Title

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Journal

Movement Disorders, 31(2)

ISSN

0885-3185

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Publication Date

2016-02-01

DOI

10.1002/mds.26476

Peer reviewed

HHS Public Access

Author manuscript

Mov Disord. Author manuscript; available in PMC 2017 February 01.

Published in final edited form as:

Mov Disord. 2016 February ; 31(2): 250–256. doi:10.1002/mds.26476.

Peripheral Synucleinopathy in Early Parkinson's Disease: Submandibular Gland Needle Biopsy Findings

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Abstract

Background—Finding a peripheral tissue biopsy site to diagnose early Parkinson's disease would be of value for clinical care, biomarker validation, and as research enrollment criteria. While autopsy and advanced Parkinson's disease studies suggest submandibular gland is an important biopsy site, there are no studies in early Parkinson's disease.

Objectives—Determine whether needle biopsy of the submandibular gland reveals Lewy type α-synucleinopathy in early Parkinson's disease.

Methods—Twenty-five early Parkinson's disease (duration < 5 years) and 10 controls underwent transcutaneous needle core biopsies of the submandibular gland. Tissue was stained for phosphorylated α-synuclein, reviewed blind to clinical diagnosis, and only nerve element staining was considered positive.

Results—Mean (Standard Deviation) age 69.5 (8.3) for Parkinson's disease group, 64.8 (8.0) years for controls, and disease duration 2.6 (1.1) years. Six Parkinson's disease and one control subject had inadequate glandular tissue. Positive staining was found in 14/19 (74%) Parkinson's disease and 2/9 (22%) control subjects. Parkinson's disease positive and negative cases did not

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There are no conflicts of interest for any author on this manuscript.

Authors' Roles: 1. Research project: A. Conception, B. Organization, C. Execution; 2. Statistical Analysis: A. Design, B. Execution, C. Review and Critique; 3. Manuscript: A. Writing of the first draft, B. Review and Critique

Adler 1A, 1B, 1C, 2A, 2C, 3A, 3B; Dugger 1A, 1B, 1C, 2C, 3B; Hentz 2A, 2B, 3B; Hinni 1A, 1C, 3B; Lott 1A, 1C, 3B; Driver-Dunckley 1C, 3B; Mehta 1C, 3B; Serrano 1C, 3B; Sue 1B, 1C, 3B; Duffy 1B, 1C, 3B; Intorcia 1C, 3B; Filon 1C, 3B; Pullen 1C, 3B; Walker 1C, 3B; Beach 1A, 1B, 1C, 2A, 2C, 3A, 3B

differ clinically. Adverse events (mainly swelling and bruising) were common (77% of cases), but were minor and transient.

Conclusions—Submandibular gland needle biopsies identified phosphorylated α-synuclein staining in 74% of early Parkinson's disease subjects. False positives may be true false positives or may represent prodromal Parkinson's disease. If confirmed in larger studies with eventual autopsy confirmation, the potential value of submandibular gland biopsies for early Parkinson's disease may be to aid in clinical trial inclusion/exclusion and eventually serve as a gold standard for biomarker studies short of autopsy confirmation.

Keywords

Parkinson's disease; submandibular gland; biopsy; synuclein

There has been great interest in the potential development of a peripheral tissue biopsy site for the diagnosis of Parkinson's disease (PD). In an extensive search for biopsy-accessible peripheral tissues that contained Lewy type α-synucleinopathy (LTS), the gastrointestinal system had the highest LTS densities and showed a rostral-caudal gradient with the submandibular gland and lower esophagus being most affected and the colon the least affected.[1] Some groups have investigated colon[2–5] and skin biopsies[5–10] with varying results. Further investigations of the submandibular gland included an autopsy feasibility study of submandibular gland needle biopsy, showing that, in large tissue sections, all autopsied PD patients (most with advanced disease) had evidence of LTS in the submandibular gland while needle biopsy of the frozen postmortem glands identified 17/19 of these.[11] Furthermore, a follow up proof-of-concept clinical study found that there was LTS present in submandibular gland needle biopsies from 9/12 clinically diagnosed advanced PD patients with disease duration >5 years.[12]

A peripheral tissue marker would be more valuable for early rather than advanced PD as the autopsy confirmed accuracy of a clinical diagnosis of early PD is much less accurate than advanced disease.[13] Therefore, the current study was designed to assess in-vivo submandibular gland needle biopsies in early PD patients (not previously studied either during life or in autopsy studies) with disease duration of < 5 years as well a control group with no clinical evidence of parkinsonism or dementia.

