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RESEARCH ARTICLE

Association of innervation-adjusted alpha-synuclein in arrector pili muscles with cardiac noradrenergic deficiency in autonomic synucleinopathies

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Abstract

Background Autonomic synucleinopathies feature deposition of the protein alpha-synuclein (AS) in neurons [e.g., Lewy body neurogenic orthostatic hypotension (nOH)] or glial cells (multiple system atrophy, MSA). AS in skin biopsies might provide biomarkers of these diseases; however, this approach would be complicated or invalidated if there were substantial loss of AS-containing nerves. We report AS content in arrector pili muscles in skin biopsies after adjustment for local innervation in patients with Lewy body nOH or MSA. Cardiac sympathetic neuroimaging by myocardial ^{18}F-dopamine positron emission tomography (PET) was done to examine pathophysiological correlates of innervation-adjusted AS.

Methods Thirty-one patients (19 Lewy body nOH, 12 MSA) underwent thoracic ^{18}F-dopamine PET and skin biopsies. AS signal intensity analyzed by immunofluorescence microscopy was adjusted for innervation by the ratio of AS to protein gene product (PGP) 9.5, a pan-axonal marker (Harvard lab site), or the ratio of AS to tyrosine hydroxylase (TH), an indicator of catecholaminergic neurons (NIH lab site).

Results The Lewy body nOH group had higher ratios of AS/PGP 9.5 or log AS/TH than did the MSA group (0.89 ± 0.05 vs. 0.66 ± 0.04, -0.13 ± 0.05 vs. -1.60 ± 0.33; $p < 0.00001$ each). All 19 Lewy body patients had AS/PGP 9.5 > 0.8 or log AS/TH > 1.2 and had myocardial ^{18}F-dopamine-derived radioactivity < 6000 nCi-kg/cc-mCi, the lower limit of normal. Two MSA patients (17%) had increased AS/PGP or log AS/TH, and two (17%) had low ^{18}F-dopamine-derived radioactivity.

Conclusions Lewy body forms of nOH are associated with increased innervation-adjusted AS in arrector pili muscles and neuroimaging evidence of myocardial noradrenergic deficiency.

Keywords Lewy bodies · Multiple system atrophy · Orthostatic hypotension · Synuclein · Tyrosine hydroxylase

Abbreviations

AS	Alpha-synuclein
^{18}F-DA	^{18}F-dopamine
MSA	Multiple system atrophy
nOH	Neurogenic orthostatic hypotension
PAF	Pure autonomic failure
PD	Parkinson disease
PGP 9.5	Protein gene product 9.5
SMA	Smooth muscle actin
TH	Tyrosine hydroxylase

Introduction

Synucleinopathies are a family of neurodegenerative diseases characterized by accumulation of the protein alpha-synuclein (AS) in neurons [42] or glial cells [39]. Autonomic synucleinopathies involve AS deposition coupled with signs or symptoms of autonomic failure—especially neurogenic orthostatic hypotension (nOH). In nOH, during orthostasis there is a failure to release the sympathetic neurotransmitter norepinephrine adequately in response to the decrease in venous return to the heart. Two general types

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of autonomic synucleinopathies involving nOH have been recognized—Lewy body nOH [as in Parkinson disease (PD), pure autonomic failure (PAF) [27], and dementia with Lewy bodies (DLB)] and multiple system atrophy (MSA), the latter identified by AS deposition in glial cytoplasmic inclusions in the brain [44].

Lewy body forms of autonomic synucleinopathy also involve AS deposition in sympathetic noradrenergic nerves. In vivo and postmortem studies have reported associations of sympathoneural AS deposition with sympathetic noradrenergic deficiency [1, 16, 25, 30, 36]. In contrast, most MSA patients do not have increased sympathoneural AS deposition [31, 47], and sympathetic innervation usually is intact [17, 37, 38].

Although cardiac sympathetic neuroimaging can establish whether nOH is associated with peripheral noradrenergic deficiency [24], this testing modality alone is imperfectly specific for differentiating Lewy body diseases from MSA, because some MSA patients have neuroimaging evidence of a myocardial sympathetic lesion [4, 37, 40]. Moreover, cardiac sympathetic neuroimaging does not provide information about synucleinopathy.

