimer's disease, DLB: dementia with Lewy bodies, ET: essential tremor, FTD: frontotemporal dementia, HC: healthy controls, MSA: multiple system atrophy, p-αSN: phosphorylated alpha-synuclein, PAF: primary autonomic failure, PAR: parkinsonisms assumed not to have αSN deposits, PD: Parkinson's disease, prox.: proximal, PSP: progressive supranuclear palsy, SEN: sensitivity, SPE: specificity, VD: vascular dementia.

controls (sensitivity of 100%).[40] Comparing the results across studies according to the antibodies used reveals that those using p-αSN antibodies showed higher specificity than those using antibodies against total or nonphosphorylated αSN by providing an ordinal yes/no result. In contrast, total αSN provided a deposition gradient that was correlated with the disease severity but was also present in control subjects. The other main reason underlying the different results appears to be the imaging method used: fluorescence microscopy may provide αSN data that are superior to those attainable using light microscopy because it can visualize specific features of small samples such as skin sections and also enhance three-dimensional characteristics on small scales by attaching fluorescent tags to antibodies attached to targeted structures and using a high-intensity light source.

Studies of clinically diagnosed patients

Twelve studies have included biopsies in patients with a clinically confirmed diagnosis of synucleinopathy, and they had various designs.[20-31] Most of the studies focused on PD patients only. Nine case–control studies compared the differences among PD patients, controls, and/or patients with other synucleinopathies or neurodegenerative diseases assumed not to have αSN deposits.[20-28] The early study of Miki et al.,[20] which was a case series of 20 PD patients, found abnormal αSN accumulation in paraffin-embedded chest skin samples in 2 (10%) of the patients, suggesting that αSN in a skin biopsy sample assessed with conventional immunohistochemistry is not a sensitive diagnostic marker. However, more-recent studies support the role of cutaneous αSN deposition as a potential biomarker for disease. The different results may be attributable to technical and methodological differences in the fixation method, the use of different antibodies (polyclonal versus monoclonal), and the analysis of cryosections with different thicknesses [thin sections (5−6 μm) versus thick sections (50−60 μm)]. These methodological differences are addressed in detail later in this review.

The sensitivity of αSN deposition in skin biopsy samples may vary depending on the underlying disease. Studies of PD patients have shown high specificities but a wide range of sensitivities: two studies found αSN deposits in only 5.2−10% of PD patients using skin samples of the chest wall and distal leg,[20,25] one study found αSN deposition in 52% of patients (16/31) using skin samples of the finger, proximal leg, distal leg, and back,[21] and one study found that 100% of PD patients had αSN deposits in biopsy samples of the volar forearm.[24] Looking at a possible length-dependent distribution, a few studies showed a proximal-to-distal gradient of cutaneous αSN deposition in PD patients: 100% of the patients had αSN depositions at the cervical site, 52−75% at the thigh, and 24−

31% at the leg.[22,29] Comparing with healthy controls and patients with essential tremor, tauopathy, or parkinsonisms assumed not to have αSN deposits, the specificity of αSN was 90% when using antibodies against total αSN and 100% when using antibodies against p-αSN.[21-24,27,28] When looking at the class of nerve fibers affected, studies involving PD, DLB, and PAF patients found αSN accumulations in autonomic fibers of the skin,[20-31] while in MSA patients they were observed mainly in unmyelinated somatosensory fibers of the sub-epidermal plexus but not in dermal autonomic fibers.[23-25] These findings suggest that the involvement of postganglionic autonomic nerve fibers contributes to autonomic symptoms in PD, DLB, and PAF, which contrasts with cutaneous autonomic fibers appearing to be preserved in MSA. Two comparative studies found that 100% of PAF patients presented with αSN depositions in autonomic nerve fibers.[29,30] The detection of cutaneous αSN deposition could therefore be a sensitive tool for PAF. Moreover, one of these studies found p-αSN deposits with an even distribution pattern in PAF, whereas all of the PD and DLB patients had a proximal-to-distal pattern.[30,31] This suggests that there are variations in the pathomechanisms underlying synucleinopathies.[22,29,31]

The various results obtained in the previous studies should be interpreted with caution, since they had quite heterogeneous designs and methodologies, as well as numerous limitations: the sample was small in many studies, most studies had a case–control design, and technical aspects of the study methodologies differed according to the biopsy site, antibodies used, the type of microscopy, tissue thickness, tissue preparation and fixation methods, and how positive results were defined. The recruited patients were diagnosed based mainly on clinical criteria, which limits the comparability of cases across studies from different referral centers. In addition, a study with a case–control design might be affected by selection bias.

