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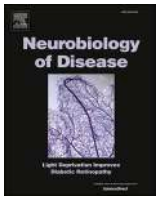

Immune landscape of the enteric nervous system differentiates Parkinson's disease patients from controls: The PADUA-CESNE cohort

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ABSTRACT

Background: Gastrointestinal dysfunction has emerged as a prominent early feature of Parkinson's Disease, shedding new light on the pivotal role of the enteric nervous system in its pathophysiology. However, the role of immune-cell clusters and inflammatory and glial markers in the gut pathogenetic process needs further elucidation.

Objectives: We aimed to study duodenum tissue samples to characterize PD's enteric nervous system pathology further. Twenty patients with advanced PD, six with early PD, and 18 matched controls were included in the PADUA-CESNE cohort.

Methods: Duodenal biopsies from 26 patients with early to advanced stage PD and 18 age-matched HCs were evaluated for the presence of surface markers (CD3+, CD4+, CD8+, CD20+, CD68+, HLA-DR), presence of misfolded alpha-synuclein and enteric glial alteration (GFAP). Correlation of immulogic pattern and clinical characteristic were analyzed.

Results: The findings validate that in patients with Parkinson's Disease, the activation and reactive gliosis are linked to the neurodegeneration triggered by the presence of misfolded alpha-synuclein in the enteric nervous system. This process intensifies from the initial to the advanced stages of the disease. The clusters of T- and B-lymphocytes in the enteric system, along with the overall expression of HLA-DR in antigen-presenting cells, exceeded those in the control group. Conversely, no differences in terms of macrophage populations were found.

Conclusions: These findings broaden our understanding of the mechanisms underlying the enteric nervous system's involvement in PD and point to the gastrointestinal system as a potential therapeutic target, especially in the early stages of the disease. Moreover, our results propose a role of T- and B-lymphocytes in maintaining inflammation and ultimately influencing alpha-synuclein misfolding and aggregation.

1. Introduction

Increasing evidence indicates that α-synuclein (α-syn) pathology in Parkinson's disease is widespread and can be detected outside the brain in the peripheral nervous system and other organs and tissues, even before motor symptoms become clinically manifest (Iranzo et al., 2023). Gastrointestinal dysfunction (Antonini et al., 2012; Rietdijk et al., 2017) is a highly prevalent early disease manifestation which has gained

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increasing interest in light of the possible pathogenetic role of the enteric nervous system (ENS), a specialized neural and glial network (Warnecke et al., 2022) embedded in the wall of the gastrointestinal tract and organized in the myenteric and submucosal plexus (Furness et al., 2014). Recent studies focused on the enteric glial cells (EGCs) disclosed a variety of complex functions, including neurotransmission facilitation, modulation of immunological responses and production of glial-derived factors influencing the intestinal barrier permeability (Natale et al., 2021; Yang et al., 2022; Montalbán-Rodríguez et al., 2024). Moreover, an abundant population of immune cells, including T- and B-lymphocytes and phagocytic cells, has been identified in the ENS, with the latter represented in the duodenum by intestinal macrophages (constituting the largest macrophages populations in the body) (Wang et al., 2019).

The peculiar heterogeneity of cellular phenotypes identified in the ENS (Natale et al., 2021), combined with the notion that dopamine is the only catecholaminergic neurotransmitter detected at this level, suggest that the disruption of this network might be particularly relevant in the context of the gastrointestinal manifestations in PD patients, and ultimately in PD pathogenesis (Natale et al., 2021).

We have documented α-syn pathology in both gastric and colonic biopsies in a group of early and advanced PD patients from our PADOVA-CESNE cohort (Emmi et al., 2023). This cohort includes patients who have been genetically characterized, with imaging, clinical and cognitive features, and peripheral biomarkers collected as part of the Study Center for Neurodegenerative disease (CESNE) at the University of Padova.

In this study, we wanted to elucidate the pattern of the immune-cell population and inflammatory and glial markers in duodenum samples to further characterize the role of ENS pathology in PD patients.