It is possible that AS buildup occurs within sympathetic nerves that are then lost. If so, then measuring total tissue AS content could invalidate tissue AS as a biomarker of nOH associated with synucleinopathy. To adjust AS for local innervation, the ratio of AS to protein gene product 9.5 (PGP 9.5, a pan-axonal marker) has been proposed [46]. Another quantitative method for innervation-adjusted AS is based on the ratio of AS to tyrosine hydroxylase (TH), a marker of sympathetic noradrenergic innervation, in regions of interest delineated by immunoreactive smooth muscle actin (SMA) [30].

In this study, cardiac sympathetic neuroimaging by ^{18}F-dopamine (^{18}F-DA) positron emission tomography (PET) was done as part of comprehensive autonomic function testing in patients with autonomic synucleinopathy. Groups of Lewy body nOH and MSA patients had skin biopsy tissues analyzed by the AS/PGP 9.5 approach at Harvard or independently by the AS/TH ratios approach at the NIH. We assessed whether results by the two quantitative methods agreed in terms of an association between increased innervation-adjusted AS and neuroimaging evidence of cardiac noradrenergic deficiency by ^{18}F-DA PET. We focused on AS/PGP 9.5 and AS/TH ratios in arrector pili (pilomotor) muscle, because this skin constituent receives only sympathetic noradrenergic innervation.

Methods

Subjects

All the patients were studied at the NIH Clinical Center after having given written informed consent to participate in research protocols approved by the Institutional Review Board of the National Institute of Neurological Disorders and Stroke. The patients had been referred for evaluation of a known or suspected autonomic synucleinopathy. nOH was confirmed by autonomic function testing that included continuous blood pressure recording associated with performance of the Valsalva maneuver [14] and orthostatic plasma catechols [13]. Secondary causes of nOH such as diabetes were excluded according to our published algorithm [24]. All the patients had been referred after standard diagnostic tests were negative.

Lewy body nOH

By the term Lewy body nOH we are referring to a family of disorders that includes pure autonomic failure (PAF), Parkinson disease with neurogenic orthostatic hypotension (PD + nOH), and dementia with Lewy bodies and nOH (DLB + nOH). Patients were classified as having PAF if they had nOH, were more than 30 years old, had no signs of central neurodegeneration, and had sympathetic noradrenergic deficiency as indicated by interventricular septal myocardial ^{18}F-DA-derived radioactivity less than 6000 nCi-kg/cc-mCi, which is the cutoff value for normality [18].

PD + nOH patients had bradykinesia and cogwheel rigidity, nOH, a putamen/occipital cortex ratio of ^{18}F-DOPA-derived radioactivity ≤ 2.4 [15], and low myocardial ^{18}F-DA-derived radioactivity. DLB + OH patients had nOH, cognitive dysfunction, dream enactment behavior or visual hallucinations, a putamen/occipital cortex ratio of ^{18}F-DOPA-derived radioactivity ≤ 2.4, and low myocardial ^{18}F-DA-derived radioactivity [33].

MSA

Patients were classified as having MSA based on nOH, progressive parkinsonism or cerebellar ataxia, and other supportive clinical or laboratory features. Supporting aspects were poor airway control manifested by dysarthria, stridor, or a history of aspiration; brainstem atrophy noted upon magnetic resonance imaging; urinary retention requiring bladder catheterization; or normal or only slightly decreased sense of smell [15].

We did not use published consensus definitions of MSA [11, 12]. These have had several weaknesses, as has been pointed out recently [43]. For instance, the second consensus statement included a poor response to levodopa as a criterion for diagnosing probable MSA [12]; however, some MSA patients have good or excellent responses to levodopa [3].

^{18}F-DA PET

^{18}F-DA PET was done as described previously [16]. Ventricular free wall ^{18}F-DA-derived radioactivity concentrations were recorded for the 5-min frame with a mid-point about 8 min after initiation of the 3-min infusion of 1 mCi of the tracer. Radioactivity concentrations in nCi/cc were adjusted for the radioactivity dose in mCi and the body mass in kg and expressed in units of nCi-kg/cc-mCi.

