Gastrointestinal Biopsy Obtained During Cancer Screening, a Biological Marker for α-Synucleinopathy?

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Abstract

The hallmark alteration in α-synucleinopathies, α-synuclein, is observed not only in the brain but also in the peripheral tissues, particularly in the intestine. This suggests that endoscopic biopsies performed for colon cancer screening could facilitate the assessment of α-synuclein in the gastrointestinal (GI) tract. Using immunohistochemistry for α-synuclein, we assessed whether GI biopsies could be used to confirm an ongoing α-synucleinopathy. Seventy-four subjects with cerebral α-synucleinopathy in various Braak stages with concomitant GI biopsies were available for study. In 81% of the subjects, α-synuclein was seen in the mucosal/submucosal GI biopsies. Two subjects with severe cerebral α-synucleinopathy and a long delay between biopsy and death displayed no α-synuclein pathology in the gut, and 11 subjects with sparse cerebral α-synucleinopathy displayed GI α-synuclein up to 36 years prior to death. The finding that there was no GI α-synuclein in 19% of the subjects with cerebral α-synucleinopathy, and α-synuclein was observed in the gut of 11 subjects (15%) with sparse cerebral α-synucleinopathy even many years prior to death is unexpected and jeopardizes the use of assessment of α-synuclein in the peripheral tissue for confirmation of an ongoing cerebral α-synucleinopathy.

Key Words: α-Synuclein, Biomarker, Gastrointestinal biopsy, Immunohistochemistry, Lewy body disease.

INTRODUCTION

α-Synucleinopathy, Lewy body disease (LBD), including Parkinson disease (PD) and dementia with Lewy bodies, among others, are incapacitating neurodegenerative disorders (1). Traditionally, LBD is considered to predominantly involve the dopaminergic neurons in the substantia nigra, with accompanying dopamine deficiency in the basal ganglia (2). Subjects with LBD display a plethora of symptoms including olfactory dysfunction, psychiatric symptoms, cognitive impairment, autonomic dysfunction, sleep disorders, weakness, and pain (2–4). Constipation is one symptom that has been reported as being associated with the development of a LBD and may occur more than 20 years before the onset of the motor symptoms (3).

The basic histological findings in LBD are the loss of dopaminergic neurons in the substantia nigra and Lewy bodies and Lewy neurites, intraneuronal inclusions, in the nerve bodies and nerve terminals (5). The main component of these inclusions is an altered α-synuclein (αS) protein that accumulates in the cells (4–6). Currently, it is acknowledged that αS pathology is not limited to the brain but is also seen in the spinal cord and in the peripheral nervous system (PNS), especially in the autonomic nervous system (4). The αS pathology seen in the PNS is considered to be related and eventually to be the cause of various nonmotor symptoms (2, 7). Braak and colleagues hypothesized that neurodegeneration in LBD is initiated in the PNS and progressively reaches the CNS (6, 8). These observations have raised the possibility that peripheral tissue biopsies could be used as in vivo diagnostic markers to identify a preclinical stage of LBD (4). Likewise, the question is whether a peripheral tissue biopsy can be used to confirm a clinically presumed diagnosis of an ongoing cerebral α-synucleinopathy.

Currently, because there are no diagnostic tests that allow for a conclusive diagnosis of a LBD, at an early stage of the disease, up to 20% of patients in the early stages are misdiagnosed (9). Since the discovery of altered αS as a constituent of hallmark lesions in LBD, that is Lewy bodies, postmortem (PM) neuropathological examination, and immunohistochemical (IHC) detection of this altered protein have become the gold standard in diagnostics (2, 5, 10, 11).

Numerous studies have addressed the detection of αS pathology in peripheral tissues, particularly in skin, intestine, and submandibular gland that are suitable for in vivo sampling (9, 12–36). Regarding the intestine, altered αS has been detected in various gastrointestinal (GI) tissue samples, ranging from the esophagus to the rectum in PD patients. Noteworthy, however, positive αS staining has also been detected in the GI tract in unimpaired subjects, probably representing subjects in a preclinical stage of LBD (18). As early as 1990, a
mass cancer screening applying colonoscopy was suggested to be used for identification of pathology in this location (37). Thus, the GI tract might be a promising peripheral tissue to assess for αS pathology as the tissue is readily available for biopsy during routine endoscopic procedures, particularly since colonoscopy is a routine screening tool in subjects aged 60 years and above in many countries (14, 38–40).