METHODS

Subjects

The study was performed by the Arizona Parkinson's Disease Consortium, whose principal members are the Mayo Clinic Arizona and Banner Sun Health Research Institute. This was a cross-sectional, controlled study with 25 PD patients (9 female) and 10 similarly-aged normal subjects (7 female), all examined by a movement disorders specialist. Inclusion criteria were a clinical diagnosis of PD with disease duration of < 5 yrs. Disease duration was based on historical data regarding when motor symptoms began, not date of diagnosis. Response to dopaminergic treatment was not a criteria for enrollment, rather the diagnosis was based on the presence of bradykinesia plus rest tremor or cogwheel rigidity as previously described.[13] Subjects were excluded if they had dementia, a bleeding diathesis,

anticoagulant treatment, were medically unable to undergo biopsy, or had a history of salivary gland disorder. Controls were defined as individuals who had no clinical evidence of a neurodegenerative disorder.

All subjects signed written informed consent obtained according to the Declaration of Helsinki and approved by the Mayo Clinic Institutional Review Board. A stipend was provided to all subjects.

Procedures

All subjects and controls had olfactory testing with the University of Pennsylvania Smell Identification Test (UPSIT-40) performed within two weeks of their biopsy.[14] All PD subjects were required to have had a previous DaT Scan or underwent a DaT scan within two weeks of their biopsy. The results of the UPSIT and the DaT Scan were not used to determine eligibility for the biopsy.

On the day of biopsy the subject met with the otolaryngologist who fully explained the biopsy procedures. The biopsies were performed as outpatient procedures in a standard examination room.

Submandibular gland biopsy

A commercially-available 16 gauge needle was used to obtain tissue cores (Max-Core Disposable Core Biopsy Instrument, Bard Medical, Covington, Georgia). Submandibular glands were localized by palpation and biopsies were performed unilaterally as previously reported.[12] Local anesthetic (0.5 cc of lidocaine) was injected into the skin overlying the submandibular gland. This was followed by a 3–5 minute waiting period to allow the anesthetic to take effect. The biopsy needle (10 cm in length, 1.29 mm diameter) was inserted transcutaneously perpendicular to the longitudinal axis of the gland, to a depth of ~22 mm. Between 3 and 6 needle cores were taken, depending on amount of tissue collected as determined by the otolaryngologist, for each subject by reinsertion of the needle at different angles through the same skin puncture site. After the procedure, pressure was used briefly to stop bleeding.

Tissue preparation

Tissue from submandibular gland needle cores was immediately placed in single standard plastic cassettes between two sponges and immersed in 70 ml of neutral-buffered 10% formalin (Fisher Scientific, Kalamazoo, MI). After 24 hours fixation at 4°C, the tissue cores were dehydrated in alcohols, infiltrated with paraffin and serial sections, positioned longitudinally, were cut at 5–7 μm with a rotary microtome. Every section was collected and mounted on a separate slide, beginning with the first appearance of tissue fragments and ending when tissue fragments were no longer visible as previously described.[12, 13, 15] To reduce the number of sections to be screened, and to protect against technical failure during staining, half of the sections were reserved and every second section was immunohistochemically stained for phosphorylated α-synuclein, using proteinase K antigen retrieval and an immunoperoxidase reaction with nickel enhancement followed by a light Neutral Red counterstain.[11] The primary antibody was a polyclonal antibody raised against

α-synuclein phosphorylated at serine 129[16] and has been demonstrated to be reactive only with the phosphorylated epitope;[17] this makes it more likely that stained peripheral structures are pathological as we have found normal control subjects (without LTS in the brain) never have immunohistochemically-positive peripheral nervous system elements.[1] Adjustment of the original protocol to better suit peripheral tissue and needle cores has been described in detail.[11]

