Lewy pathology of the gastrointestinal (GI) tract has potential as a biomarker for the diagnosis of synucleinopathies such as Parkinson’s disease (PD) and idiopathic rapid eye movement sleep disorder (iRBD). However, low sensitivity limits its use in clinical practice. In a previous autopsy study, evaluation of multiple sections increased the positivity rate, and earlier in vivo studies reported high sensitivity using a method called ‘whole-mount staining’. Therefore, this study aimed to determine whether evaluation of a larger tissue volume increases the sensitivity of detecting alpha-synuclein (AS) pathology in the GI tract in patients with synucleinopathy.

MATERIALS & METHODS

Participants and specimen selection

Patients in this study were selected from those who partici-
pated in previous studies of PD and iRBD. We selected patients with PD or iRBD, and controls who had both proximal and distal marginal blocks of the GI tract archived in the pathology bank. Therefore, two formalin-fixed paraffin-embedded blocks were collected per participant. This study was approved by the Institutional Review Board of Seoul National University Hospital (IRB No. H-1409-043-608). The requirements for a waiver of informed consent were met, and a waiver was granted.

**Immunohistochemistry**

A total of five serial 3-μm sections were obtained from each surgical block for immunohistochemistry (IHC). The paraffin sections were mounted on a glass slide, dewaxed, rehydrated, and incubated with primary antibodies on automated machines as previously described. A primary antibody against phosphorylated AS (pAS) (1/1,000 anti-pAS at serine 129 monoclonal Ab [EP1536Y]; Abcam ab51253, Cambridge, UK) was used in conjunction with the Leica Bond Max (A33030) system, in accordance with the manufacturer's instructions. Bound antibodies were detected using the Bond Polymer Refine Detection system (Leica Biosystems, Wetzlar, Germany).

**Pathologic evaluation**

To avoid bias, the raters were blinded to the clinical information of the participants. All stained slides were scanned with a Leica Slide Scanner (Aperio AT2, Leica Biosystems). Anonymized digital slides were evaluated using the Pathologic Slide Viewing Software (Aperio ImageScope ver. 12.4, Leica Biosystems).

pAS positive findings were defined conservatively as in the previous study: 1) pAS IHC showing definite and clear staining such as ‘dots and fiber’ or ‘Lewy body-like staining’ pattern, as the consensus paper suggested and 2) localization in neural structures confirmed with anatomic inspection. pAS-positive findings were semiquantitatively rated as grade 1, 2, or 3, which corresponded to sparse, moderate, or frequent in the findings were semiquantitatively rated as grade 1, 2, or 3, and the subjects were not randomly selected. Therefore, descriptive analyses were mainly conducted. For group comparison, nonparametric tests were used because of the small number of participants. All statistical analyses were conducted with SPSS ver. 26.0.0.0 (IBM Corp., Armonk, NY, USA).

**RESULTS**

Nine patients (4 PD and 5 iRBD) and five controls were selected. In previous studies, pAS was positive in 5/9 patients (55.6%) and 1/5 controls (20.0%). In an extensive evaluation of 10 slides per patient, the positivity rate increased to 8/9 patients (88.9%), but the rate remained the same (20.0%) in controls (p = 0.023; Table 1 and Figure 1).

In the detailed analysis of 5 slides from the proximal and distal blocks in both the patient and control groups, samples from 6 of 9 subjects with pAS positivity (66.7%) were positive in both proximal and distal blocks (Supplementary Table 1 in the online-only Data Supplement and Figure 1). Moreover, positivity was present in all 5 serial sections in 80.0% of positively stained blocks (12/15); three blocks of P02 and C02 were exceptions.

Regarding the severity of pAS positivity, severe density (3+) was found in both PD and iRBD patients. When the results were discordant between proximal and distal blocks (P06, P08, and C02), only mild density (1+) was found in the positive block. Similarly, when pAS-positive findings were not present in all 5 serial sections (P02 and C02), only mild density (1+) was seen. Finally, there was a severity gradient in the distal block of P01.