In this study, we assessed whether αS pathology was readily detected in GI biopsies. The subjects that included had displayed αS pathology in the brain at PM neuropathological assessment. The goal was to identify whether the pathology observed in the GI biopsy was in line with the pathology observed in the brain. In parallel, we conducted a critical assessment of the methods applied.

MATERIALS AND METHODS

This study was carried out on tissue obtained PM and on archived surgical samples. The subjects and/or their relatives had given their consent for the use of the tissue and the study was approved by the local ethical committee (Dnr 2011/286) and updated in 2015.

The autopsies were carried out following standard procedures. The PM delay was noted, the brain was removed, weighed, and placed in 4% buffered formalin. After 2–5 days of fixation, the grossing was carried out; the severity of arteriosclerosis was registered (none, mild, moderate/severe); and macroscopic lesions observed were noted on the coronal sections. Standard sections of brain tissue were sampled and placed in commercial mega cassettes for further fixation in 4% buffered formalin (41). After an additional 2 weeks of fixation, the automatic paraffin embedding procedure was initiated.

During a period of 10 years, 972 complete autopsies in including a neuropathological investigation were performed. Out of these, 216 subjects (22%) had displayed αS pathology in the brain to some extent, thus fulfilling the primary selection criterion. The assessment of αS pathology in the brain was carried out applying IHC stains (Table 1). Currently recommended consensus criteria, that is Braak stage for αS, ranging from stage 1 to stage 6 and McKeith criteria brainstem, limbic and neocortical stage, were followed to assess the severity of αS pathology seen in the brains (6, 41, 42).

Out of the cohort of 216 subjects with αS pathology in the brain, those subjects that had a GI sample taken during their life and/or those with GI sampling performed during the autopsy were identified. This search was feasible due to a Laboratory Investigation System and long-term storage of biopsy/surgical samples at the Uppsala University Hospital. Overall, 74 subjects (34%) were identified, fulfilling the second selection criterion. The mean age of the study cohort at death was 80.2 ± 0.9 (mean ± SEM); the age ranged from 62 to 95 years. Most of the subjects were male 45 (61%). Moreover, 50% of the whole cohort had displayed cognitive impairment; 36% of these were females and 14% were males.

A general description of the archived material available in this retrospective study is provided in Table 2. In 43 out of the 74 subjects, a GI tissue sample was obtained prior to death; in 27 subjects, the GI samples were obtained during autopsy; and in 4 subjects, both premortem and PM materials were available. The number of GI tissue samples for each subject varied from 1 to 9. The time delay between GI organ sampling and autopsy ranged from 0 to 36 years.

An archived hematoxylin and eosin (H&E)-stained section from the GI tract was assessed to evaluate the tissue quality. Samples where nerve elements were not clearly observed in the H&E-stained sections were also included. This approach was applied, as in the mucosa and even in the scarce submucosa; nerve structures are seldom clearly observed in H&E staining. The presence of inflammation, necrosis, or neoplasm was not an exclusion criterion, provided that sufficient “healthy” tissue was present in the tissue sample.

About 4-μm-thick consecutive sections were cut from the selected GI tissue blocks and stained with the αS antibodies listed in Table 1. A dichotomous approach was chosen while assessing the αS IHC stains (none = negative; sparse, moderate, and frequent = positive) to secure high reproducibility. A specific template was created detailing different αS staining patterns in different parts of the GI organ wall (mucosa, submucosa, and muscle layer).

Data were analyzed using the methods of descriptive and analytical statistics. Categorical data are presented as n (%) and analyzed by chi-square contingency table test or Fisher exact test. All tests were two-tailed. The level of significance was set at 0.05. Statistical analysis was performed using the IBM SPSS Statistics for Windows (Version 21.0, IBM Corp., Armonk, NY).

RESULTS

Out of the 74 subjects, 10 (13.5%) displayed αS pathology in the brain to an extent of Braak stage 1, 1 subject Braak stage 2 (1%), 7 Braak stage 3 (10%), 35 Braak stage 4 (47%), 14 Braak stage 5 (19%), and 7 subjects Braak stage 6 (10%). Thus, 18 subjects (24%) were in the brainstem stage of αS pathology, 35 (47%) in the limbic, and 21 (28%) were in the neocortical stage of αS pathology (6, 42).