2. Materials and methods

2.1. Subjects

Clinical data of these patients have been reported previously (Emmi et al., 2023).

Twenty (20) patients (12 males; mean age 65.2 years, 95% CI 61.4 to 69.0; mean disease duration 11.3 years, 95% CI 9.0 to 13.6) with advanced PD who required initiation of Levodopa Carbidopa Intestinal Gel (LCIG) infusion were enrolled for the study. In advanced patients, an average of four 3-mm^3 duodenal-wall biopsies were sampled in a district topographically unrelated to percutaneous endoscopic gastrostomy with jejunal extension PEG-J placement site. Early PDs also underwent a diagnostic endoscopy with biopsy collection.

In addition, we also investigated 6 early untreated PD subjects (3 males; mean age 63.2 years, 95% CI 50.5 to 76.0; mean disease duration 2.7 years, 95% CI -0.5 to 6.0) with disease duration <5 years. Early PD patients voluntarily underwent screening diagnostic endoscopy with biopsy collection.

Duodenal biopsies from 18 subjects comparable for age and sex (9 males; median age 68.5 yrs., range 54–86 yrs., 95% CI 63.8 to 73.4) undergoing screening diagnostic endoscopy were included as healthy controls (HCs). HCs had no history of neurologic, psychiatric, or other major medical illnesses.

Patients undergoing systemic anti-inflammatory or immunomodulant/immunosuppressive therapies, as well as patients with recent vaccination history were excluded. Anti-platelet treatments (aspirin, clopidogrel) were interrupted 7 days prior to the procedure.

The study protocol received approval by the ethical committee for clinical experimentation of Padova Province (Prot. n. 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.

2.2. Clinical assessment

A complete neurological and neuropsychological examination was performed on all PD patients. Motor involvement was assessed using the Italian version of the Movement Disorder Society Unified Parkinson's Disease Rating Scale (MDS-UPDRS) (Antonini et al., 2013), while disease staging was evaluated using the Hoehn and Yahr. Moreover, the MDS-non motor rating scale (MDS-NMS) (Chaudhuri et al., 2020) was employed to investigate possible coexistent non-motor symptoms in PD patients. Cognitive assessment was performed as previously reported and included the Montreal Cognitive Assessment (MoCA) scale activities of daily living (ADL) and instrumental ADL (IADL) and the Parkinson's Disease-Cognitive Functional Rating Scale (PD-CFRS) (Fiorenzato et al., 2024; Garon et al., 2024).

The levodopa equivalent dose (LEDD) was calculated according to a previous protocol (Jost et al., 2023).

2.3. 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 CD3 (Polyclonal Rabbit Anti-Human, Citrate Buffer HIER, dilution 1:200, Dako Omnis, Code Number: GA503), CD4 (Monoclonal Mouse Anti-Human, dilution 1:200; Leica Byosystems, Code Number: CD4–368-L-CE), CD8 (Monoclonal Mouse Anti-Human, dilution 1:200; Leica Byosystems, Code Number: CD8-4B11-L-CE), CD20 (Monoclonal Mouse Anti-Human, Citrate Buffer HIER, dilution 1:200 Clone KP1, Dako Omnis, Code Number: M0814) and CD68 (Monoclonal Mouse Anti-Human, EDTA Buffer HIER, IHC dilution 1:5000, IF dilution 1:500, Clone L26, Dako Omnis, Code Number: M0756), HLA-DR (Monoclonal Rabbit Anti-Human, Citrate Buffer HIER, dilution 1:50 Clone: LN-3, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), Aggregated αSyn (Monoclonal Mouse Anti-Human, dilution 1:5000, Clone 5G4, Millipore), Glial Fibrillary Acidic Protein (GFAP, Monoclonal Rabbit Anti-Human, dilution 1:1000, Dako Omnis), and β-III-Tubulin Antibody (Polyclonal Rabbit Anti-Human, dilution 1:300, BioLegend) was performed as previously reported (Emmi et al., 2023). Antigen retrieval was performed on a PT-Link Dako Antigen retrieval station using a citrate buffer at pH 6 solution at 96° for 15 min.