Slide Examination

Slides were examined blind to clinical diagnosis, DaT scan, and smell test results. Slides were initially screened by a PhD trained neuropathologist (BND), who recorded the slide numbers of all slides with staining features thought to represent possible specific and positive staining of nerve fibers, as well as those with staining considered to be non-specific and/or artifactual. Then a board-certified MD, PhD neuropathologist (TGB) examined all slides that had been marked as possibly positive as well as a random selection of slides marked negative or marked as having non-specific and/or artifactual staining. As previously published, non-specific staining was defined as epithelial cell cytoplasmic staining.[11] In some cases, where the number of initially-marked slides was less than 25% of the total slide number, additional slides were randomly examined so that at least 25% of stained slides were re-examined. In other cases, where initial examination found only one positive slide, adjacent unstained slides were stained to determine the consistency of the finding. Positive slides were defined as those with immunoperoxidase staining that was morphologically consistent with neuronal fibers.

Statistics

The proportion of subjects with a positive biopsy was compared between groups by using the Pearson chi-square test. Confidence intervals for sensitivity and specificity were calculated by using the exact binomial method. Other subject characteristics were compared between groups by using the two-sample *t* test or the Pearson chi-square test. The Fisher exact test was used instead of the Pearson chi-square test when the minimum expected cell count was less than five.

RESULTS

Demographics

The PD group was an average of five years younger than the control group and a smoking history was less frequent, although neither were significantly different (Table 1). The mean (SD) duration of PD was 2.6 (1.1) years at the time of biopsy. Seventeen of the PD patients were on levodopa or a dopamine agonist, four were on MAO-B inhibitor monotherapy, and four were medication naive.

Submandibular gland biopsy findings

All 25 PD and 10 control subject biopsies were performed unilaterally. There was no difference in the mean number of needle cores taken per subject and the mean aggregate microscopically-identified gland area was no greater in the Parkinson's disease group (Table 1). Initial microscopic examination of the submandibular gland tissue cores showed the

variable presence of skin, subcutaneous tissue, striated muscle and salivary gland tissue. Biopsies from 7 subjects (2 Control, 5 PD) had focal or multifocal chronic inflammatory mononuclear cell infiltrates, mostly within the stroma and often around ducts. A total of 8 of the subjects (7 PD and 1 Control) had a submandibular gland maximal aggregate area of <2 mm^2 and were arbitrarily defined, prior to unblinding, as being insufficient for determining whether LTS was present. Despite this criterion, one subject, who when unblinded was found to be in the PD group, judged to have insufficient tissue was still found to be LTS positive and is included in the LTS positive group analysis.

Overall, 14 (74%) of 19 PD subjects (18 with sufficient glandular tissue and one with insufficient tissue) had biopsies that were positive for LTS (Figure 1), with two or more slides showing stained structures morphologically consistent with nerve fibers (Figure 2). Positive fibers were present within nerve fascicles in the stromal connective tissue, adjacent to or within the walls of small arteries and arterioles, and interweaving amongst parenchymal serous gland cells. The two slides did not need to be adjacent sections, and no subject had only one positive slide. Of the 9 controls with adequate glandular tissue 2 (22%) had LTS (Figure 1). Of the four PD subjects that were treatment naïve, two were LTS positive and two were LTS negative.

The mean number of slides screened (BND), and then reexamined (TGB), did not significantly differ for the PD and control groups (Table 2). Maximal aggregate glandular area also did not differ between groups (Table 2). The total number of slides reviewed did not significantly differ between LTS positive and LTS negative groups although there was a trend for the LTS positive group to have more slides reviewed by the neuropathologist (Table 3). The mean number of positive slides in the PD group was 6.4 ± 4.5 (Table 2). The prevalence of chronic inflammation did not differ between the two diagnostic groups (4/12 for the negative group and 3/15 for the positive group, $p = 0.66$). In the LTS positive control cases, one had 17/17 slides positive and the other had 2/22 positive. The mean maximal glandular area was 27% lower in the LTS positive group compared to the LTS negative group.

Non-specific staining of keratin was seen in skin and subcutaneous tissue in the biopsy samples of PD and controls.