While reassessing available archived H&E-stained GI samples, some were excluded due to repeated samplings taken from the same region within 1 year. The oldest sample was always included. When a colorectal sample was available from a patient, other samples obtained from the GI channel were excluded. Thus, from a total of 144 available archived GI samples, 97 samples from 74 patients were chosen for this study (Table 2). These 97 samples were stained with all 3 αS and the p62 antibody. The following IHC staining patterns were identified as specific: 1, diffuse and/or granular staining in nerves and/or ganglia; 2, stained dots, not clearly characterized as positivity within various nonneuronal cells (epithelial or other); 3, fibers, and 4, globules, that is, coarse, Lewy-body-like aggregates (Fig. 1). Staining patterns such as perivascular/vascular walls staining, epithelial cells and other nonneuronal cells, and lac-ygranular staining were considered nonspecific (Fig. 1). The αS labeling outcomes in the study cohort of LBD subjects are summarized in Table 3.

The best performing antibody was αS^{LBD509}, which was positive in more than 80% of cases. This outcome was not significantly influenced by whether the sample was obtained...
during life or PM. Regarding αS\textsuperscript{LB509}, the large intestine seems to be the organ most frequently affected.

For the purpose of this study, all samples containing muscle layer of the intestine wall were considered as full thickness tissue samples regardless of whether they contained mucosa and/or submucosa. Furthermore, an IHC staining was carried omitting the primary antibody on 3 samples with substantial αS labeling independent of antibody used and no labeling was observed on these cases.

We compared mucosa ± submucosa (m/s) samples with full thickness (ft) tissue samples. All 4 antibodies performed well in both ft and m/s samples, and no significant differences were observed (αS\textsuperscript{KM51} ft 54% vs m/s 42%, p = 0.33; αS\textsuperscript{5G4} ft 49% vs m/s 48%, p = 0.97; αS\textsuperscript{LB509} ft 84% vs m/s 77% p = 0.50; P62 ft 65% vs m/s 48%, p = 0.15).

The staining outcome was assessed in relation to the Braak stages. Group A (Braak stages 1–3) included 18 subjects; and group B (Braak stages 4–6) included 56 subjects. When all 74 subjects were included, regardless of the tissue type, group A did not differ significantly from group B regarding the staining outcome.

When only ft tissue samples were included, the αS\textsuperscript{LB509} positivity difference was close to significant (p = 0.07) when comparing group A with group B subjects. Out of the 8 (with ft biopsy) group A subjects, 63% were positive compared to 89% out of the 35 (with ft biopsy) group B subjects.

When comparing the labeling outcome independent of the antibody used in the different regions of the GI tract, no differences were observed. Only 3 subjects (4%) had samples removed from several locations of the GI channel on the same

### TABLE 1. Antibodies

| Antibody               | Clone   | Source                                      | Dilution | Pretreatment           |
|------------------------|---------|---------------------------------------------|----------|------------------------|
| Synuclein full length  | KM51*   | Leica Biosystems, Werzlar, Germany, No. NCL-L-ASYN | 1:100    | CB pH 6\textsuperscript{1}+80% FA 5 min |
| Synuclein aa115-122    | LB509   | Abcam, Cambridge, United Kingdom, ab27776   | 1:20 000 | CB pH 6\textsuperscript{1}+80% FA 5 min |
| Synuclein aa44-57      | 5G4     | Kem-en-tech, Taastrup, Denmark, No. 847-0102004001 | 1:5000   | CB pH 6\textsuperscript{1}+80% FA 5 min |
| P62                    | Clone 3, lck ligand | BD Bioscience, Franklin Lakes, NJ, No. 610833 | 1:500    | CB pH 6\textsuperscript{1} |

CB, citrate buffer; FA, formic acid.
*Used for postmortem brain assessment.
†Autoclave.