Differences between αSN and p-αSN according to clinical measures

Several studies have evaluated the correlations of cutaneous synuclein deposition with disease severity and autonomic dysfunction. One study yielded correlations between the αSN ratio and the score on the Hoehn and Yahr scale or measures of autonomic function tests, which suggested that greater αSN deposition was associated with greater autonomic dysfunction and more-advanced stages of PD.[26] The same group recently described that αSN deposition is present also in early stages of the disease and when PD has been diagnosed recently (within 0.5−3 years), even among PD patients without autonomic failure, which indicates that cutaneous synuclein deposition may be present also during the premotor stages.[27] Those authors also found that αSN ratios were higher in indi-

viduals with autonomic failure and were correlated with the severity of PD, suggesting that the αSN ratio can provide a sensitive and specific biomarker for PD patients.[27] Other studies have investigated if αSN deposition is correlated with disease severity and autonomic dysfunction, quantifying p-αSN deposition as the percentage of autonomic structures or nerve bundles showing positive staining, or a more-sophisticated index of total p-αSN staining.[22,24,29] However, those studies did not demonstrate a correlation between deposits of p-αSN and clinical or nuclear imaging measures including the disease duration, motor involvement, autonomic dysfunction graded on the Composite Autonomic Scoring Scale, or the heart-to-mediastinum denervation ratio obtained from nuclear imaging.[22,24,29] Therefore, while a correlation of p-αSN with a clinical measure remains to be identified, the total αSN ratio may be appropriate for evaluating the disease severity or autonomic dysfunction in PD patients based on cutaneous synuclein depositions. Although questions remain, cutaneous synuclein deposition might reflect the disease stage or degree of autonomic failure in synucleinopathies. Future studies are needed to evaluate the associations of disease severity and autonomic dysfunction with cutaneous αSN deposition.

METHODOLOGICAL DIFFERENCES IN DETECTING CUTANEOUS SYNUCLEIN DEPOSITION

Another important aspect to consider when comparing the results from the above-mentioned studies is methodological differences in how cutaneous αSN deposits are detected. Those differences are primarily the locations where biopsy samples are obtained from, the thickness of tissue sections, fixation techniques, and staining protocols (i.e., the selection of the detection antibody, secondary antibodies, and amplification systems), in addition to differences in study designs and the characteristics of the included subjects. We discuss these methodological and technical differences in more detail below.

Differences in tissue fixation
Autopsy samples are routinely fixed in formalin or formaldehyde solution for more than 24 hours and then embedded in paraffin, whereas *in vivo* samples are typically fixed in a 2% paraformaldehyde or Zamboni's solution (paraformaldehyde mixed with picric acid and NaOH) for 18−24 hours for the optimal detection of peripheral nerve fibers. Overfixation with formalin can impair the ability to quantify the peripheral nerve density and also to detect αSN.[40]

Differences in tissue section thicknesses
There are also differences in the methods used to section fixed skin biopsy tissue samples. Autopsy samples are generally cut into 5- to 6-μm-thick tissue sections from paraffin blocks, while *in vivo* samples are cut into 50- to 60-μm-thick sections. αSN is deposited within nerves in the dermal layer of the skin (i.e., in vasomotor nerve fibers of the blood vessels, sudomotor nerve fibers of the sweat glands, and pilomotor nerve fibers of the pilomotor muscles), and is therefore spread out across this structure with dimensions of approximately 2 mm. Since a section that is 5−6 μm thick has only one-tenth the sampling region of a section that is 50−60 μm thick, it is far less likely to contain dermal nerve fibers in which potential αSN depositions could be detected.[40]

The thickness of tissue sections also has a profound effect on the detection rate of studies involving *in vivo* samples. The overall sensitivity of immunostaining has been higher in studies using 50-μm-thick sections than in those involving sections that are 10−20 μm thick. Important data for clarifying this problem has come from the recent study of Wang et al.,[41] who compared the detection of p-αSN in cutaneous autonomic nerve fibers across *in vivo* samples with section thicknesses of 10, 20, and 50 μm. This study, which has only been reported on abstract form, found that immunostaining with 50-μm-thick tissue sections was superior to using sections that were 20 or 10 μm thick for detecting of p-αSN in PD.[41] This difference is probably due to the quantity of intact nerve fibers in each section.

