20. Hoche F, Guell X, Vangel MG, Sherman JC, Schmahmann JD. The cerebellar cognitive affective/Schmahmann syndrome scale. Brain 2017;141(1):248–270. https://doi.org/10.1093/brain/awx317

21. Zahorec R. Neutrophil-to-lymphocyte ratio, past, present and future perspectives. Bratislava Med J 2021;122(7):474–488. https://doi.org/10.4149/BLL_2021_078

22. Balta S, Ozturk C. The platelet-lymphocyte ratio: a simple, inexpensive and rapid prognostic marker for cardiovascular events. Platelets 2015;26(7):680–681. https://doi.org/10.3109/09537104.2014.979340

23. Chen J-H, Zhai E-T, Yuan Y-J, et al. Systemic immune-inflammation index for predicting prognosis of colorectal cancer. World J Gastroenterol 2017;23(34):6261–6272. https://doi.org/10.3748/wjg.v23.i34.6261

24. Zinellu A, Collu C, Nasser M, et al. The aggregate index of systemic inflammation (AISI): a novel prognostic biomarker in idiopathic pulmonary fibrosis. J Clin Med 2021;10(18):4134. https://doi.org/10.3390/jcm10184134

25. Ren H, Liu X, Wang L, Gao Y. Lymphocyte-to-monocyte ratio: a novel predictor of the prognosis of acute ischemic stroke. J Stroke Cerebrovasc Dis 2017;26(11):2595–2602. https://doi.org/10.1016/j.jstrokecerebrovasdis.2017.06.019

26. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc, Ser B 1995;57(1):289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

27. Presta I, Vismara M, Novellino F, et al. Innate immunity cells and the neurovascular unit. Int J Mol Sci 2018;19(12):3856. https://doi.org/10.3390/ijms19123856

28. Novellino F, Donato A, Malara N, Madrigal JLM, Donato G. Complete blood cell count-derived ratios can be useful biomarkers for neurological diseases. Int J Immunopathol Pharmacol 2021;35:1–4. https://doi.org/10.1177/20587384211048264

29. Hansen M, Zeddies S, Meinders M, et al. The RNA-binding protein ATXN2 is expressed during Megakaryopoiesis and may control timing of gene expression. Int J Mol Sci 2020;21(3):967. https://doi.org/10.3390/ijms21030967

30. Kalinowska A, Losy J. PECAM-1, a key player in neuroinflammation. Eur J Neurol 2006;13(12):1284–1290. https://doi.org/10.1111/j.1468-1331.2006.01640.x

31. Velázquez-Pérez L, Rodríguez-Labrada R, Laffita-Mesa JM. Prodromal spinocerebellar ataxia type 2: prospects for early interventions and ethical challenges. Mov Disord 2017;32(5):708–718. https://doi.org/10.1002/mds.26969

32. An P, Zhou X, Du Y, et al. Association of neutrophil-lymphocyte ratio with mild cognitive impairment in elderly Chinese adults: a case-control study. Curr Alzheimer Res 2020;16(14):1309–1315. https://doi.org/10.2174/1567205017666200103110521

33. Ramos-Cejudo J, Johnson AD, Beiser A, et al. The neutrophil to lymphocyte ratio is associated with the risk of subsequent dementia in the Framingham heart study. Front Aging Neurosci 2021;13, 773984. https://doi.org/10.3389/fnagi.2021.773984

34. Hou J-H, Ou Y-N, Xu W, Zhang P-F, Tan L, Yu J-T. Association of peripheral immunity with cognition, neuroimaging, and Alzheimer's pathology. Alzheimer's Res Ther 2022;14(1):29. https://doi.org/10.1186/s13195-022-00968-y

35. Warren KN, Beason-Held LL, Carlson O, et al. Elevated markers of inflammation are associated with longitudinal changes in brain function in older adults. J Gerontol, Ser A 2018;73(6):770–778. https://doi.org/10.1093/gerona/glx199

36. Paul S, Dansithong W, Figueroa KP, Scoles DR, Pulst SM. Staufen1 links RNA stress granules and autophagy in a model of neurodegeneration. Nat Commun 2018;9(1):3648. https://doi.org/10.1038/s41467-018-06041-3

37. Ma Y, Wang X, Luo W, et al. Roles of emerging RNA-binding activity of cGAS in innate antiviral response. Front Immunol 2021;12(November):1–16. https://doi.org/10.3389/fimmu.2021.741599

Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Duodenal alpha-Synuclein Pathology and Enteric Gliosis in Advanced Parkinson's Disease

Aron Emmi, PhD,[1,2,3] Michele Sandre, PhD,[2,3] Francesco Paolo Russo, MD, PhD,[3,4] Giulia Tombesi, PhD,[5] Federica Garrì, MD,[2] Marta Campagnolo, MD, PhD,[2,3] Miryam Carecchio, MD, PhD,[2,3] Roberta Biundo, PhD,[2,3,6] Gaya Spolverato, MD, PhD,[3,4] Veronica Macchi, MD, PhD,[1,3] Edoardo Savarino, MD, PhD,[3,4] Fabio Farinati, MD,[3,4] Piero Parchi, MD, PhD,[7,8,9] Andrea Porzionato, MD, PhD,[1,3] Luigi Bubacco, PhD,[3,5] Raffaele De Caro, MD,[1,3] Gabor G. Kovacs, MD, PhD,[10,11,12] and Angelo Antonini, MD, PhD[2,3*]

[1]*Institute of Human Anatomy, Department of Neuroscience, University of Padova, Padova, Italy* [2]*Parkinson and Movement Disorders Unit, Centre for Rare Neurological Diseases, Padua Neuroscience Center (PNC), Department of Neuroscience,*

***Correspondence to:** Professor. Angelo Antonini, Center for Neurodegenerative Disease Research (CESNE), University of Padua, Via Giustiniani 3, 35121 Padova, Italy; E-mail: angelo.antonini@unipd.it

Aron Emmi and Michele Sandre contributed equally to this work.

Relevant conflicts of interest/financial disclosures: A.A. has received compensation for consultancy and speaker-related activities from UCB, Boehringer Ingelheim, Ever Pharma, General Electric, Britannia, AbbVie, Kyowa Kirin, Zambon, Bial, Theravance Biopharma, Jazz Pharmaceuticals, Roche, and Medscape; has received research support from Bial, Lundbeck, Roche, Angelini Pharmaceuticals, Horizon 2020 Grants 825785 and 101016902, Ministry of Education University and Research Grant ARS01_01081, Cariparo Foundation, and Movement Disorders Society for NMS Scale validation; and has served as consultant for Boehringer–Ingelheim for legal cases on pathological gambling. G.G.K. has served as an advisor for Biogen; has received royalty for 5G4 synuclein antibody and publishing royalties from Wiley, Cambridge University Press, and Elsevier; has received grants from Edmond J. Safra Philanthropic Foundation, Rossy Family Foundation, The Michael J. Fox Foundation, Parkinson Canada, and Canada Foundation for Innovation. All other authors declare no conflict of interest.