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

2.4. 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) (Emmi et al., 2021). Specimens were digitally scanned at 20× magnification and an average of 3 ± 1 non-overlapping counting fields were defined and loaded into ImageJ software for semi-automatic immunoreactivity quantification. A Maximum Entropy Threshold was applied and manually adjusted for each section to discern immunopositivity elements from background and negative tissue. An expert morphologist performed quality control of the applied threshold by overlying the thresholded images to the original photomicrographs. Particle analysis was employed with an 8-infinity μm threshold to define immunoreactive elements quantity and total area occupied within the digital image (Supplementary Fig. 5). Counting fields for each sample were treated as repeated measures and averaged per subject.

Table 1
Clinical and demographical data of the study cohort.

	Healthy controls ($N = 18$)		Early PD ($N = 6$)		Advance stage PD with PEG-J ($N = 20$)		
	Median	2.5–97.5 P	Median	2.5–97.5 P	Median	2.5–97.5 P	*P* value
Sex (Male%)	50%		50%		60%		0.8000
Age at biopsy (years)	68.5	54.0–86.0	63	53.0–70.0	64	48.0–79.0	0.4570
Age at diagnosis (years)			59.5	49.0–66.0	54	34.0–67.0	0.1610
Disease duration (years)			3	1.0–6.0	13	5.0–22.0	**0.0004**
LEDD at baseline			425	400.0–450.0	1246	750.0–2588.0	0.0066
DAED at baseline			0	0.0–0.0	195	0.0–360.0	0.0530
MDS-UPDRS-I			4.5	1.0–19.0	12	6.0–24.0	0.0680
MDS-UPDRS-II			8.5	4.0–12.0	17.5	2.0–37.0	0.0189
MDS-UPDRS-III			20	12.0–31.0	32.5	10.0–58.0	0.0550
MDS-UPDRS-IV			0	0.0–2.0	8	3.0–13.0	**0.0003**
H&Y > 2			0%		50%		0.0800
Motor phenotype % (TD/PIGD/mixed)				75% / 25% / 0%		32% / 58% /10%	0.2594
MDS-NMS (Tot. score)			37	24.0–185.0	135.5	17.0–301.0	0.2311
MDS-NMS (Non-motor fluct. Tot. Score)			1	0.0–22.0	14	0.0–76.0	0.6520
PD-CFRS			1	1.0–1.0	1	0.0–9.0	0.7920
PDQ8			6	6.0–6.0	13	0.0–21.0	0.4166
MoCA (Corrected score)			25.6	23.6–28.0	24.0	16.8–30.0	0.1710
Cognitive status (II –level) (% NC / MCI)				100% / 0%		56% / 44%	0.1884
ADL			5.5	5.0–6.0	5	2.0–6.0	0.6840
IADL			8	8.0–8.0	5	2.0–8.0	0.0270

Note. Demographic comparison between healthy controls, early-stage PD, and advanced-stage PD was conducted using Kruskal-Wallis ANOVA. Within PD subgroups, comparisons were made using the Mann-Whitney U test. An adjusted P value of 0.003, following Bonferroni correction, was considered significant. LEDD: levodopa daily dose; DAED: dopamin agonist daily dose; MDS-UPDRS. Movement Disorder Society Unified Parkinson's Disease Rating Scale; H&Y: Hoehn and Yahr; TD: Tremor dominant; PIGD: Postural instability and gait disorders; MDS-NMS: Movement Disorder Society non motor scale; PD-CFRS: Cognitive Functional Rating Scale; PDQ8: Parkinson's Disease Questionnaire-8; MoCA: Montreal Cognitive Assessment; ADL: Activities of Daily Living; IADL: Instrumental Activities of Daily Living Scale.