UPSIT and DaT Scan Results

There was significant olfactory dysfunction comparing the PD and control groups (Table 1) while there was no difference between the LTS positive and LTS negative PD groups (Table 3). The two LTS positive controls had normal olfaction with UPSIT scores of 35 and 36. All PD cases had an abnormal DaT scan while controls did not have DaT scans.

Side effects from biopsy

Side effects (mainly swelling and bruising) were common (27/35, 77% of all biopsy subjects), but were minor and transient. The most common side effect was mild-moderate swelling, present in 22 subjects. Bruising was reported by five subjects, three reported sore throat, and two drainage from the biopsy site. No serious adverse events occurred. There was no difference in the occurrence of side effects in the PD subjects compared to the

controls. Every subject received follow-up phone calls to assess for side effects and those with side effects had resolution within 1–2 days of the biopsy.

DISCUSSION

This study, when considered together with previous studies,[1, 11–13] presents firm evidence that transcutaneous needle biopsies of the submandibular gland may be used to identify Lewy-type synucleinopathy in patients with PD, even early PD. It is important to clarify that this study, nor previous studies, address the issue of biopsies for prodromal, or pre-motor, Parkinson's disease. A previous biopsy study found that 75% of advanced PD patients, mean disease duration of 11.8 years, had LTS in their submandibular gland.[12] That previous study was a proof-of-concept study and did not have autopsy confirmation[12] and was in turn based on an autopsy feasibility study showing that LTS was present in large tissue blocks of submandibular gland from 19/19 PD cases, while a simulated needle biopsy from these same 19 post-mortem glands found positivity in 17 (89%).[11] The submandibular gland has been the focus of the biopsies as all the autopsied cases, published by multiple groups[1, 18] had positive LTS in the submandibular gland, it is readily accessible to percutaneous biopsy, and unlike the parotid gland there is not a major neurovascular bundle that could potentially be damaged. Ways to increase the yield of glandular tissue, possibly by using ultrasound guidance, need to be considered.

As the diagnostic accuracy for clinical PD, as confirmed by autopsy, in patients with PD disease duration of ≥ 5 years at first visit is ~88%,[13] the finding of LTS in submandibular gland biopsies from patients with advanced PD was not surprising but served to indicate promise for the method.[12] The present study clearly demonstrates that LTS is also present in the submandibular glands of a majority of early PD patients (74%) that had adequate glandular tissue present. Due to the lack of an effective gold standard for the clinical diagnosis of early PD, it is not possible, at this time, to know the true rate of false positives and false negatives. False negatives are expected as the needle may miss LTS-affected tissue. The autopsy feasibility study indicated that needle core biopsies may be expected to miss approximately 10% of subjects with submandibular gland LTS.[11] False positives may not actually be false positive as autopsy studies have shown that 10–20% of clinically normal elderly subjects have incidental Lewy body disease.[19] The LTS positive controls in this study did not have REM sleep behavior disorder nor hyposmia (UPSIT= 35 and 36 in these cases), non-motor features that often precede motor PD.[20, 21] DaT scans were not performed on the control subjects to know if they have an abnormality that might suggest a neurodegenerative disorder. Given the abundant positive nerve fibers (17/17 slides positive) in one case, this subject may well have incidental Lewy body disease, or "prodromal" PD. If true then that subject would not be a false positive in terms of peripheral synuclein staining in a subject with brain synuclein. It is possible that the control case with only two positive slides may have been a false positive due to artifactual staining that mimicked a true positive morphology. As all LTS positive and negative PD cases had an abnormal DaT scan, these cases all are likely have a CNS neurodegenerative disorder but not necessarily PD or a synucleinopathy.[22–25] As two of the LTS negative PD subjects had normal UPSIT scores it is possible they did not have PD. To meaningfully calculate sensitivity and specificity, and to determine the disease status of the tentative false positive and false negative cases, it will

be necessary to follow all subjects neurologically for several years to establish a more secure clinical PD diagnosis and/or to eventual autopsy and neuropathological diagnosis. However, the aggregate evidence from the combined autopsy and biopsy studies summarized here provides an indication that submandibular gland needle biopsy may be an improvement over standard neurological examination in the diagnostic accuracy of early PD, especially given the lack of a definitive biomarker.