### TABLE 2. Description of the Gastrointestinal (GI) Tissue Samples

| Sampling Strategy                  | Number of Patients | GI Location          | Number of Samples | Delay between GI Sampling and Death in Months (Mean ± SEMs) |
|------------------------------------|--------------------|----------------------|-------------------|----------------------------------------------------------|
| GI samples obtained during life    | 43                 | Ventricle            | 8                 | 6.5 ± 2.3                                                |
|                                    |                    | Small intestine      | 9                 | 7.8 ± 2.6                                                |
|                                    |                    | Appendix             | 1                 |                                                         |
|                                    |                    | Large intestine      | 37                | 9.9 ± 1.7                                                |
| GI samples obtained at autopsy     | 27                 | Esophagus            | 5                 |                                                         |
|                                    |                    | Ventricle            | 7                 |                                                         |
|                                    |                    | Small intestine      | 6                 |                                                         |
|                                    |                    | Appendix             | 0                 |                                                         |
|                                    |                    | Large intestine      | 9                 |                                                         |
| GI samples obtained “in vivo” and postmortem from 4 subjects | 4                  | Large intestine      | 2                 | Subject 1: sample at autopsy and 10 years prior to death |
|                                    |                    | Large intestine      | 2                 | Subject 2: sample at autopsy and 17 years prior to death |
|                                    |                    | Various GI locations | 2                 | Subject 3: sample from ventricle at autopsy and small intestine 6 years prior to death |
|                                    |                    | Various GI locations | 9                 | Subject 4: samples from esophagus, ventricle, small and large intestine at autopsy and large intestine 4 years prior to death |
| In total                           | 74                 |                      | 97                |                                                         |
date. One male subject, age at death 74 years, Braak stage 5, displayed αS\textsuperscript{LB509} pathology in a sample obtained close to death from the small intestine, appendix, and large intestine. The second male subject, age at death 88 years, Braak stage 3, displayed αS\textsuperscript{LB509} pathology in a sample obtained during life 13 years prior to death from the ventricle and small intestine. The third male subject, age at death 77 years, Braak stage 5, displayed αS\textsuperscript{LB509} pathology in a sample obtained at autopsy from the esophagus, ventricle, duodenum, and rectum, whereas the jejunum, ileum, and colon were negative.

Table 4 provides a summary of the staining outcomes in the GI samples in the 15 subjects with the longest duration between GI sampling and death. The GI samples were obtained 8–36 years prior to death. In 13 out of the 15 subjects with Braak stages ranging from 1 to 6, αS\textsuperscript{LB509} pathology was detected in the GI sample. There were 3 subjects in Braak stage 1, with concomitant αS\textsuperscript{LB509} pathology in a GI sample obtained 10, 11, and 36 years prior to death. Contrary to the above, there were 2 subjects in Braak stages 4 and 5 lacking αS\textsuperscript{LB509} pathology in the GI sample, obtained 12 respective 23 years prior to death.

DISCUSSION

To the best of our knowledge, this study is the first to investigate GI biopsies obtained as long as 36 years prior to death in subjects that displayed α-synucleinopathy at PM examination. We only selected subjects with verified αS pathology in the brain, ranging from Braak stages 1–6 and observed αS pathology in the GI in as many as 81% of the 74 subjects. Our results are in line with previous publications reporting that αS pathology is present in the GI tract in 40%–100% of individuals with LBD (12, 15, 17, 21, 23, 28, 36). The reported incidences of αS in the GI differ, and the results are difficult to compare as both the extent of pathology in the brain, that is Braak stage, and the duration between the GI biopsy and death...
are not harmonized, that is not the same. Because these incidences are generally relatively high, they support the use of GI αS assessment as a biomarker to confirm ongoing α-synucleinopathy.

It is well known and acknowledged that the IHC technique and the type of antibody used significantly influence the assessment outcome (41, 43). Regarding the GI biopsies, we also assessed the performance of different αS antibodies. Some studies have shown that both phosphorylated and nonphosphorylated αS can be used, whereas others have preferred using phosphorylated αS (16, 22, 25, 27, 30). Due to the differing outcomes reported, we tested several commonly used αS antibodies (αSLB509, αSKM51, and αSG4) and an antibody directed to ubiquitin-binding protein p62 (44, 45). We observed that the antibody αSLB509 performed the best, that is 81% of the subjects in our cohort displayed pathology in the gut. In previous studies, while assessing the brain, the αSG4 was reported as being more reliable due to limited unspecific labeling of physiological αS43. We did not observe excessive unspecific labeling with αSLB509 in the gut. This is probably related to the likelihood that the extent of physiological αS in the brain exceeds that what is seen in the gut. However, it is difficult, if not impossible, to have reliable comparisons regarding the performance of different antibodies due to significant differences in the tissue characteristics, that is biopsy versus PM tissue, PM time, fixative, and fixation time, among others. Thus, one always needs to be cautious regarding false negative outcomes and repeat the staining when this is suspected.