**DISCUSSION**

This study demonstrates that pAS positivity increases with increasing tissue volume examined, which is consistent with previous studies. Beach et al. reported that the pAS positivity rate in the GI tract increased from 11/17 (64.7%) to 14/15 (93.3%) when multiple slides and 80-μm frozen sections were examined in their large-scale autopsy study. Lebouvier et al. also reported good results (72%–80%, pAS positivity) using whole-mount staining, which involves microdissecting the submucosa from the mucosa in the biopsied colon tissue and mounting and staining the whole submucosa at once. The method allowed us to evaluate a large submucosal tissue volume. This study also showed that mild AS accumulation with the semiquantitative grade 1+ could be inconsistently rated within the proximal and distal sites of one organ and consecutive sections taken from a single site. These findings could be explained by the multifocal
The distribution of AS accumulation in the GI tract, which has a greater influence on the pathologic evaluation of milder disease stages. Therefore, an extensive volume of large full-depth pathologic specimens is required to achieve the maximal positivity rate, but this is not feasible in clinical practice. These results further support the fundamental limit of biopsies that contain only a small portion of mucosal and submucosal layers from the intestinal wall.

There were no definite differences in the distribution and severity between patients with iRBD and PD, although iRBD is a prodromal stage of PD. Previous studies have reported pAS-positive rates of 62.5% and 58.3% in the stomach specimens of patients with iRBD and PD, respectively. Several studies have reported that the sensitivity of detecting AS accumulation is higher in patients with iRBD than in PD using specimens from the colon, submandibular gland, and skin. These results support the notion that AS accumulation in the enteric nervous system precedes the progression of Lewy pathology from the periphery. Therefore, it supports the gut-to-brain progression model presented in Braak's hypothesis. In contrast, one patient with iRBD (P09) showed no positivity on extensive examination. The negative result of one control subject (C01) whose initial result was positive can be explained by the different definitions of AS positivity. The previous definition of AS positivity allowed a diffuse staining pattern that the initial staining result for this subject showed. Later, we used a more conservative definition based on evidence from subsequent studies.

Table 1. pAS immunostaining results of extensive tissue (5 slides per block) compared with original staining findings

| Group | ID | Diagnosis | PD subtype | Duration of disease onset to operation (yr) | Specimen | pAS result | Distal block | Proximal block |
|-------|----|-----------|------------|--------------------------------------------|----------|------------|-------------|---------------|
|       |    |           |            |                                            |          | Initial*  | Extensive  | Highest grade | N of positive slides | Initial*  | Extensive  | Highest grade | N of positive slides |
| Patient | P01 | PD | TD | 1 | Stomach | (+) | (+) | (+) | (+) | 3+ | 5 | (+) | (+) | 3+ | 5 |
| Patient | P02 | PD | TD | -2 | Proximal colon | (+) | (+) | (-) | (+) | 1+ | 1 | (+) | (+) | 1+ | 4 |
| Patient | P03 | iRBD |       | -2 | Stomach | (+) | (+) | (-) | (+) | 3+ | 5 | (+) | (+) | 2+ | 5 |
| Patient | P04 | iRBD |       | -1 | Esophagus | (+) | (+) | (+) | (+) | 3+ | 5 | (+) | (+) | 3+ | 5 |
| Patient | P05 | iRBD |       | 0 | Stomach | (+) | (+) | (+) | (+) | 2+ | 5 | (+) | (+) | 3+ | 5 |
| Patient | P06 | PD | TD | 5 | Proximal colon | (-) | (+) | (-) | (+) | 1+ | 5 | (-) | (-) | 0 | 0 |
| Patient | P07 | PD | PIGD | 1 | Stomach | (-) | (+) | (-) | (-) | 0 | 0 | (-) | (-) | 0 | 0 |
| Patient | P08 | iRBD |        | -1 | Stomach | (+) | (+) | (+) | (+) | 3+ | 5 | (-) | (-) | 1+ | 5 |
| Patient | P09 | iRBD |        | -3 | Stomach | (-) | (+) | (-) | (-) | 0 | 0 | (-) | (-) | 0 | 0 |
| Control   | C01 | Proximal colon | (-) | (+) | (+) | 0 | 0 | (-) | (-) | 0 | 0 | (-) | (-) | 0 | 0 |
| Control   | C02 | Proximal colon | (-) | (+) | (-) | 0 | 0 | (-) | (-) | 1+ | 2 | (-) | (-) | 0 | 0 |
| Control   | C03 | Stomach | (-) | (-) | (-) | 0 | 0 | (-) | (-) | 0 | 0 | (-) | (-) | 0 | 0 |
| Control   | C04 | Stomach | (-) | (-) | (-) | 0 | 0 | (-) | (-) | 0 | 0 | (-) | (-) | 0 | 0 |
| Control   | C05 | Stomach | (-) | (-) | (-) | 0 | 0 | (-) | (-) | 0 | 0 | (-) | (-) | 0 | 0 |

*Initial result is retrieved from immunostaining data of previous studies which stained one slide per block. pAS, phosphorylated alpha-synuclein; PD, Parkinson's disease; iRBD, idiopathic rapid eye movement sleep disorder; TD, tremor-dominant; PIGD, postural instability gait disturbance.