While the number of slides that were reviewed by the neuropathologist may be considered by some to be excessive, there are multiple medical conditions that require pathologist review of large slide numbers to make the diagnosis. It was not the purpose of this study to determine a streamlined diagnostic test procedure but rather to determine the presence of peripheral synuclein staining in early PD biopsies. Further statistical analysis of these results may determine the minimal number of slides required to achieve an acceptable sensitivity.

It is important to consider the potential value of a submandibular gland tissue biopsy for early PD. First, many studies of disease-modifying agents, as well as symptomatic agents, for PD have failed in human trials. One reason for trial failure may be enrollment of subjects that do not have PD. Given the inaccuracy of the clinical diagnosis for early PD,[13] using a LTS positive needle biopsy of the submandibular gland as an inclusion criterion for a clinical trial may reduce the overall number of patients needed in the study, result in a higher observed effect size and increase the probability of finding a significant treatment effect. Submandibular gland biopsy may also prove useful as a new gold standard, short of autopsy, for validation of other candidate biomarkers, although further data on this procedure is needed. It will be important, however, for tissue biopsy or any other biomarker of early PD to follow the subjects clinically to determine whether they follow a typical clinical course, and ultimately, to autopsy to determine whether or not the subject indeed had PD.

Acknowledgments

This study was funded by the Michael J. Fox Foundation for Parkinson’s Research. The funders had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication. Dr. Charles Adler had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Dr. Charles Adler and Mr. Joseph Hentz from Mayo Clinic Arizona conducted and are responsible for data analysis.

Dr. Adler has received consulting fees from Allergan, Abbvie, Acadia, Ipsen, Lilly, Lundbeck, Merz, and Teva, and received research funding from Avid Radiopharmaceuticals, the Michael J Fox Foundation for Parkinson’s Research, and the NIH/NINDS.

Dr. Dugger received research funding from Avid Radiopharmaceuticals, the Arizona Biomedical Research Commission, CurePSP Foundation, Arizona Alzheimer’s Research Consortium, Essential Tremor Foundation, Michael J. Fox Foundation for Parkinson’s Research, NIH/NINDS, and National Alzheimer’s Coordinating Center.

Mr. Hentz received research funding from the American Heart Association, Caridian BCT, Consolidated Anti-Aging Foundation, International Essential Tremor Foundation, and NIH.

Dr. Hinni reports nothing to disclose.

Dr. Lott reports nothing to disclose.

Dr. Driver-Dunckley has received consulting fees from Teva and Merz, and received research funding from Ipsen, the Michael J Fox Foundation for Parkinson’s Research, and the NIH/NINDS.

Dr. Serrano has received research funding from Avid Radiopharmaceuticals and is paid to conduct neuropathological services for Navidea Biopharmaceuticals and Janssen Research and Development.

L. Sue receives research funding from Navidea Biopharmaceuticals, Janssen Research and Development, the Michael J Fox Foundation for Parkinson's Research, and the NIH/NINDS/NIA.

A. Duffy reports research funding from the Michael J Fox Foundation for Parkinson's Research, Arizona Alzheimer's Consortium, Avid Radiopharmaceuticals, Adamas Pharmaceuticals, Inc., Genentech Incorporated, Pfizer, and the NIH/NINDS/NIA.

A. Intorcia receives research funding from Navidea Biopharmaceuticals, Janssen Research and Development, the Michael J Fox Foundation for Parkinson's Research, and the NIH/NINDS/NIA.

J. Filon receives research funding from Navidea Biopharmaceuticals, Janssen Research and Development, the Michael J Fox Foundation for Parkinson's Research, and the NIH/NINDS/NIA.

J. Pullen receives research funding from Navidea Biopharmaceuticals, Janssen Research and Development, the Michael J Fox Foundation for Parkinson's Research, and the NIH/NINDS/NIA.

Dr. Walker receives research funding from the National Institutes of Health, Michael J Fox Foundation for Parkinson's Research, and the Arizona Alzheimer's Consortium.

Dr. Beach receives consulting fees from GE Healthcare and Avid Radiopharmaceuticals, has received research funding from Avid Radiopharmaceuticals is paid to conduct neuropathological services for Navidea Biopharmaceuticals and Janssen Research and Development and receives research funding from the Michael J Fox Foundation for Parkinson's Research, and the NIH/NINDS/NIA.