Full financial disclosures and author roles may be found in the online version of this article.

Received: 29 November 2022; **Revised:** 26 January 2023; **Accepted:** 3 February 2023

Published online 27 February 2023 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/mds.29358

University of Padova, Padova, Italy [3]*Center for Neurodegenerative Disease Research (CESNE), Department of Neuroscience, University of Padova, Padova, Italy* [4]*Department of Surgery, Oncology and Gastroenterology, Padova University Hospital, Padova, Italy* [5]*Department of Biology, University of Padova, Padova, Italy* [6]*Department of General Psychology, University of Padova, Padova, Italy* [7]*Department of Pathobiology and Laboratory Medicine, University of Toronto, Toronto, Ontario, Canada* [8]*IRCCS, Istituto delle Scienze Neurologiche di Bologna, Bologna, Italy* [9]*Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy* [10]*Tanz Centre for Research in Neurodegenerative Disease, University of Toronto, Toronto, Ontario, Canada* [11]*Departments of Laboratory Medicine and Pathobiology and Medicine, University of Toronto, Toronto, Ontario, Canada* [12]*Laboratory Medicine Program & Krembil Brain Institute, University Health Network, Toronto, Ontario, Canada*

ABSTRACT: Background: The role of the gut-brain axis has been recently highlighted as a major contributor to Parkinson's disease (PD) physiopathology, with numerous studies investigating bidirectional transmission of pathological protein aggregates, such as α-synuclein (αSyn). However, the extent and the characteristics of pathology in the enteric nervous system have not been fully investigated.

Objective: We characterized αSyn alterations and glial responses in duodenum biopsies of patients with PD by employing topography-specific sampling and conformation-specific αSyn antibodies.

Methods: We examined 18 patients with advanced PD who underwent Duodopa percutaneous endoscopic gastrostomy and jejunal tube procedure, 4 untreated patients with early PD (disease duration <5 years), and 18 age- and -sex-matched healthy control subjects undergoing routine diagnostic endoscopy. A mean of four duodenal wall biopsies were sampled from each patient. Immunohistochemistry was performed for anti-aggregated αSyn (5G4) and glial fibrillary acidic protein antibodies. Morphometrical semiquantitative analysis was performed to characterize αSyn-5G4^{+} and glial fibrillary acidic protein–positive density and size.

Results: Immunoreactivity for aggregated α-Syn was identified in all patients with PD (early and advanced) compared with controls. αSyn-5G4^{+} colocalized with neuronal marker β-III-tubulin. Evaluation of enteric glial cells demonstrated an increased size and density when compared with controls, suggesting reactive gliosis.

Conclusions: We found evidence of synuclein pathology and gliosis in the duodenum of patients with PD, including early de novo cases. Future studies are required to evaluate how early in the disease process duodenal pathology occurs and its possible contribution to levodopa effect in chronic patients. © 2023 The Authors. *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society.

Key Words: neuropathology; alpha-synuclein; gastrointestinal biopsies; enteric nervous system; Parkinson's disease

Introduction

In Parkinson's disease (PD), the role of the gut-brain axis has been greatly highlighted by recent developments in both clinical and preclinical research.[1-5]

There is growing interest in the detection of α-synuclein (αSyn) aggregates, the histological hallmark of PD, in peripheral tissues,[6] including the gastrointestinal (GI) tract.[7-10] Considering the involvement of the enteric nervous system in the prodromal stages of PD and its relationship with gut motility,[11,12] the detection of αSyn aggregation and its deposition in gut tissues is of great relevance.[13] Recent animal models suggested a possible bidirectional transmission of αSyn pathology that may originate either in the enteric nervous system and spread toward the brain, or begin in the brain and then spread toward the periphery; this has driven the discussion about the so-called gut-brain axis.[2,14,15] These findings have been supported by studies on human postmortem samples,[10] while in vivo human studies reported heterogeneous findings in GI biopsies (mainly gastric and colonic).[16-18] Phosphorylated αSyn (p-αSyn) at serine 129 residue has been considered the most reliable marker to distinguish pathological deposits from physiological protein. However, a consensus has not been reached so far on which antibody (anti-αSyn or anti–p-αSyn) should be used in peripheral tissues to distinguish patients with PD from controls,[9,19,20] although antibodies specific for αSyn aggregates have been used in a single study on colonic mucosa with promising results.[21] Specifically, the antibody, clone 5G4, can detect αSyn aggregates targeting the sequence encompassing amino acids 46–53 of αSyn.[22,23] Recently, in an in vitro comparative analysis of several αSyn targeting antibodies, αSyn-5G4 showed high conformational specificity and strong immunoreactivity for all forms of αSyn aggregates with no reaction toward αSyn monomers[24] (Fig. S1). Furthermore, αSyn-5G4 immunohistochemistry was more reliable in identifying αSyn aggregates across synucleinopathies compared with other αSyn antibodies and was also able to detect astrocytic and oligodendroglial αSyn inclusions in Lewy body disease.[23,25]

Furthermore, considerable attention has been drawn to enteric glial cells (EGCs), which may play a critical role in the cross-talk between inflammation and neurodegeneration. According to available studies, EGCs participate in the regulation of GI functions, playing a key role in the pathophysiology of GI disorders.[26,27] More recently, EGCs have emerged as critical players in regulating GI function in PD, because higher levels of expression for enteric glial markers (such as glial fibrillary acidic protein [GFAP] and SOX10) were reported in the GI tract of patients with PD, suggesting enteric glial reaction. Conversely, levels of glial markers were negatively related to PD disease duration,

suggesting that EGC reaction is more relevant at disease onset and decreases over time,[28-30] but the precise mechanism by which EGCs contribute to PD pathogenesis remains to be elucidated.

In this study, we aimed to investigate the histopathological changes in the enteric nervous system by characterizing both αSyn aggregates and enteric glial responses in duodenum biopsies of patients with PD with extensive clinical and demographical documentation.

Subjects and Methods

Subjects

Eighteen patients (12 males, 6 females; mean age, 65.2 years, 95% confidence interval [CI] 61.4–69.0 years; mean disease duration, 11.3 years; 95% CI, 9.0–13.6 years) with advanced PD who required initiation of levodopa carbidopa intestinal gel infusion were enrolled for the study.[31]

All patients underwent percutaneous endoscopic gastrostomy with jejunal extension placement; an average of four 3-mm^3 duodenal-wall biopsies were sampled in a topographically unrelated district to percutaneous endoscopic gastrostomy with jejunal extension placement. Along with the routine clinical assessment (Movement Disorder Society–Unified Parkinson's Disease Rating Scale Parts I–IV and Hoehn & Yahr scales), the Wexner Constipation Score[32] was also calculated (Table S1).

In addition, we also investigated four early untreated subjects with PD (three males and one female; mean age, 63.2 years, 95% CI, 50.5–76.0 years; mean disease duration, 2.7 years, 95% CI, 0.4–5.1 years) with disease duration <4 years. Patients with early PD voluntarily underwent screening diagnostic endoscopy with biopsy collection.