There was a small portion of mucosal and submucosal layers from the intestinal wall, which showed a greater influence on the pathologic evaluation of milder disease stages. Therefore, an extensive volume of large full-depth pathologic specimens is required to achieve the maximal positivity rate, but this is not feasible in clinical practice. These results further support the fundamental limit of biopsies that contain only a small portion of mucosal and submucosal layers from the intestinal wall.

There were no definite differences in the distribution and severity between patients with iRBD and PD, although iRBD is a prodromal stage of PD. Previous studies have reported pAS-positive rates of 62.5% and 58.3% in the stomach specimens of patients with iRBD and PD, respectively. Several studies have reported that the sensitivity of detecting AS accumulation is higher in patients with iRBD than in PD using specimens from the colon, submandibular gland, and skin. These results support the notion that AS accumulation in the enteric nervous system precedes the progression of Lewy pathology from the periphery. Therefore, it supports the gut-to-brain progression model presented in Braak's hypothesis. In contrast, one patient with iRBD (P09) showed no positivity on extensive examination. The negative result of one control subject (C01) whose initial result was positive can be explained by the different definitions of AS positivity. The previous definition of AS positivity allowed a diffuse staining pattern that the initial staining result for this subject showed. Later, we used a more conservative definition based on evidence from subsequent studies.

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| Group | ID | Diagnosis | PD subtype | Duration of disease onset to operation (yr) | Specimen | pAS result | Distal block | Proximal block |
|-------|----|-----------|------------|--------------------------------------------|----------|------------|-------------|---------------|
| Patient | P01 | PD | TD | 1 | Stomach | (+) | (+) | (+) | (+) | 3+ | 5 | (+) | (+) | 3+ | 5 |
| Patient | P02 | PD | TD | -2 | Proximal colon | (+) | (+) | (-) | (+) | 1+ | 1 | (+) | (+) | 1+ | 4 |
| Patient | P03 | iRBD |       | -2 | Stomach | (+) | (+) | (-) | (+) | 3+ | 5 | (+) | (+) | 2+ | 5 |
| Patient | P04 | iRBD |       | -1 | Esophagus | (+) | (+) | (+) | (+) | 3+ | 5 | (+) | (+) | 3+ | 5 |
| Patient | P05 | iRBD |       | 0 | Stomach | (+) | (+) | (+) | (+) | 2+ | 5 | (+) | (+) | 3+ | 5 |
| Patient | P06 | PD | TD | 5 | Proximal colon | (-) | (+) | (-) | (+) | 1+ | 5 | (-) | (-) | 0 | 0 |
| Patient | P07 | PD | PIGD | 1 | Stomach | (-) | (+) | (-) | (-) | 0 | 0 | (-) | (-) | 0 | 0 |
| Patient | P08 | iRBD |        | -1 | Stomach | (+) | (+) | (+) | (+) | 3+ | 5 | (-) | (-) | 1+ | 5 |
| Patient | P09 | iRBD |        | -3 | Stomach | (-) | (+) | (-) | (-) | 0 | 0 | (-) | (-) | 0 | 0 |
| Control   | C01 | Proximal colon | (-) | (+) | (+) | 0 | 0 | (-) | (-) | 0 | 0 | (-) | (-) | 0 | 0 |
| Control   | C02 | Proximal colon | (-) | (+) | (-) | 0 | 0 | (-) | (-) | 1+ | 2 | (-) | (-) | 0 | 0 |
| Control   | C03 | Stomach | (-) | (-) | (-) | 0 | 0 | (-) | (-) | 0 | 0 | (-) | (-) | 0 | 0 |
| Control   | C04 | Stomach | (-) | (-) | (-) | 0 | 0 | (-) | (-) | 0 | 0 | (-) | (-) | 0 | 0 |
| Control   | C05 | Stomach | (-) | (-) | (-) | 0 | 0 | (-) | (-) | 0 | 0 | (-) | (-) | 0 | 0 |

*Initial result is retrieved from immunostaining data of previous studies which stained one slide per block. pAS, phosphorylated alpha-synuclein; PD, Parkinson's disease; iRBD, idiopathic rapid eye movement sleep disorder; TD, tremor-dominant; PIGD, postural instability gait disturbance.
The staining results for this subject confirmed that the current definition of pAS positivity used in this study was more reliable than the previous one because there were no positive findings in this subject, including the ‘diffuse staining’ pattern.