Interestingly, we observed that αS pathology was readily assessed in both ft and in m/s samples. This result is of

### TABLE 3. Samples with α-Synuclein (αS) or p62 Labeling in the Gastrointestinal (GI) Tissue

| Sampling Strategy | GI Location | Total Number | αS*- or p62*-Positive GI Samples in Numbers (n) and Percent (%) |
|-------------------|-------------|--------------|---------------------------------------------------------------|
|                   |             |              | ab KM51 n (%) | ab 5G4 n (%) | ab LB509 n (%) | ab p62 n (%) |
| Tissue obtained during life | Ventricle | 8 | 4 (50) | 2 (25) | 5 (63) | 2 (25) |
| | Small intestine | 9 | 4 (44) | 7 (78) | 8 (89) | 7 (78) |
| | Large intestine | 29 | 16 (55) | 16 (55) | 25 (86) | 20 (69) |
| Total | | 46 | 24 (52) | 25 (54) | 38 (83) | 29 (63) |
| Tissue obtained at autopsy | Esophagus | 5 | 3 (60) | 4 (80) | 4 (80) | 4 (80) |
| | Ventricle | 7 | 4 (57) | 4 (57) | 5 (71) | 4 (57) |
| | Small intestine | 7 | 3 (43) | 1 (14) | 5 (71) | 4 (57) |
| | Large intestine | 9 | 2 (22) | 2 (22) | 8 (89) | 2 (22) |
| Total | | 28 | 12 (43) | 11 (39) | 22 (77) | 14 (50) |
| Total all samples | | 74 | 36 (49) | 36 (49) | 60 (81) | 46 (62) |

*Antibodies (ab) as listed in Table 1.

### TABLE 4. Fifteen Subjects with the Longest Time between Biopsy from the Gastrointestinal Tract (GI), Large Intestine, and Neuropathological Investigation at Death

| Time between Biopsy and Death in Years | Age at Death | Gender | Extent of αS in Brain Braak Stage | αS in GI Antibody LB509 | Tissue Type* |
|---------------------------------------|--------------|--------|----------------------------------|------------------------|-------------|
| 8                                     | 77           | Male   | 5                                | 1                      | 1           |
| 9                                     | 85           | Male   | 4                                | 1                      | 2           |
| 10                                    | 93           | Female | 1                                | 1                      | 2           |
| 11                                    | 74           | Male   | 5                                | 1                      | 2           |
| 11                                    | 68           | Male   | 1                                | 1                      | 2           |
| 12                                    | 95           | Male   | 4                                | 0                      | 2           |
| 13                                    | 83           | Male   | 4                                | 1                      | 2           |
| 16                                    | 74           | Male   | 3                                | 1                      | 2           |
| 17                                    | 87           | Female | 4                                | 1                      | 2           |
| 21                                    | 92           | Male   | 4                                | 1                      | 2           |
| 21                                    | 92           | Male   | 6                                | 1                      | 2           |
| 23                                    | 81           | Female | 5                                | 0                      | 2           |
| 23                                    | 74           | Male   | 4                                | 1                      | 2           |
| 28                                    | 89           | Male   | 4                                | 1                      | 1           |
| 36                                    | 83           | Female | 1                                | 1                      | 2           |

*Tissue type, 1, full thickness; 2, mucosa; 3, submucosa.
interest because the colon cancer screening biopsies are of m/s type. Similar results have also been reported by others (12, 16, 17, 28). It is noteworthy that contradictory observations have also been reported, leading to the conclusion that the m/s samples are insufficient to reliably assess αS pathology (15, 23, 46). The observed differences are probably related to the tissue characteristics and methodology used. Our m/s samples were surgical biopsies and thus lacked many of the alterations that are common in PM material.