Duodenal biopsies from 18 subjects comparable for age and sex (9 males, 9 females; mean age, 68.6 years, 95% CI, 63.8–73.4 years) undergoing screening diagnostic endoscopy were included as healthy control subjects (HCs). Notably, HCs were further evaluated clinically and interviewed to exclude any manifestation suggestive of PD or any other neurological disorder.

The study protocol received approval by the ethical committee for clinical experimentation of Padua province (protocol no. 0034435, 08/06/2020). Informed consent for the use of biological samples was obtained from all patients. All procedures on human tissue samples were carried out in accordance with the Declaration of Helsinki.

Tissue Processing and Staining

Tissue samples were fixed in phosphate-buffered 4% paraformaldehyde, embedded in paraffin, and sectioned at the microtome (5-μm slices). Single- and double-marker immunoperoxidase staining for aggregated αSyn (monoclonal mouse, clone 5G4; Millipore), GFAP (monoclonal rabbit; Dako Omnis), S100β (recombinant polyclonal antibody, clone 16HCLC; ThermoFisher Scientific), SOX10 (monoclonal antibody, clone 20B7; eBioscience), β-III-tubulin (rabbit polyclonal; BioLegend), PGP9.5 (rabbit polyclonal, ab15503; Abcam), and neurofilament heavy polypeptide (RabMab Rabbit mAb, clone EPR20020; Abcam) was performed on a Dako EnVision Autostainer station according to the manufacturer's recommendations. Antigen retrieval was performed on a PT-Link Dako Antigen retrieval station using citrate buffer at pH 6 solution at 96°C for 15 minutes, followed by 1 minute in 95% formic acid for the αSyn-5G4 antibody.

To validate and choose the suitable neuronal marker in duodenum, we compared anti-β-III-tubulin, anti-PGP9.5, and anti-Neurofilament heavy polypeptide antibodies in peripheral tissues (duodenum and skin) of the same case (Fig. S4).

Immunoperoxidase staining was repeated at least three times to assure reaction consistency and was independently evaluated by three morphologists blind to the clinical findings. Controversies were resolved by consensus.

Morphometrical Quantification

Photomicrographs were acquired under a Leica DM4500B microscope (Leica Microsystems) connected to a Leica DFC320 high-resolution digital camera (Leica Microsystems) and a computer equipped with software for image acquisition (QWin; Leica Microsystems) and analysis (ImageJ).[33-35] Whole-section photomicrographs were acquired at 5× magnification, while an average of four 20× magnification photomicrographs per available sample were acquired as counting fields and loaded into ImageJ software for semiautomatic immunoreactivity quantification.

The area of the sections was quantified by manually drawing the boundaries of the specimens. A maximum entropy threshold was applied and manually adjusted for each section to discern immunopositive elements from background and negative tissue. Quality control of the applied threshold was performed by an expert morphologist by overlaying the thresholded images to the original photomicrographs. Particle analysis was done with a 0 to infinity px threshold to define immunopositive elements quantity and total area occupied within the digital image. For GFAP staining, the number of immunopositive elements was divided by the total area of the sample to obtain a semiquantitative measure of immunoreactive density within each section. For αSyn-5G4 staining, because immunoreactive structures did not present as distinct elements with defined

boundaries, total immunoreactive area (μm^2) and % of immunoreactive area (A%) were estimated per counting field. Counting fields for each available sample were treated as repeated measures and averaged per single subject.

Immunofluorescence and Confocal Microscopy

Fluorescent immunohistochemistry was performed manually. Antigen retrieval was performed on deparaffinized tissue as in immunoperoxidase staining methods. Tissue autofluorescence was quenched with a 50 mM NH_4Cl solution for 10 minutes. Sections were treated with permeabilization and blocking solution (15% vol/vol goat serum, 2% wt/vol bovine serum albumin, 0.25% wt/vol gelatin, 0.2% wt/vol glycine in phosphate-buffered saline) containing 0.5% Triton X-100 for 90 minutes before incubation of primary antibodies. Primary antibodies were diluted in blocking solution and incubated at 4°C overnight. Alexa Fluor plus 488 goat anti-mouse secondary antibody (A32723; Thermo Fisher Scientific) and Alexa Fluor plus 568 anti-rabbit secondary antibody (A-11011; Thermo Fisher Scientific) were diluted 1:200 in blocking solution as described earlier and incubated for 60 minutes at room temperature. Hoechst 33258 was used for nuclear staining (dilution: 1:10,000 in phosphate-buffered saline; Invitrogen) for 10 minutes. Slides were mounted and coverslipped with Mowiol solution (Novabiochem).[36] Confocal immunofluorescence z-stack images were acquired on a Leica SP5 Laser Scanning Confocal Microscope using HC PL FLUOTAR 20×/0.50 Dry or HCX PL APO lambda blue 40X/1.40 oil objectives. Images were acquired at a 16-bit intensity resolution over 2048 × 2048 pixels. z-stack images were converted into digital maximum intensity z-projections, processed, and analyzed using ImageJ software.

The antibodies used for immunofluorescence were the following: mouse anti-aggregated αSyn clone 5G4 (MABN389, 1:1000; Sigma-Aldrich), rabbit GFAP (1:1000; Dako Omnis), mouse GFAP (1:1000; Genetex); S100β (recombinant polyclonal antibody, clone 16HCLC; ThermoFisher Scientific), SOX10 (monoclonal antibody, clone 20B7; eBioscience), and rabbit β-III-tubulin (Poly18020; 1:10,000; BioLegend).

Statistical Analyses

Statistical analyses and visualizations were performed using GraphPad Prism v.9. Nonparametric data were analyzed with Mann–Whitney *U* test. Pearson's correlation analysis has been used to assess possible correlations between αSyn expression in duodenum (after passing Kolmogorov–Smirnov normality test) and clinical characteristics, including motor and non-motor scales, cognitive assessments, and main non-motor symptoms of PD. Values are indicated as the median, with significance as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