In conclusion, examination of a large tissue volume increased the sensitivity of detecting AS accumulation in the GI tract. This study implies a fundamental limit of biopsied tissue and highlights the complexity of pathologic progression of synucleinopathy.

**Supplementary Materials**

The online-only Data Supplement is available with this article at https://doi.org/10.14802/jmd.22042.

**Conflicts of Interest**

The authors have no financial conflicts of interest.

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REFERENCES

1. Shin C, Park SH, Yun JY, Shin JH, Yang HK, Lee HJ, et al. Fundamental limit of alpha-synuclein pathology in gastrointestinal biopsy as a pathologic biomarker of Parkinson’s disease: comparison with surgical specimens. Parkinsonism Relat Disord 2017;44:73-78.

2. Beach TG, Adler CH, Sue LI, Velders L, Lue L, White III CL, et al. Multi-organ distribution of phosphorylated alpha-synuclein histopathology in subjects with Lewy body disorders. Acta Neuropathol 2010;119:689-702.

3. Lebouvier T, Chaumette T, Damier P, Coron E, Touchefeu Y, Veignaud S, et al. Pathological lesions in colonic biopsies during Parkinson’s disease. Gut 2008;57:1741-1743.

4. Lebouvier T, Poulet H, Coron E, Drouard A, NGuyen JM, Roy M, et al. Colonic neuropathology is independent of olfactory dysfunction in Parkinson’s disease. J Parkinsons Dis 2011;1:389-394.

5. Lebouvier T, Neunlist M, Bruley des Varannes S, Coron E, Drouard A, NGuyen JM, et al. Colonic biopsies to assess the neuropathology of Parkinson’s disease and its relationship with symptoms. PLoS One 2010;5:e12728.

6. Shin C, Park SH, Shin JH, Yun JY, Yang HK, Lee HJ, et al. Gastric synucleinopathy as prodromal pathological biomarker in idiopathic REM sleep behaviour disorder. J Neurol Neurosurg Psychiatry 2021;92:450-451.

7. Beach TG, Corbillé AG, Letournel F, Kordower JH, Kremer T, Munoz DG, et al. Multicenter assessment of immunohistochemical methods for pathological alpha-synuclein in sigmoid colon of autopsied Parkinson’s disease and control subjects. J Parkinsons Dis 2016;6:761-770.

8. Shin C, Park SH, Yun JY, Shin JH, Yang HK, Lee HJ, et al. Alpha-synuclein in staining in non-neural structures of the gastrointestinal tract is non-specific in Parkinson disease. Parkinsonism Relat Disord 2018;55:15-17.

9. Chahine LM, Beach TG, Brumm MC, Adler CH, Coffey CS, Mosovsky S, et al. In vivo distribution of α-synuclein in multiple tissues and biofluids in Parkinson disease. Neurology 2020;95:e1267-e1284.

10. Sprenger FS, Stefanova N, Gelpi E, Seppi K, Navarro-Otano J, Offner E, et al. Enteric nervous system α-synuclein immunoreactivity in idiopathic REM sleep behavior disorder. Neurology 2015;85:1761-1768.

11. Vilas D, Iranzo A, Tolosa E, Aldecoa I, Berenguer J, Vilaseca I, et al. Assessment of α-synuclein in submandibular glands of patients with idiopathic rapid-eye-movement sleep behaviour disorder: a case-control study. Lancet Neurol 2016;15:708-718.

12. Al-Qassabi A, Tsaos TS, Racolta A, Kremer T, Cañamero M, Belousov A, et al. Immunohistochemical detection of synuclein pathology in skin in idiopathic rapid eye movement sleep behavior disorder and parkinsonism. Mov Disord 2021;36:895-904.

13. Braak H, de Vos RA, Bohl J, Del Tredici K. Gastric alpha-synuclein immunoreactive inclusions in Meissner’s and Auerbach’s plexuses in cases staged for Parkinson’s disease-related brain pathology. Neurosci Lett 2006;396:57-72.