The authors thank the Parkinson's disease patients and control subjects who were willing to undergo biopsies for this study. We thank Dr. Haruhiko Akiyama for generously contributing the antibody against phosphorylated α-synuclein.

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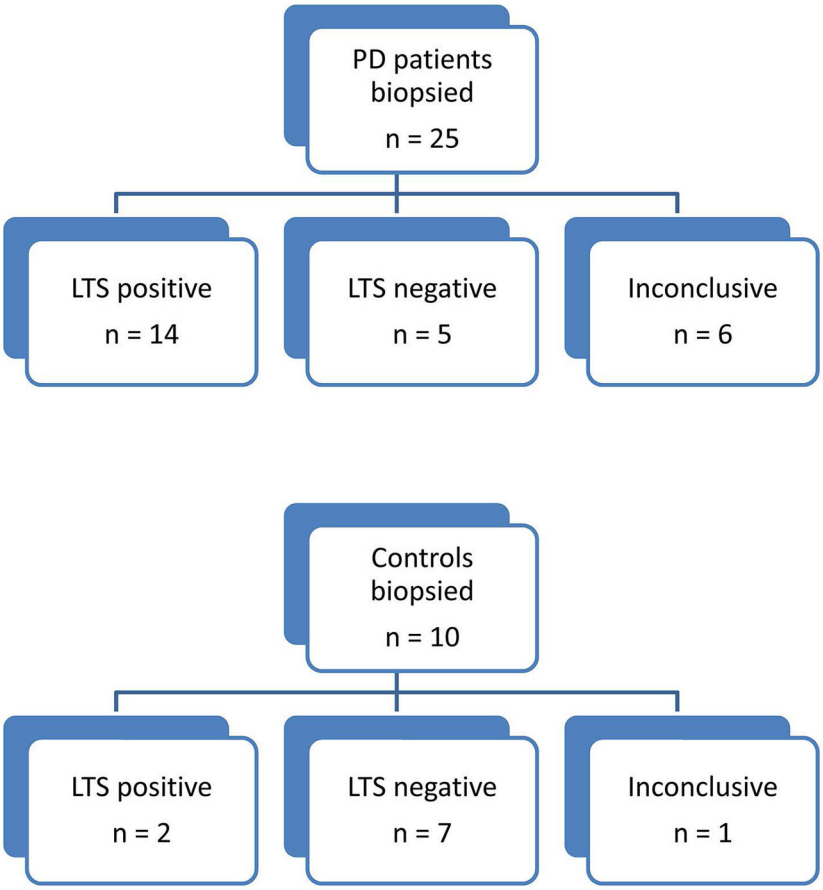

Figure 1.
Flow sheets for the number of PD patients and Controls that had a submandibular gland biopsy and the biopsy results.

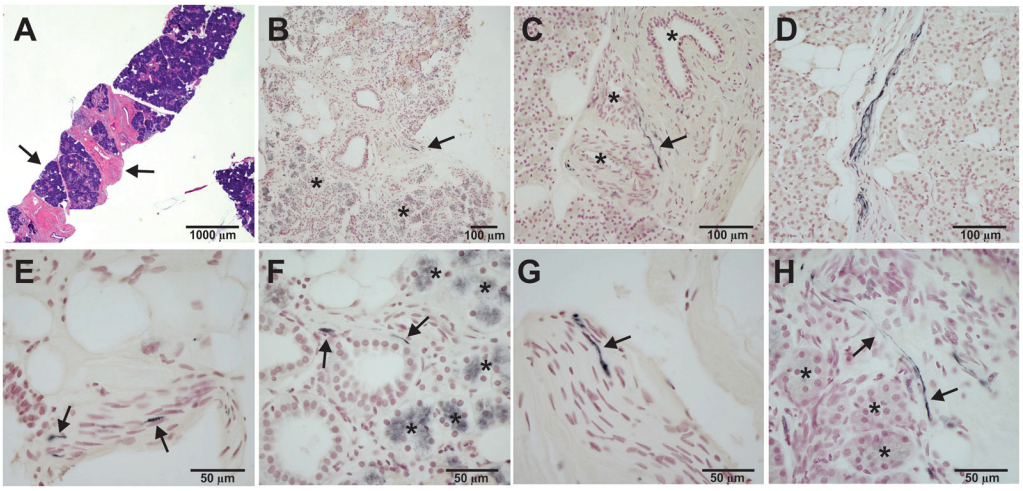

Figure 2. Photomicrographs of needle core tissue from submandibular gland biopsies of subjects with Parkinson's disease