Results

αSyn Pathology

αSyn-5G4 immunoreactive elements detected in duodenal specimens were classified according to morphological criteria into four groups: (1) compact and globular immunoreactivities (Fig. 1A,B); (2) granular cellular immunoreactivities (Fig. 1C,D), mostly resembling cross-reactivity with resident mast cells; and (3) dot-like immunoreactivities (Fig. 1E,F), which we interpreted as either cross-reaction with lipopigment staining in the duodenal mucosa or as actual aggregated αSyn deposits if colocalizing with neuronal structures via immunofluorescent staining/double-labeled immunoperoxidase staining. These three morphologies were found in both patients with PD and HCs. The fourth morphology was observed as thread-like immunoreactivities (Fig. 1G,H), which represented the most reliable immunoreactivity type to discern patients with PD from HCs. Immunofluorescent staining (Fig. 1I1,I2) and double-immunoperoxidase staining (Fig. 1J,K) confirmed the colocalization between αSyn-5G4 threaded immunoreactivities and β-III-tubulin, a pan-neuronal and neuritic marker, indicating aggregated αSyn deposits in duodenal nerve fibers of the mucosa and submucosa. Similar thread-like immunoreactivities were also found in the duodenal mucosa and submucosa of subjects with early PD (Fig. 1L,M). Non-colocalizing immunoreactivity types 1–3 are displayed in Figure S3. Regardless of morphology, immunoreactivity for aggregated αSyn was absent (4/18) or barely detectable (14/18) (with predominant globular or cellular immunoreactive morphology) in HCs (Fig. 1N,O), whereas all duodenal samples collected from both early and advanced PD patients were characterized by marked (22/22; 100%) immunoreactivity for aggregated αSyn (Fig. 1P,Q), displaying one or more thread-like reactivities. Semiautomatic morphometric quantification for the burden of aggregated αSyn immunoreactivity demonstrated statistically significant higher immunoreactive tissue area in patients with advanced PD compared with HCs ($^{****}P < 0.0001$; PD: mean % area: 1.58%, 95% CI, 1.40–1.75; HCs: mean % area: 0.18%, 95% CI, 0.09–0.26) (Fig. 1R) and also in patients with early PD when compared with age-matched HCs ($^{**}P < 0.01$; patients with early PD: mean % area: 0.80%, 95% CI, 0.13–1.47; age-matched HCs: mean % area: 0.18%, 95% CI, 0.05–0.31) (Fig. 1S).

We then defined the criteria for diagnosing PD-related αSyn pathology with two parameters: (1) an average morphometric area of aggregated αSyn burden in at least three counting fields ≥0.26% (upper 95% CI

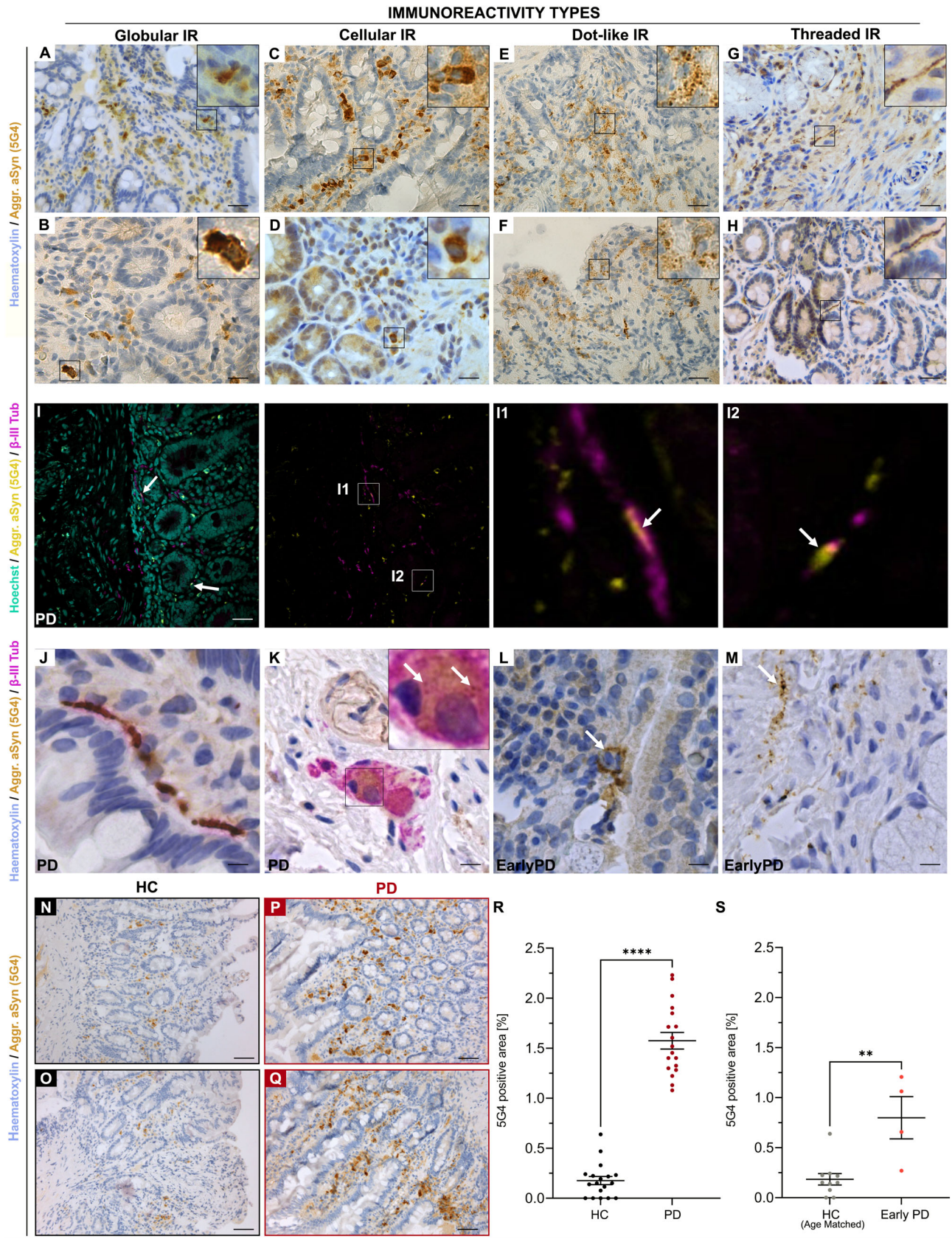

FIG. 1. Legend on next page.

of mean in HCs) of the total counting field (A% > 0.26), and/or (2) at least a single unequivocal thread-like profile detected by αSyn-5G4.

Application of the first parameter alone demonstrated a sensitivity of 100.00% (95% CI, 81.47–100.00%), specificity of 83.33% (95% CI, 58.58–96.42%), positive predictive value of 85.71% (95% CI, 68.11–94.40%), and negative predictive value of 100% for discerning manifest PD from HC. The calculated accuracy was thus equal to 91.67% (95% CI, 77.53–98.25%).

Application of the second parameter alone demonstrated a sensitivity of 100.00% (95% CI, 81.47–100.00%), specificity of 94.44% (95% CI, 72.71–99.86%), positive predictive value of 94.74% (95% CI, 72.82–99.18%), and negative predictive value of 100.00% for discerning manifest PD from HC. The calculated accuracy was thus equal to 97.22% (95% CI, 85.47–99.93%).

The combination of both parameters demonstrated a sensitivity of 100.00% (95% CI, 81.47–100.00%), specificity of 100.00% (95% CI, 81.47–100.00%), positive predictive value of 100.00%, and negative predictive value of 100.00% for discerning manifest PD from HC. The calculated accuracy was thus equal to 100.00% (95% CI, 85.47–99.93%) and was higher than the single parameters. Summary estimates of 5G4 immunohistochemistry as a diagnostic marker for PD in duodenal biopsies are reported in Table S2.