Skin biopsies

Three-millimeter punch skin biopsies were taken from the lateral distal leg or nape of the neck [30]. Skin biopsy samples were placed in Zamboni fixative solution and kept at 4 °C for about 18–20 h, washed with Sorensen's phosphate buffer (133 mM, pH 7.6), and placed in 20% glycerol for cryoprotection. Skin biopsy samples sent to the Harvard laboratory were obtained from the lateral aspect of the leg; all but two samples assayed at the NIH site were from the nape of the neck, a location offering the advantage of consistently abundant arrector pili muscles. Amounts of AS do not differ importantly between the two body locations [35]. At both laboratory sites, the samples were analyzed by personnel who were blinded as to the individual clinical diagnosis.

At the Harvard laboratory site, fluorescent immunohistochemical staining was performed on 50-µm-thick sections using previously published methods for visualizing AS and PGP 9.5 [45, 46]. The primary antibody to PGP 9.5 was from UltraClone (Isle of Wight, UK) and for AS from Chemicon (EMD Millipore, Gibbstown, NJ, USA). The AS antibody was polyclonal and recognized multiple binding sites from amino acids 111–131 [10]. The tissue sections were imaged by confocal microscopy (Zeiss LSM5 Pascal Exciter; Carl Zeiss, Thornwood, NY, USA). The average number of nerve fibers intersecting five horizontal lines across the width of the pilomotor muscle in 3-mm-thick confocal images were reported in fibers/mm. To calculate AS/PGP 9.5 ratios, the percent of nerve fibers containing AS were expressed as a percent of the total nerve density as measured by PGP 9.5 in arrector pili muscles.

At the NIH laboratory site, skin biopsy samples were sliced into 8–10-µm-thick sections (Histoserv, Germantown, MD, USA). Immunolabeling protocols were applied to identify TH using rabbit anti-TH antibody (Pel-Freez Biologicals, Rogers, AR, USA), AS using mouse IgG_1 monoclonal anti-AS (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and alpha SMA using mouse IgG_{2a} monoclonal anti-SMA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as published [30]. Regions of interest were placed around arrector pili muscles. Average densities of AS, SMA, and TH within the regions of interest were tabulated for each visualized constituent.

During the course of the study, we switched from leg to nape of the neck as the site of skin biopsies, since the latter site would more reliably yield samples containing arrector pili muscles, which receive purely noradrenergic innervation. The samples assayed at the Harvard site were obtained before the switch in biopsy location.

Other clinical laboratory tests

The 40-item University of Pennsylvania Smell Identification Test (UPSIT, Sensonics International, Haddon Heights, NJ, USA) was administered. Clinical magnetic resonance imaging (MRI) was done in most patients, and findings of cortical atrophy (cortical tissue shrinkage or cerebroventricular or sulcal enlargement) by unblinded radiologists were tabulated.

Data analysis and statistics

Mean values (± 1 SEM) for ^{18}F-DA-derived radioactivity, AS/PGP 9.5 ratios, and AS/TH ratios in arrector pili muscles were compared between the Lewy body and MSA groups by independent-means *t* tests. Because the variance of AS/TH ratios increases with the mean value, the log of the AS/TH ratio (log AS/TH) was used for statistical purposes to avoid violation of the assumption of homogeneity of variance. Pearson correlation coefficients were calculated for relationships between values of continuous variables. For analyzing frequencies, Fisher's exact test was used. A p value less than 0.05 defined statistical significance.

Results

The Lewy body nOH group included 11 men and 8 women [mean age 70.8 ± (SEM) 1.9 years], and the MSA group included 9 men and 3 women (mean age 64.1 ± 2.2 years). Among the Lewy body group, there were 8 patients with PAF, 6 PD + nOH, 2 PAF that evolved into dementia with Lewy bodies and nOH (DLB + nOH), 2 PD + nOH + DLB, and 1 DLB + nOH. Among the MSA group, 2 were considered to have a purely cerebellar form and the remainder a parkinsonian or mixed form. The Lewy body group was older than the MSA group ($p = 0.032$ by independent means *t* test). The Lewy body nOH group had a lower

mean UPSIT score than did the MSA group (18.3 ± 1.4 vs. 30.2 ± 1.4, $p = 0.00001$). Of the 19 Lewy body patients, 13 (68%) were anosmic. The Lewy body group had a higher frequency of MRI evidence of cortical atrophy (14/18 = 78% vs. 3/11 = 27%, $p = 0.006$).