The αS hypothesis suggests that aggregation of αS is initiated in the CNS in the GI tract and/or the olfactory organs with consequent propagation of protein alteration towards the CNS (8, 47). Furthermore, a GI gradient with a higher percentage of αS in the esophagus and stomach when compared with the small and large intestine, has been suggested. In addition, some have observed a higher density of pathology in the proximal compared to the distal parts of the large intestine (15, 19). In the present relatively large cohort, we had 3 subjects in which we were able to assess the regional distribution of the αS pathology in various locations of the GI tract, that is samples obtained on the same day from different locations in the GI tract. We observed that some locations were positive whereas others were negative, and the distribution of positivity was haphazard. Lack of regional preference, that is gradient of αS pathology in the gut, has also been reported by others (22, 34, 35). When the regional preference was assessed by others as above, the GI samples in general were obtained from different patients. Thus, based on our results, we cannot state whether a certain GI region is more prone to display αS pathology.

Here, we were able to assess the influence of time on the progression of αS pathology. There were 15 subjects for whom 8–36 years had passed from the time of GI sampling to death. At death αS was observed in the brain to various extents, ranging from Braak stages 1–6. In 13 subjects, the αS pathology was indeed observed in the gut biopsy, whereas there were 2 subjects in Braak stages 4 and 5 that were negative in the gut, 12 and 23 years prior to death, respectively. Contrary to the above, there were 3 subjects all in Braak stage 1 at death but with verified GI αS pathology 10, 11, and 36 years prior to death. These results are surprising and suggest that the impact of the premortem obtained GI biopsy as a biomarker might be questionable. The first outcome, negative GI samples and significant brain pathology, might be explained by a sampling deficit or by decrease in the αS load in the periphery with the duration of the disease, as previously suggested (48). Noteworthy, however, an increase in GI αS pathology with progression of PD have also been published (14). The question of where the αS alteration is initiated in the CNS or PNS and how it progresses or if it can be initiated simultaneously in several different locations has previously been debated (23). Our results are in line with other studies that have failed to detect a time association between αS labeling in the GI tract and clinical symptoms of a brain disease (22). Thus, our results are more in line with the presumption that the pathological alteration can be initiated in several different locations within the neuraxis.

The protein αS has been reported to be seen in the GI channel by many, but there are very few studies assessing αS in the PNS in the preclinical/prodromal phase of PD. The identification of subjects in the preclinical/prodromal phase of sporadic α-synucleinopathy is impossible. It has been reported that only 30% of subjects with αS in their brain have a clinical diagnosis, that is thus representing cases of the category incidental α-synucleinopathy (49). In one study, the presence of αS in the GI was observed 2–5 years before the clinical diagnosis of PD, and αS aggregates have been seen in the PNS in subjects with neuropathologically confirmed incidental LBD (18, 23). To reliably assess αS in the GI in the preclinical stage of an α-synucleinopathy, retrospective studies in line with ours need to be carried out. As many as 11 of our subjects displayed sparse brain pathology, that is Braak stages 1 and 2 and displayed no clinical signs of disease. In 9 of these subjects (82%), αS pathology was seen in the gut several years prior to death with a range of 0–36 years (mean ± SEM 8.2 ± 3.97 years). These results certainly suggest that assessment of αS in a GI biopsy could be used as a biomarker in order to identify subjects in the prodromal phase of a LBD.

While assessing 74 subjects with neuropathologically verified LBD, GI αS pathology was seen in 81% of the subjects. The αS pathology was readily seen in endoscopically obtained m/s GI samples while applying the IHC methodology and the commercial αS-LB509 antibody. These 2 observations support the use of colon cancer screening biopsies as an in vivo biomarker to verify the ongoing α-synucleinopathy. Surprisingly, 2 of our subjects with a long delay between biopsy and death and relatively severe LBD (Braak stages 4 and 5) displayed no αS pathology in the GI biopsy; some with sparse extent of brain pathology (Braak stages 1 and 2) had displayed GI αS pathology up to 36 years prior to death. These 2 observations suggest that the use of this approach, αS assessment in a GI biopsy as a biomarker, might be unsecure. In some of our subjects with GI pathology, the outcome might have been an ongoing α-synucleinopathy, whereas the patients never develop a disease even after 10, 11, and 36 years. Thus, in conclusion, even if the methodology is available and reliable, and could easily be implemented on routinely obtained colon cancer screening samples, we cannot recommend the use of this approach as a biomarker due to the limited knowledge of the initiation site, progression, and evolution of the pathology. Further studies, both retrospective and prospective, including PM assessment of the brain and peripheral organs are certainly needed.

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