GFAP Analysis and Enteric Gliosis

GFAP-immunoreactive elements presented as discrete, round, immunoreactive cells localized predominantly within the duodenal mucosa and submucosa (Fig. 2A, C, high-magnification insets). S100β immunoperoxidase and immunofluorescent staining (Fig. 2G–L) showed colocalization between GFAP and S100β, indicating a contextual expression of these markers in duodenal EGCs of the mucosa and submucosa. Although we detected instances of $GFAP^+/S100\beta^-$ EGCs, we found no instance of $GFAP^-/S100\beta^+$ structures. SOX10 immunoperoxidase and immunofluorescent staining (Fig. 2M–R) showed sparse and sporadic cellular immunoreactivities in the duodenal mucosa and submucosa. Similarly to S100β, we found numerous instances of $GFAP^+/SOX10^-$ EGCs and rare instances of $GFAP^-/SOX10^+$ cells. These findings suggest that GFAP represents a broad marker for EGCs in the human duodenal mucosa and submucosa and was thus used for morphometrical quantification.

Morphometrical analyses demonstrated both increased EGC density (PD mean: 95.2 ± 32.6; HC mean: 45.8 ± 14.9; Fig. 2E) and increased cell size (PD mean: 28.2 ± 1.4 μm^2; HC mean: 25.1 ± 1.2 μm^2; Fig. 2F) in patients with advanced PD compared with HCs ($^{****}P < 0.0001$ for both cell density and size), suggestive of local reactive gliosis.

Spearman's rank correlation analysis between αSyn-5G4 immunoreactive area, EGC density, and EGC cell size in the duodenum in patients with advanced PD showed a strong correlation ($R_s = 0.751$, $^{****}P < 0.0001$ and $R_s = 0.741$, $^{****}P < 0.0001$, respectively; $n = 36$).

Correlation with Clinical Data

Pearson's correlation analysis was performed between αSyn-5G4 immunoreactive areas and collected clinical data. Remarkably, a good correlation was found comparing αSyn-5G4 immunoreactive area and patients' years of disease ($R = 0.526$; $^{*}P < 0.05$; $n = 22$). No other correlation was found with sex, motor severity, global cognitive scales' score, quality of life, and Movement Disorder Society–Unified Parkinson's Disease Rating Scale score. Interestingly, we found a trend of increased aggregated αSyn distribution in patients who complained of constipation ($P = 0.058$) (Wexner Constipation Score); however, validation of this finding requires a larger cohort as a prospective assessment with validated instruments. Similarly, no significant correlation was observed with the other variables examined, including other GI disturbances.

Discussion

In this study, we documented marked immunoreactivity for aggregated αSyn and morphological changes in EGC suggestive of reactive gliosis in the duodenum of patients with PD, including a small number with early PD. These data expand our knowledge on the

FIG. 1. αSyn-5G4 immunohistochemistry in duodenal biopsies. (**A–I**) 5G4 immunoreactivity types encountered in the duodenal mucosa. Globular immunoreactivities (**A**, **B**) and cellular reactivities (**C**, **D**), found in both patients with Parkinson's disease (PD) and healthy control subjects (HCs), likely represent antibody cross-reactivity with resident mast cells. (**E**, **F**) Dot-like reactivities can be either ascribed to lipopigment deposits in the gastrointestinal (GI) mucosa or represent actual 5G4 aggregated synuclein deposits in nerve fibers fragmented within the sectioning plane. (**G–K**) Threaded (thread-like) reactivities encountered predominantly in patients with PD indicate α-synuclein aggregates in nerve fibers of the GI tract, as demonstrated by double-label immunofluorescent staining for 5G4 aggregated synuclein and β-III-tubulin (**G**, **H**); a pan-neuronal and neuronal marker (**I1**, **I2**) double-label immunoperoxidase staining showed a similar pattern of colocalization at the level of β-III-tubulin-positive nerve fibers (**J**) and neurons in the submucosa (**K**). $5G4^+$ thread-like reactivities were also detected in patients with early PD (**L**, **M**). Overall, αSyn-5G4 immunoreactivity was lower in HCs (**N**, **O**) compared with patients with PD (**P**, **Q**). Morphometrical quantification followed by Mann–Whitney nonparametric test shows statistically significant differences between advanced PD and HC ($^{****}P < 0.0001$; $n = 18$ for HC and $n = 18$ for PD) (**R**), as well as patients with early PD and age-matched HCs ($^{**}P < 0.01$; $n = 10$ for HC-age matched and $n = 4$ for early PD) (**S**).