Skin biopsies from 13 patients were assayed at the Harvard site, 12 at the NIH site, and 6 at both sites. In most patients, skin biopsies and ^{18}F-DA scanning were done during the same inpatient stay, and the mean intervals from skin biopsies to ^{18}F-DA scanning did not differ between the two compared groups (0.1 ± 0.5 vs. 0.2 ± 0.2 years).

Mean ^{18}F-DA-derived radioactivity in the left ventricular free wall was lower in the Lewy body nOH group (2865 ± 953 vs. 9386 ± 1113 nCi-kg/cc-mCi, $p < 0.00001$). All 19 Lewy body patients had AS/PGP 9.5 > 0.8 or log AS/TH > 1.2 and had myocardial ^{18}F-DA-derived radioactivity < 6,000 nCi-kg/cc-mCi (Fig. 1). Representative images are shown in Fig. 2. Two MSA patients (17%) had increased AS/PGP or log AS/TH, and two (17%) had low ^{18}F-DA-derived radioactivity. One patient with autopsy-proven MSA and no Lewy bodies in the brain had both low ^{18}F-DA-derived radioactivity and a value for AS/PGP 9.5 within the range of Lewy body nOH patients (arrow in Fig. 1).

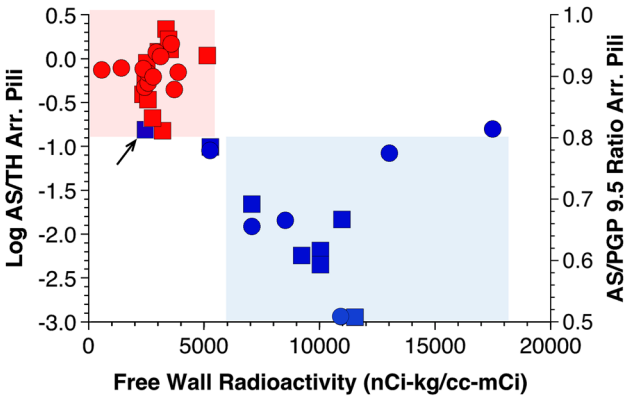

Fig. 1 Individual values for log AS/TH and AS/PGP 9.5 ratios in arrector pili muscles from skin biopsies vs. left ventricular free wall ^{18}F-DA-derived radioactivity in patients with nOH. Circles show results for log AS/TH assayed at the NIH laboratory sites and squares for AS/PGP 9.5 assayed at the Harvard laboratory site. Red markers indicate Lewy body diseases and blue multiple system atrophy (MSA). The arrow indicates a data point from 1 MSA patient with low ^{18}F-DA-derived radioactivity and a AS/PGP 9.5 ratio within the range of patients with Lewy body nOH. The pink zone indicates the range of data in Lewy body nOH patients. The light blue zone indicates the range of data for patients with ^{18}F-DA-derived radioactivity above 6000 nCi-kg/cc-Ci, the cutoff value for defining normality. *AS* alpha-synuclein, *^{18}F-DA* ^{18}F-dopamine, *MSA* multiple system atrophy, *PGP 9.5* protein gene product 9.5, *TH* tyrosine hydroxylase. Note that all patients with Lewy body nOH have low ^{18}F-DA-derived radioactivity and increased AS/TH or AS/PGP 9.5 ratios

Discussion

We report that in patients with nOH in the setting of an autonomic synucleinopathy, innervation-adjusted AS in arrector pili muscles is associated with neuroimaging evidence of cardiac noradrenergic deficiency. Similar results were obtained by two different methods for quantifying AS adjusted for sympathetic innervation, carried out independently by two different collaborating laboratories of the Autonomic Rare Diseases Clinical Research Consortium.

Previous studies using observational or semiquantitative methods reported augmented immunoreactive AS in skin biopsies in PAF [8, 41], PD [8, 10, 35, 47], and DLB [2], but not in MSA [6, 47]. These studies did not adjust AS deposition for local innervation density. Major strengths of the present study were the quantitative approaches for measuring innervation-adjusted AS deposition and internal replication of the main results using different assay methods.