14. Beach TG, Adler CH, Sue LI, Shill HA, Driver-Dunckley E, Mehta SH, et al. Vagus nerve and stomach synucleinopathy in Parkinson’s disease, incidental Lewy body disease, and normal elderly subjects: evidence against the “body-first” hypothesis. J Parkinsons Dis 2021;11:1833-1843.

15. Horsager J, Andersen KB, Knudsen K, Fedorova TD, Okke N, et al. Brain-first versus body-first Parkinson’s disease: a multimodal imaging case-control study. Brain 2020;143:3077-3088.

16. Borghammer P, Van Den Berge N, Vagus nerve and stomach synucleinopathy in Parkinson’s disease, incidental Lewy body disease, and normal elderly subjects: evidence against the “body-first” hypothesis. J Parkinsons Dis 2021;11:1833-1843.

17. Forsen H, Rask L, Juhlin D, Kivipelto M. AIMS-15 cognitive test in Parkinson’s disease: a comparison between Mattis Dementia Rating Scale and AIMS-15 cognitive test. J Neurol Neurosurg Psychiatry 2010;81:836-839.

18. Postuma RB, Pelletier A, Gagnon JF, Montplaisir J. Evolution of prodromal Parkinson’s disease: a hypothesis. J Parkinsons Dis 2019;9(s2):S281-S295.

19. Forsen H, Rask L, Juhlin D, Kivipelto M. AIMS-15 cognitive test in Parkinson’s disease: a comparison between Mattis Dementia Rating Scale and AIMS-15 cognitive test. J Neurol Neurosurg Psychiatry 2010;81:836-839.

20. Postuma RB, Pelletier A, Gagnon JF, Montplaisir J. Evolution of prodromal multiple system atrophy from REM sleep behaviour disorder: a descriptive study. J Parkinsons Dis 2022;12:983-991.
Supplementary Table 1. Detailed pAS immunostaining results with semi-quantitative grades of extensive tissue (5 slides per block)

| Group | ID  | Distal block | Proximal block |
|-------|-----|--------------|----------------|
|       |     | Extensive   | Distal 1 | Distal 2 | Distal 3 | Distal 4 | Distal 5 | Extensive | Proximal 1 | Proximal 2 | Proximal 3 | Proximal 4 | Proximal 5 |
| Patient | P01 | (+) | 3+ | 3+ | 2+ | 2+ | 1+ | (+) | 3+ | 3+ | 3+ | 3+ | 3+ |
| Patient | P02 | (+) | 0 | 0 | 0 | 1+ | 0 | (+) | 1+ | 1+ | 1+ | 1+ | 0 |
| Patient | P03 | (+) | 3+ | 2+ | 3+ | 3+ | 3+ | (+) | 2+ | 2+ | 1+ | 2+ | 2+ |
| Patient | P04 | (+) | 2+ | 2+ | 3+ | 3+ | 2+ | (+) | 3+ | 3+ | 2+ | 3+ | 2+ |
| Patient | P05 | (+) | 1+ | 2+ | 2+ | 2+ | 2+ | (+) | 3+ | 3+ | 3+ | 3+ | 3+ |
| Patient | P06 | (+) | 1+ | 1+ | 1+ | 1+ | 1+ | (-) | 0 | 0 | 0 | 0 | 0 |
| Patient | P07 | (+) | 3+ | 3+ | 3+ | 3+ | 3+ | (+) | 3+ | 3+ | 3+ | 3+ | 3+ |
| Patient | P08 | (-) | 0 | 0 | 0 | 0 | 0 | (+) | 1+ | 1+ | 1+ | 1+ | 1+ |
| Patient | P09 | (-) | 0 | 0 | 0 | 0 | 0 | (-) | 0 | 0 | 0 | 0 | 0 |
| Control | C01 | (-) | 0 | 0 | 0 | 0 | 0 | (-) | 0 | 0 | 0 | 0 | 0 |
| Control | C02 | (-) | 0 | 0 | 0 | 0 | 0 | (+) | 1+ | 0 | 1+ | 0 | 0 |
| Control | C03 | (-) | 0 | 0 | 0 | 0 | 0 | (-) | 0 | 0 | 0 | 0 | 0 |
| Control | C04 | (-) | 0 | 0 | 0 | 0 | 0 | (-) | 0 | 0 | 0 | 0 | 0 |
| Control | C05 | (-) | 0 | 0 | 0 | 0 | 0 | (-) | 0 | 0 | 0 | 0 | 0 |

pAS, phosphorylated alpha-synuclein.