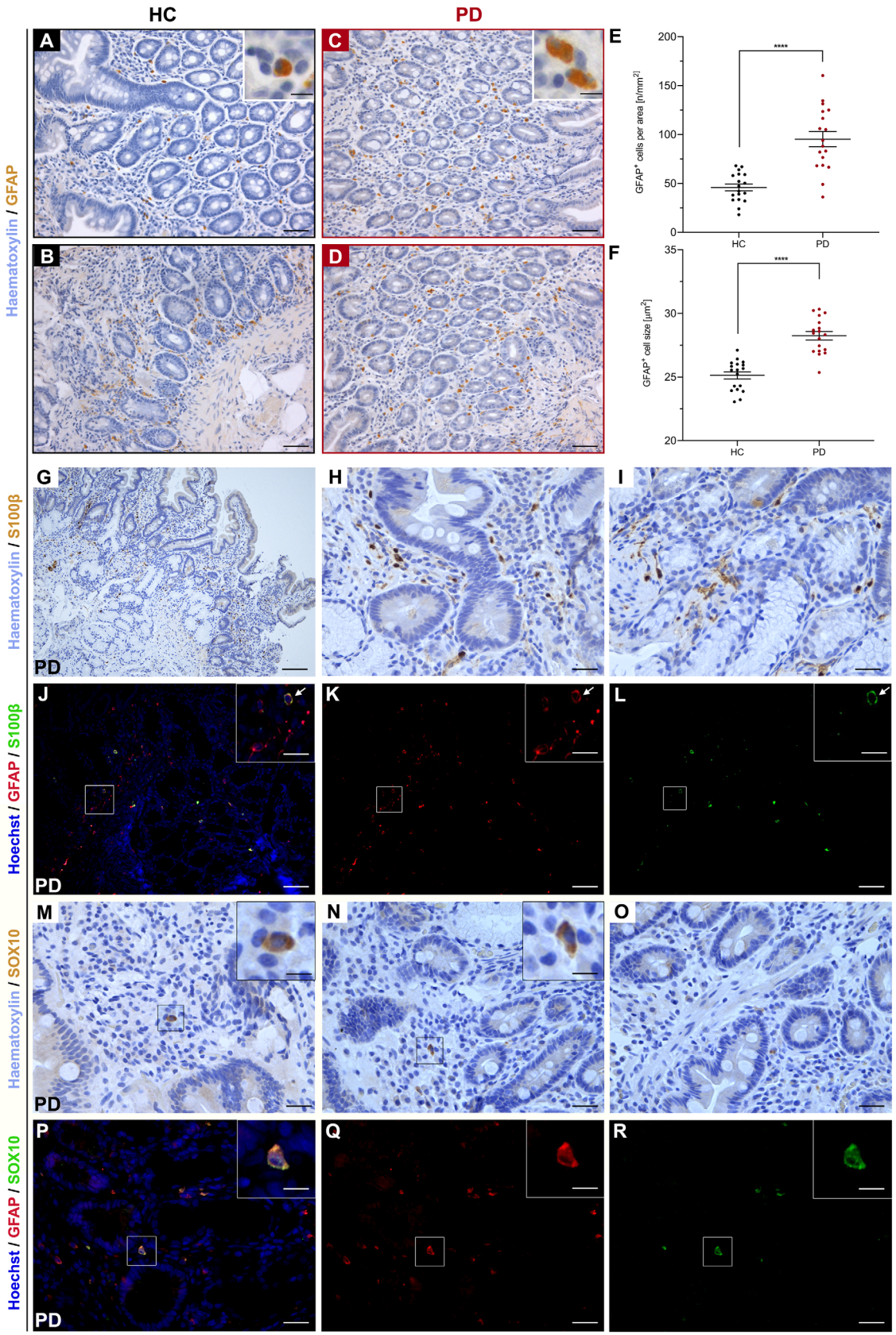

FIG. 2. Glial fibrillary acidic protein (GFAP)-immunoperoxidase-stained duodenal biopsies. (**A**, **B**) Healthy control subjects (HCs). (**C**, **D**) Patients with Parkinson's disease (PD). Scale bars: 15 µm; 50 µm (high-magnification insets). (**E**, **F**) Mann–Whitney nonparametric test shows statistically significant differences between PD and HC in terms of both enteric glial cell size (**E**) and density (**F**) (***$P < 0.001$, ****$P < 0.0001$; n = 18 for HC and n = 18 for PD). Enteric glial cells of the duodenal mucosa also express S100β (**G**–**I**) and SOX10 (**M**–**O**). Both markers colocalize with GFAP$^+$ cells (**J**–**L**, **P**–**R**), which represent the broadest marker for enteric glial cells in the human duodenal mucosa.

involvement of the enteric nervous system in PD and suggest the duodenum as a possible target for disease detection in early PD. Unlike previous studies in the literature, we investigated αSyn pathology in anatomically defined regions of the human duodenum, focusing on a poorly investigated tract of the GI system and at the same time reducing sampling-site variability often affecting studies conducted on the colonic mucosa. Furthermore, we investigated the role of EGC in PD while also validating GFAP as a marker for enteric gliosis.

Our results also indicate that the αSyn-5G4 conformation-specific αSyn antibody is a reliable marker of pathology in PD. Although low or barely detectable immunoreactivity for αSyn-5G4 was also found in HC, marked differences with patients with PD were demonstrated by morphometrical quantification. Furthermore, morphological evaluation demonstrated thread-like immunoreactivities as PD-specific findings; immunofluorescent staining confirmed colocalization between αSyn aggregates and neuronal markers (Figs. 1 and S5), indicating synuclein aggregates in nerve fibers of the duodenal mucosa and submucosa. αSyn-5G4 antibody is reported to be specific for αSyn aggregates, because monomeric synuclein is not detectable in vitro.[24] Conversely, p-αSyn antibodies are not reliable to discern between patients with PD and control subjects in gastric and colonic mucosa specimens, thus significantly limiting their usefulness.[37,38] Unlike previous studies, we have examined an anatomically defined region of the small intestine, the duodenum, greatly restricting the sampling-site variability present in colonic mucosa studies. Moreover, the left half of the transverse colon and the descending colon receive parasympathetic innervation from the sacral nerves deriving from the spinal cord, and not from the vagus nerve, and thus are inherently biased and likely not relevant to the brain-gut hypothesis of αSyn transmission. The localization and density of αSyn aggregates in the average aging population have not been documented to date, with low quantities of aggregated αSyn possibly representing a normal finding in aging subjects, as seen in this small cohort.

GFAP-immunoreactive cell density and size were also higher in patients with PD compared with HCs, suggesting enteric gliosis. However, immunofluorescent staining for αSyn-5G4 and GFAP antibodies did not show colocalization between markers (Fig. S2). Conversely, αSyn-5G4 colocalization with neuronal marker β-III-tubulin was found, indicating a preferential site for αSyn aggregates. Challis et al.[39] evidenced increased myenteric EGCs volume and count in a mouse model of αSyn preformed fibrils duodenal inoculation, similar to our in vivo findings. According to the authors, αSyn-preformed fibril inoculation induces reactive gliosis in response to fibrils seeding, thus linking αSyn pathology to EGCs reaction. Our study suggests a localized response of EGCs in in vivo PD patients in line with the current animal model studies, which is also supported by the strong correlation between EGC values and aggregated αSyn; however, the mechanisms underlying this require further investigation in humans, with regard to inflammatory and immunity processes involved.[40] Furthermore, we validated GFAP as a marker for enteric gliosis by performing double-immunofluorescent staining with S100β and SOX10, which have been previously used to investigate EGCs in both human and animal models.[41]

There was no correlation between patients' clinical characteristics, including cognitive scales and αSyn-5G4 distribution in the duodenum, suggesting that peripheral pathology is a disease biomarker but may not reflect phenotypic variability. Inclusions of patients at various disease stages may provide further insight into the temporal dynamics of aggregated αSyn in the GI tract, especially in the premotor phase of the disease.

Limitations of this study include the relatively small sample size and few patients with early-stage PD. In contrast, the strength of this study includes the prospective and systematic assessment of the patients and the extensive recording of clinical data, which made the correlations more reliable.

In conclusion, our data suggest that significant synuclein pathology occurs in the duodenum of patients with PD. For diagnostic evaluation, a combination of a morphometric method (standardized for each laboratory), followed by multicentric studies to evaluate inter-rater variability, and detection of a single thread-like αSyn-5G4 immunoreactivity is recommended. Future studies are warranted to confirm these findings in prodromal subjects, ie, with REM behavior disorders, and evaluate individuals with other synucleinopathies, in particular, multiple system atrophy. ■

Acknowledgments: The project was supported by "Segala award" from the Italian Neurological Society. We thank all the donors and their families. Open Access Funding provided by Universita degli Studi di Padova within the CRUI-CARE Agreement.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. Klingelhoefer L, Reichmann H. Pathogenesis of Parkinson disease—the gut–brain axis and environmental factors. Nat Rev Neurol 2015; 11(11):625–636. https://doi.org/10.1038/nrneurol.2015.197
2. Kim S, Kwon S-H, Kam T-I, et al. Transneuronal propagation of pathologic α-synuclein from the gut to the brain models Parkinson's disease. Neuron 2019;103(4):627–41.e7. https://doi.org/10.1016/j.neuron.2019.05.035