Polyclonal versus monoclonal antibodies
As mentioned above, immunohistochemical studies measuring total αSN use polyclonal antibodies, whereas those measuring p-αSN use monoclonal antibodies. The selection of antibodies significantly affects the results obtained: 4 of 12 studies involving clinically diagnosed subjects used antibodies against total αSN, with the remaining 8 using antibodies against p-αSN (Table 2). Although there have been no direct comparisons, performing immunohistochemical staining with antibodies against total αSN may provide higher sensitivity compared to using anti-p-αSN antibodies. Previous studies observed p-αSN deposits intermittently within cutaneous nerve fibers, and detected them inside small dermal nerve bundles or in autonomic small fibers innervating blood vessels, usually in the deeper layers of the dermis.[21,22] These p-αSN deposits could be missed if deeper layers are not analyzed and if the p-αSN deposits are not colocalized with a panaxonal marker such as protein gene product (PGP) 9.5. In contrast, total αSN deposits can be identified more easily than p-αSN deposits. However, αSN deposits are also present in healthy controls, albeit in smaller amounts, and therefore require quantification to distinguish between healthy and diseased states. Thus, p-αSN is more specific to synucleinopathy and the

choice of detection antibody against p-αSN or total αSN can have a large effect on study outcomes.

Background artifacts

Nonspecific background noise and staining artifacts are inevitable in most immunohistochemical studies, and will depend on the choice of polyclonal or monoclonal antibodies, type of staining methods used, fixation technique, tissue thickness, and use of light or immunofluorescence microscopy. A recent unpublished study observed a low-intensity signal of staining artifacts and background noise in certain areas around autonomic structures in sections in which p-αSN-positive nerve fibers were not colocalized with PGP-9.5-positive fibers, irrespective of the tissue thickness.[41] These artifacts could be misinterpreted as p-αSN-immunoreactive fibers. Therefore, applying a double-staining method to p-αSN with the panaxonal marker PGP 9.5 could be very helpful for decreasing the rates of false-positive and false-negative results.

CONCLUSION

This review of a combination of postmortem and *in vivo* studies allows us to draw the following conclusions: first, cutaneous αSN quantified in skin biopsy samples provides high specificity and good sensitivity for the detection of synucleinopathies. A high specificity is important for the diagnostic value of a biomarker, especially in the early stages of a disease when clinical uncertainties may make a diagnosis difficult. It is particularly interesting that the recent study demonstrated p-αSN deposition in skin nerves in 75% of patients with idiopathic REM-sleep behavior disorder, suggesting that a skin biopsy can be considered a safe and sensitive procedure for detecting αSN pathology in the prodromal stage of synucleionpathies.[42]

Second, a skin biopsy performed to detect αSN is a relatively easy, repeatable, and minimally invasive technique compared to obtaining samples from other organs.

Third, there are still several unanswered questions about the optimal methods, biopsy sites, antibodies, fixation methods, and tissue thickness to utilize when detecting αSN. Determining the diagnostic accuracy of cutaneous αSN pathology may require evaluations of the sensitivity and specificity of immunohistochemical staining in skin samples from neuropathologically proven subjects. There are practical difficulties associated with obtaining samples from autopsied patients. In addition, it is not adequate to identify the diagnostic accuracy of cutaneous αSN pathology for some clinically diagnosed patients. Moreover, the results of published studies are conflicting. It can be assumed that the diversity of methodologies is the main reason behind the conflicting results

from published studies. It might be plausible to determine the optimal method by comparing the results obtained when applying different methods to the same samples.

Some authors recently tested the efficacies of different immunohistochemical methods in detecting αSN pathology in gastrointestinal specimens in identifying the technique with the highest sensitivity and specificity.[43,44] One of these studies examined four methods in archived colonic biopsy samples obtained from PD patients and controls,[43] and the other assessed seven methods applied to sigmoid colon sections obtained from autopsied PD and control subjects.[44] The stained sections were graded by blinded judging. The findings of these studies suggest that obtaining multiple and full-thickness samples and using well-trained raters to judge slides are other important factors for increasing the diagnostic accuracy.[44] Most importantly, the utility of different immunohistochemical methods should be evaluated for the detection of cutaneous αSN pathology in identical samples, and the stained sections should be judged in a blind manner by well-trained raters in order to determine whether it could be a reliable biomarker for synucleinopathies. A standardized methodology is essential to increase the diagnostic value and widen the clinical application of skin biopsies in the detection of αSN. After a standardized protocol for detecting cutaneous αSN deposition has been established, its clinical and scientific value should be verified and compared with preexisting diagnostic tests or tools.

Fourth, the studies reported on to date have not revealed any distinctive features of the specific subtypes of synucleinopathies, except in the case of MSA, in which cutaneous αSN deposition was observed mainly in unmyelinated somatosensory fibers of subepidermal plexus, and not in dermal autonomic fibers, which contrasts with the findings for other subtypes. Further studies are also needed to address this issue.

There are numerous methodological challenges to staining and quantifying αSN in skin biopsy samples, and standardized methods still need to be developed and tested. However, the measurement of cutaneous αSN could serve as a useful biomarker for synucleinopathies if standardized protocols can be established.

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