We did not use an antibody to AS phosphorylated at Ser129. The specificity of anti-phosphorylated AS clones has been questioned because of cross-reactivity with other proteins [5]. This may be relevant to MSA. Previous studies about phosphorylated AS in skin biopsies from patients with MSA reported either no phosphorylated AS signal or else signal in non-autonomic fibers [7, 9, 26, 28, 47]. No published article has described increased phosphorylated AS in sympathetic noradrenergic nerves in MSA.

PAF diagnosed clinically by nOH, no identified secondary cause, and absence of evidence of central neurodegeneration can evolve into MSA [32]. Our definition of PAF also included evidence of sympathetic noradrenergic deficiency, as reported originally in 1977 when the disease was called primary orthostatic hypotension [48] and confirmed later by our group [19, 22]. In our experience, all PAF patients have low myocardial ^{18}F-dopamine-derived radioactivity, indicating cardiac noradrenergic deficiency [20], whereas most MSA patients do not [15]. This implies that in a patient with primary nOH, the finding of normal cardiac noradrenergic innervation excludes PAF. In the present study, no patient with an initial diagnosis of PAF evolved to MSA during follow-up.

Our results confirm that myocardial ^{18}F-DA-derived radioactivity is highly sensitive but imperfectly specific in distinguishing Lewy body forms of nOH from other causes of nOH such as MSA [4, 21, 29, 40]. The additional information provided by analyzing skin biopsies for innervation-adjusted AS in arrector pili muscles seems to enhance the accuracy of the clinical laboratory approach for distinguishing Lewy body nOH from MSA. Nevertheless, one of the MSA patients had both low ^{18}F-DA-derived radioactivity and an AS/PGP 9.5 ratio in arrector pili muscle within the range of Lewy body nOH patients. In this patient,