3. Chung HK, Ho H-A, Pérez-Acuña D, et al. Modeling α-synuclein propagation with preformed fibril injections. J Mov Disord 2019;12(3):139–151. https://doi.org/10.14802/jmd.19046
4. Cersosimo MG, Raina GB, Pecci C, et al. Gastrointestinal manifestations in Parkinson's disease: prevalence and occurrence before motor symptoms. J Neurol 2013;260(5):1332–1338. https://doi.org/10.1007/s00415-012-6801-2
5. Espay AJ, Kalia LV, Gan-Or Z, et al. Disease modification and biomarker development in Parkinson disease. Neurology 2020;94(11):481–494. https://doi.org/10.1212/wnl.0000000000009107
6. Chahine LM, Beach TG, Brumm MC, et al. In vivo distribution of α-synuclein in multiple tissues and biofluids in Parkinson disease. Neurology 2020;95(9):e1267–e1284. https://doi.org/10.1212/wnl.0000000000010404
7. Ma L-Y, Liu G-L, Wang D-X, et al. Alpha-synuclein in peripheral tissues in Parkinson's disease. ACS Chem Nerosci 2019;10(2):812–823. https://doi.org/10.1021/acschemneuro.8b00383
8. Sánchez-Ferro Á, Rábano A, Catalán MJ, et al. In vivo gastric detection of α-synuclein inclusions in Parkinson's disease. Mov Disord 2015;30:517–524.
9. Barrenschee M, Zorenkov D, Böttner M, et al. Distinct pattern of enteric phospho-alpha-synuclein aggregates and gene expression profiles in patients with Parkinson's disease. Acta Neuropathol Commun 2017;5(1):1. https://doi.org/10.1186/s40478-016-0408-2
10. Gelpi E, Navarro-Otano J, Tolosa E, et al. Multiple organ involvement by alpha-synuclein pathology in Lewy body disorders. Mov Disord 2014;29(8):1010–1018. https://doi.org/10.1002/mds.25776
11. Singh A, Dawson TM, Kulkarni S. Neurodegenerative disorders and gut-brain interactions. J Clin Investig 2021;131(13):e143775. https://doi.org/10.1172/jci143775
12. Fleming MA, Ehsan L, Moore SR, et al. The enteric nervous system and its emerging role as a therapeutic target. Gastroenterol Res Pract 2020;2020:1–13. https://doi.org/10.1155/2020/8024171
13. Borghammer P. The α-synuclein origin and connectome model (SOC model) of Parkinson's disease: explaining motor asymmetry, non-motor phenotypes, and cognitive decline. J Parkinsons Dis 2021;11:455–474. https://doi.org/10.3233/JPD-202481
14. Borghammer P, Van Den Berge N. Brain-first versus gut-first Parkinson's disease: a hypothesis. J Parkinsons Dis 2019;9(s2):S281–S295. https://doi.org/10.3233/JPD-191721
15. Leclair-Visonneau L, Neunlist M, Derkinderen P, et al. The gut in Parkinson's disease: bottom-up, top-down, or neither? Neurogastroenterol Motil 2020;32(1):e13777. https://doi.org/10.1111/nmo.13777
16. Stokholm MG, Danielsen EH, Hamilton-Dutoit SJ, et al. Pathological α-synuclein in gastrointestinal tissues from prodromal Parkinson disease patients.Ann Neurol 2016;79(6):940–949.
17. Beck G, Hori Y, Hayashi Y, et al. Detection of phosphorylated alpha-synuclein in the muscularis propria of the gastrointestinal tract is a sensitive predictor for Parkinson's disease. Parkinson's Dis 2020;2020:1–8. https://doi.org/10.1155/2020/4687530
18. Ruffmann C, Bengoa-Vergniory N, Poggiolini I, et al. Detection of alpha-synuclein conformational variants from gastro-intestinal biopsy tissue as a potential biomarker for Parkinson's disease. Neuropathol Appl Neurobiol 2018;44(7):722–736. https://doi.org/10.1111/nan.12486
19. Harapan BN, Frydrychowicz C, Classen J, et al. No enhanced (p-) α-synuclein deposition in gastrointestinal tissue of Parkinson's disease patients. Parkinsonism Relat Disord 2020;80:82–88. https://doi.org/10.1016/j.parkreldis.2020.08.020
20. Fricova D, Harsanyiova J, Kralova TA. Alpha-synuclein in the gastrointestinal tract as a potential biomarker for early detection of Parkinson's disease. Int J Mol Sci 2020;21(22):8666. https://doi.org/10.3390/ijms21228666
21. Skorvanek M, Gelpi E, Mechirova E, et al. α-Synuclein antibody 5G4 identifies manifest and prodromal Parkinson's disease in colonic mucosa. Mov Disord 2018;33(8):1366–1368. https://doi.org/10.1002/mds.27380
22. Kovacs GG, Breydo L, Green R, et al. Intracellular processing of disease-associated α-synuclein in the human brain suggests prion-like cell-to-cell spread. Neurobiol Dis 2014;69:76–92.
23. Kovacs GG, Wagner U, Dumont B, et al. An antibody with high reactivity for disease-associated α-synuclein reveals extensive brain pathology. Acta Neuropathol 2012;124(1):37–50.
24. Kumar ST, Jagannath S, Francois C, et al. How specific are the conformation-specific α-synuclein antibodies? Characterization and validation of 16 α-synuclein conformation-specific antibodies using well-characterized preparations of α-synuclein monomers, fibrils and oligomers with distinct struct. Neurobiol Dis 2020;146:105086. https://doi.org/10.1016/j.nbd.2020.105086
25. Sorrentino ZA, Goodwin MS, Riffe CJ, et al. Unique α-synuclein pathology within the amygdala in Lewy body dementia: implications for disease initiation and progression. Acta Neuropathol Commun 2019;7(1):142. https://doi.org/10.1186/s40478-019-0787-2
26. Sharkey KA. Emerging roles for enteric glia in gastrointestinal disorders. J Clin Investig 2015;125(3):918–925. https://doi.org/10.1172/jci76303
27. Grubišić V, Gulbransen BD. Enteric glia: the most alimentary of all glia. J Physiol 2017;595(2):557–570. https://doi.org/10.1113/jp271021
28. Clairembault T, Kamphuis W, Leclair-Visonneau L, et al. Enteric GFAP expression and phosphorylation in Parkinson's disease. J Neurochem 2014;130(6):805–815. https://doi.org/10.1111/jnc.12742
29. Clairembault T, Leclair-Visonneau L, Neunlist M, et al. Enteric glial cells: new players in Parkinson's disease? Mov Disord 2015;30(4):494–498. https://doi.org/10.1002/mds.25979
30. Benvenuti L, D'Antongiovanni V, Pellegrini C, et al. Enteric glia at the crossroads between intestinal immune system and epithelial barrier: implications for Parkinson disease. Int J Mol Sci 2020;21(23):9199. https://doi.org/10.3390/ijms21239199
31. Antonini A, Odin P, Pahwa R, et al. The long-term impact of levodopa/carbidopa intestinal gel on 'off'-time in patients with advanced Parkinson's disease: a systematic review. Adv Ther 2021;38(6):2854–2890. https://doi.org/10.1007/s12325-021-01747-1
32. Agachan F, Chen T, Pfeifer J, et al. A constipation scoring system to simplify evaluation and management of constipated patients. Dis Colon Rectum 1996;39(6):681–685. https://doi.org/10.1007/bf02056950
33. Porzionato A, Guidolin D, Emmi A, et al. High-quality digital 3D reconstruction of microscopic findings in forensic pathology: the terminal pathway of a heart stab wound*. J Forensic Sci 2020;65(6):2155–2159. https://doi.org/10.1111/1556-4029.14497
34. Emmi A, Porzionato A, Contran M, et al. 3D reconstruction of the morpho-functional topography of the human vagal trigone. Front Neuroanat 2021;15:663399. https://doi.org/10.3389/fnana.2021.663399
35. Emmi A, Antonini A, Sandre M, et al. Topography and distribution of adenosine A2A and dopamine D2 receptors in the human subthalamic nucleus. Front Neurosci 2022;16:945574. https://doi.org/10.3389/fnins.2022.945574
36. Emmi A, Stocco E, Boscolo-Berto R, et al. Infrapatellar fat pad-synovial membrane anatomo-functional unit: microscopic basis for Piezo1/2 mechanosensors involvement in osteoarthritis pain. Front Cell Dev Biol 2022;10:886604. https://doi.org/10.3389/fcell.2022.886604
37. Visanji NP, Marras C, Kern DS, et al. Colonic mucosal α-synuclein lacks specificity as a biomarker for Parkinson disease. Neurology 2015;84(6):609–616. https://doi.org/10.1212/wnl.0000000000001240
38. Chung SJ, Kim J, Lee HJ, et al. Alpha-synuclein in gastric and colonic mucosa in Parkinson's disease: limited role as a biomarker. Mov Disord 2016;31(2):241–249. https://doi.org/10.1002/mds.26473
39. Challis C, Hori A, Sampson TR, et al. Gut-seeded α-synuclein fibrils promote gut dysfunction and brain pathology specifically in aged mice. Nat Neurosci 2020;23(3):327–336. https://doi.org/10.1038/s41593-020-0589-7