Section A was stained with hematoxylin and eosin. All other sections were stained with an immunohistochemical method for phosphorylated α-synuclein and then counterstained with Neutral Red (see Methods). A) Typical needle core. Arrow on left points to a region of glandular tissue while arrow on right points to an area of stromal connective tissue. B) Arrow points to an immunoreactive nerve fiber within a stromal nerve fascicle. Asterisks indicate non-specific immunoperoxidase staining of gland cell cytoplasm. C) Immunoreactive nerve fiber adjacent to two small blood vessels (two lower asterisks). A small duct is also close by (upper asterisk is within duct lumen). D) Several immunoreactive nerve fibers running in parallel within a stromal nerve fascicle. E) Immunoreactive nerve fibers (arrows) within a stromal nerve fascicle. F) Immunoreactive nerve fibers adjacent to a small duct. Asterisks indicate non-specific immunoperoxidase staining of gland cell cytoplasm. G) Immunoreactive nerve fiber (arrows) within a stromal nerve fascicle. H) Immunoreactive nerve fibers (arrows) adjacent to unstained glandular epithelial cells (asterisks).

Table 1

Demographics of all Parkinson's disease and Control Cases

	Parkinson's disease N=25	Control N=10	95% CI Difference	P
Age at biopsy, years	69.8 (8.2)	64.7 (7.5)	−1.0 to 11.2	.10
Male	16 (64%)	3 (30%)	−0.04 to 0.64	.13
Family history of Parkinson's disease	5 (20%)	0 (0%)	−0.11 to 0.41	.29
Smoking history	8 (32%)	6 (60%)	−0.61 to 0.10	.15
UPSIT Score	18.4 (8.1)	35.7 (4.1)	−22.9 to −11.8	<.001

Data expressed as mean (SD or percentage)

Table 2

Biopsy specifics for Parkinson's disease and Control Cases

	Parkinson's disease N=25	Control N=10	95% CI Difference	P
Number of tissue core biopsies	4.72 (0.68)	4.60 (0.84)	−0.43 to 0.67	.66
Maximal Total Gland area, mm^2	5.8 (4.8)	8.2 (5.8)	−6.2 to 1.5	.22
Total slides screened by PhD-trained neuropathologist	64 (13)	65 (12)	−10 to 9	.95
Total slides examined by board-certified neuropathologist	23 (14)	20 (11)	−7 to 13	.53
Number of positive cases	14	2		
Number of positive slides in LTS positive subjects Mean (SD), N	6.4 (4.5), 14	9.5 (10.6), 2	−11.5 to 5.4	.45

Data expressed as mean (SD)

Table 3

Results for Parkinson's disease LTS positive vs. Parkinson's disease LTS negative cases

	Parkinson's disease LTS positive N=14	**Parkinson's disease LTS negative N=5**	**95% CI Difference**	**P**
Age at biopsy, years	70.4 (6.9)	64.6 (12.7)	−3.6 to 15.4	.21
Male	9 (64%)	3 (60%)	−0.41 to 0.54	>.99
Smoking history	4 (29%)	2 (40%)	−0.60 to 0.34	>.99
UPSIT score	15.4 (5.9)	19.6 (9.7)	−11.9 to 3.5	.27
Number of tissue core biopsies	4.86 (0.66)	4.00 (0.84)	−0.72 to 0.84	.8
Maximal Total Gland area, mm^2	6.8 (4.9)	9.3 (1.1)	−7.1 to 2.3	.29
Total slides screened by PhD-trained neuropathologist	67.6 (10.7)	59.0 (8.7)	−2.7 to 20.0	.13
Total slides examined by board-certified neuropathologist	29.3 (15.4)	15.6 (1.8)	−1.2 to 28.5	.07

Data expressed as mean (SD)