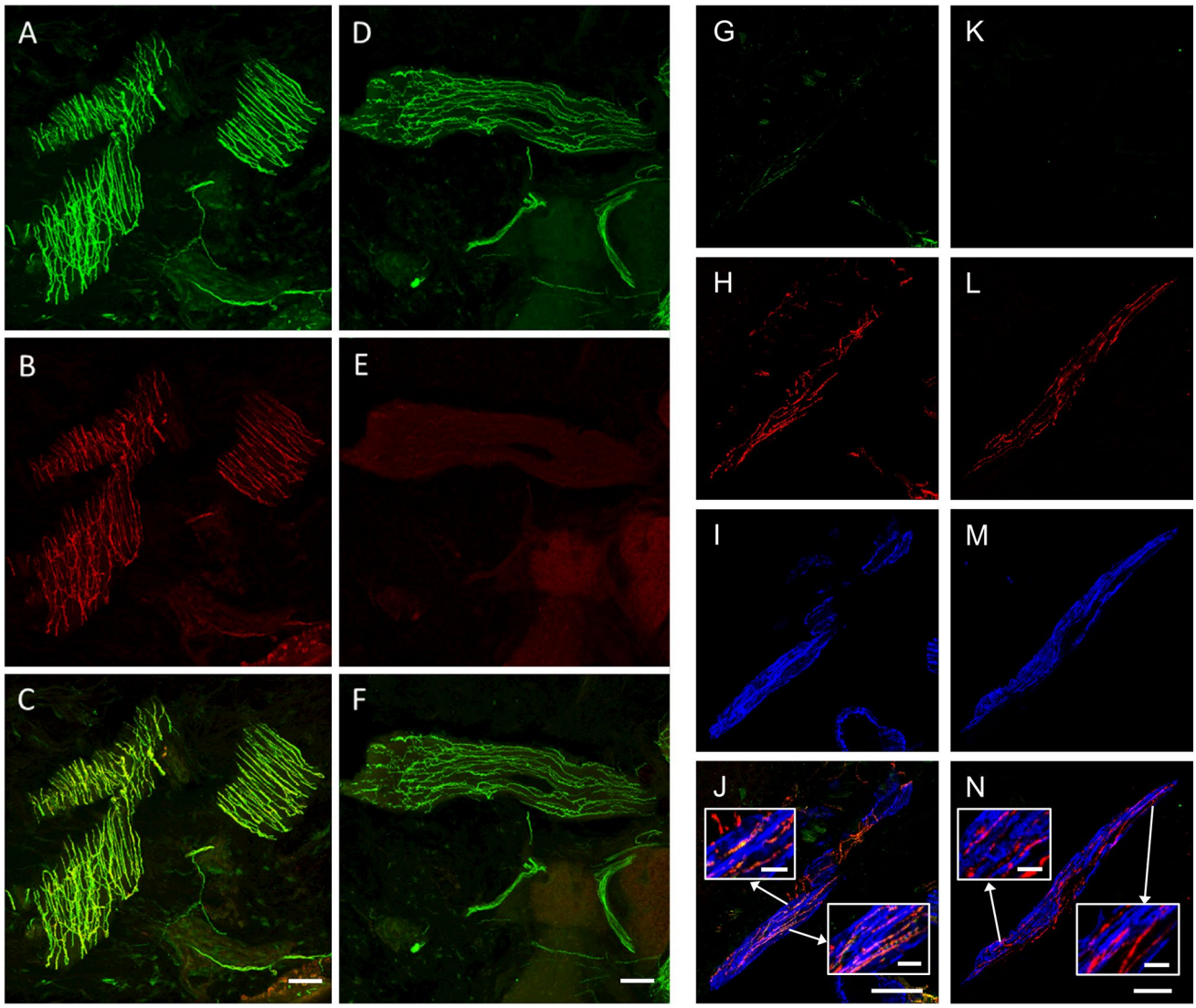

Fig. 2 Representative microscopic images of immunoreactive alpha-synuclein (AS) and PGP 9.5 (**a**–**f**) and of AS, tyrosine hydroxylase (TH), and smooth muscle actin (SMA) (**g**–**n**) in arrector pili muscles from patients with neurogenic orthostatic hypotension. **a**–**c**, **g**–**j** are from patients with Parkinson disease, and **d**–**f**, **k**–**n** from patients with multiple system atrophy (MSA). **a**, **d** Immunoreactive PGP 9.5 (green), **b**, **e** AS (red), and **c**, **f** the merged images of PGP 9.5 and AS. Note AS in PGP 9.5-containing fibers in the PD patient (**c**) and only background AS in the MSA patient (**e**). **g**, **k** Immunoreactive AS (green), **h**, **l** TH (red), **i**, **m** SMA (blue), and **j**, **n** the merged images of AS, TH, and SMA, where yellow indicates overlap of AS with TH. Note AS in TH-containing fibers in the PD patient (**j**) and not in the MSA patient (**n**). Scale bars: **a**–**f** 100 µm; **g**–**n** 50 µm (insets 10 µm)

postmortem assessments revealed profound myocardial norepinephrine deficiency and no Lewy bodies in the brain [4]. The results reported here in this patient indicate that MSA can entail cardiac noradrenergic deficiency and sympathetic intraneuronal synucleinopathy, without intracerebral Lewy bodies.

Lewy body forms of nOH are known to be associated with severe olfactory dysfunction [23, 34], whereas in MSA, the sense of smell is normal or only modestly decreased in most patients. In our study, the majority of patients with Lewy body nOH were anosmic, indicating associations among cardiac noradrenergic deficiency, olfactory dysfunction, and AS deposition in sympathetic noradrenergic nerves in autonomic synucleinopathies.

The Lewy body nOH group had a higher frequency of cortical atrophy noted upon MRI than did the MSA group. Our study was not designed appropriately to determine whether this group difference indicated cognitive dysfunction in Lewy body nOH as a whole, because the Lewy

body nOH group included patients with a variety of diagnoses, including DLB.

Limitations

The focus of our study was on the association between innervation-adjusted AS in skin biopsies with results of cardiac sympathetic neuroimaging in patients with Lewy body nOH or MSA. The study did not include a comparison group of healthy control subjects, which would be required for formal assessment of diagnostic accuracy of innervation-adjusted AS, cardiac noradrenergic deficiency, or a combination of the two abnormalities in identifying Lewy body forms of nOH. Because of the absence of healthy controls, it is possible that Lewy body forms of nOH and MSA both involve increased AS deposition in arrector pili muscles, but with the increase larger in the former group.

Whether the results apply to Lewy body diseases without nOH or to other forms of nOH that do not involve synucleinopathy (e.g., autoimmune autonomic ganglionopathy) cannot be determined from the present data.

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