40. Sandre M, Emmi A, Tombesi G, et al. Alpha-synuclein pathology and enteric glia in advanced Parkinson's disease: a study from gastrointestinal biopsies. J Neurol Sci 2021;429:119460. https://doi.org/10.1016/j.jns.2021.119460

41. Boesmans W, Lasrado R, Vanden Berghe P, et al. Heterogeneity and phenotypic plasticity of glial cells in the mammalian enteric nervous system. Glia 2015;63(2):229–241. https://doi.org/10.1002/glia.22746

Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Pallidal Beta Activity Is Linked to Stimulation-Induced Slowness in Dystonia

Roxanne Lofredi, MD,[1,2*] Ute Scheller, MD,[1,3] Aurika Mindermann, cand.med.,[1] Lucia K. Feldmann, MD,[1,2] Joachim K. Krauss, MD,[4] Assel Saryyeva, MD,[4] Gerd-Helge Schneider, MD,[5] and Andrea A. Kühn, MD[1,6]

[1]*Department of Neurology, Movement Disorders and Neuromodulation Unit, Charité – Universitätsmedizin Berlin, Berlin, Germany* [2]*Berlin Institute of Health, Berlin, Germany* [3]*Department of Neurology, Georg-August-Universität Göttingen, Göttingen, Germany* [4]*Department of Neurosurgery, Medizinische Hochschule Hannover, Hannover, Germany* [5]*Department of Neurosurgery, Charité – Universitätsmedizin Berlin, Berlin, Germany* [6]*Exzellenzcluster – NeuroCure, Charité – Universitätsmedizin Berlin, Berlin, Germany*

ABSTRACT: Background: Pallidal deep brain stimulation (DBS) effectively alleviates symptoms in dystonia patients, but may induce movement slowness as a side-effect. In Parkinson's disease, hypokinetic symptoms have been associated with increased beta oscillations (13–30 Hz). We hypothesize that this pattern is symptom-specific, thus accompanying DBS-induced slowness in dystonia.

Methods: In 6 dystonia patients, pallidal rest recordings with a sensing-enabled DBS device were performed and tapping speed was assessed using marker-less pose estimation over 5 time points following cessation of DBS.

Results: After cessation of pallidal stimulation, movement speed increased over time ($P < 0.01$). A linear mixed-effects model revealed that pallidal beta activity explained 77% of the variance in movement speed across patients ($P = 0.01$).

Conclusions: The association between beta oscillations and slowness across disease entities provides further evidence for symptom-specific oscillatory patterns in the motor circuit. Our findings might help DBS therapy improvements, as DBS-devices able to adapt to beta oscillations are already commercially available. © 2023 The Authors. *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society.

Key Words: beta oscillations; bradykinesia; deep brain stimulation; dystonia; GPi

***Correspondence to:** Dr. Roxanne Lofredi, Department of Neurology, Campus Mitte, Charité Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany; E-mail: roxanne.lofredi@charite.de

Funding agencies: Dr. Lofredi is participant in the BIH Charité Clinician Scientist Program funded by the Charité-Universitätsmedizin Berlin, and the Berlin Institute of Health at Charité (BIH), and Fellow of the SPOKES Wellcome Trust Funded Translational Partnership. The work was supported by Deutsche Forschungsgemeinschaft (424778381-TRR 295 Retune).

Received: 8 September 2022; **Accepted:** 30 January 2023

Published online 20 February 2023 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/mds.29347

Introduction

Dystonia is a hyperkinetic movement disorder that responds well to deep brain stimulation (DBS) of the globus pallidus internus (GPi).[1-3] Recordings from DBS-electrodes revealed that exaggerated low frequency (LF) oscillations (3–12 Hz) in the GPi correlate with dystonic symptom severity[4] and are reduced by acute DBS along with symptom alleviation.[5] More recently, pulse generators with sensing capacity became available, demonstrating the stability over time of pallidal LF-activity as biomarker of dystonic symptoms as well as its suppression by long-term DBS.[6] Given that both beneficial and unwanted DBS-effects may develop only after days or even months, the possibility of chronic recordings is of particular interest in dystonia. DBS-induced parkinsonian signs (e.g., decreased movement speed) are such slowly developing side-effects that are reversible upon cessation of pallidal DBS.[7-11] Although it is still unknown whether DBS-induced movement slowing in dystonia is accompanied by a specific oscillatory pattern, movement speed[12,13] and bradykinesia[14] in Parkinson's disease (PD) patients have been shown to correlate with subcortical activity mostly confined to the low beta band (Lbeta) (13–20 Hz).[15] One previous study showed that dopamine-depleting medication increases pallidal Lbeta