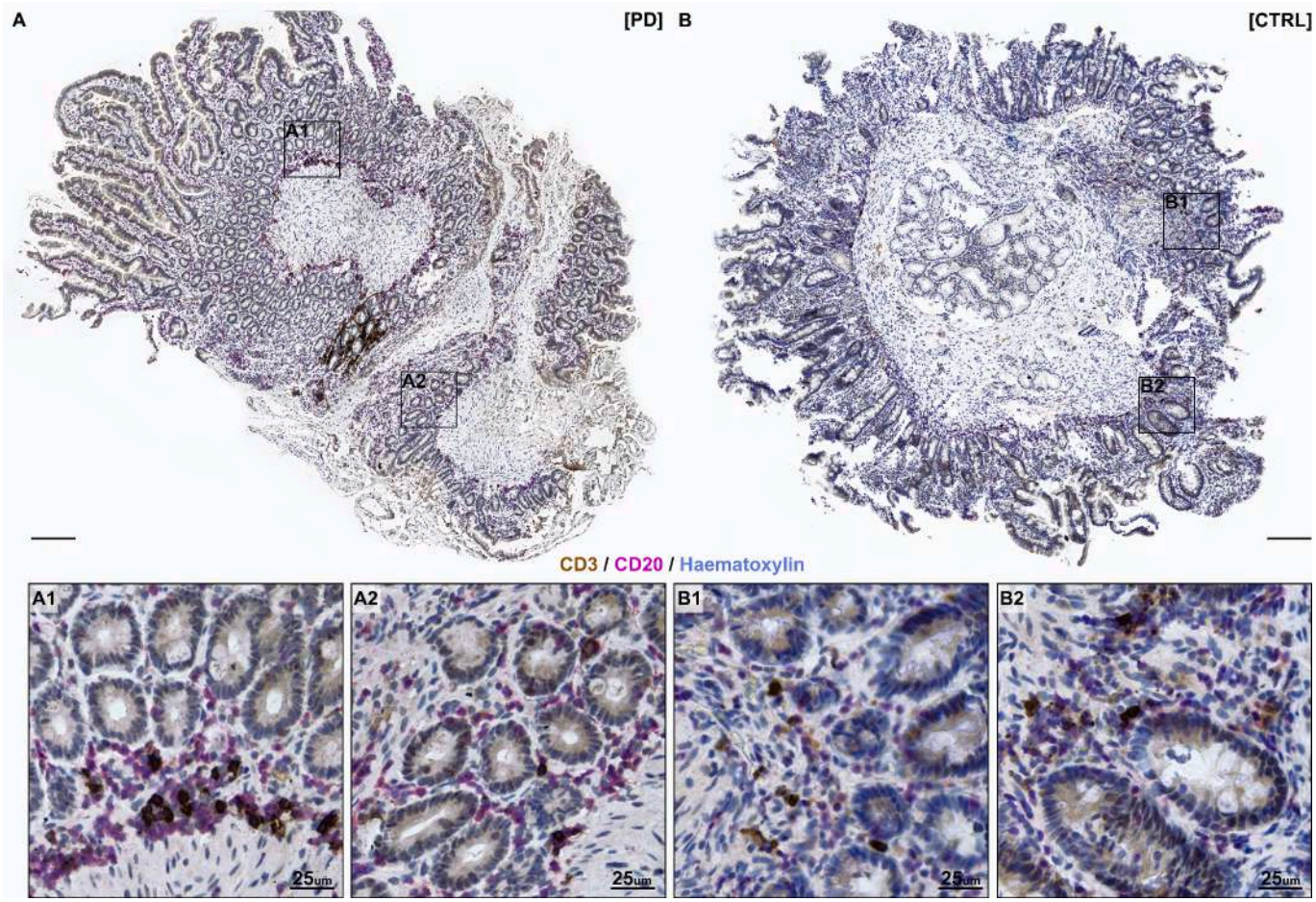


Fig. 1. CD3 (brown) and CD20 (magenta) immunohistochemistry of duodenal biopsies in Parkinson's Disease patients (A) and Healthy Controls (B). Magnifications (A1–2, B1–2) display cellular reactivities of t-lymphocytes (CD3) and b-lymphocytes (CD20). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

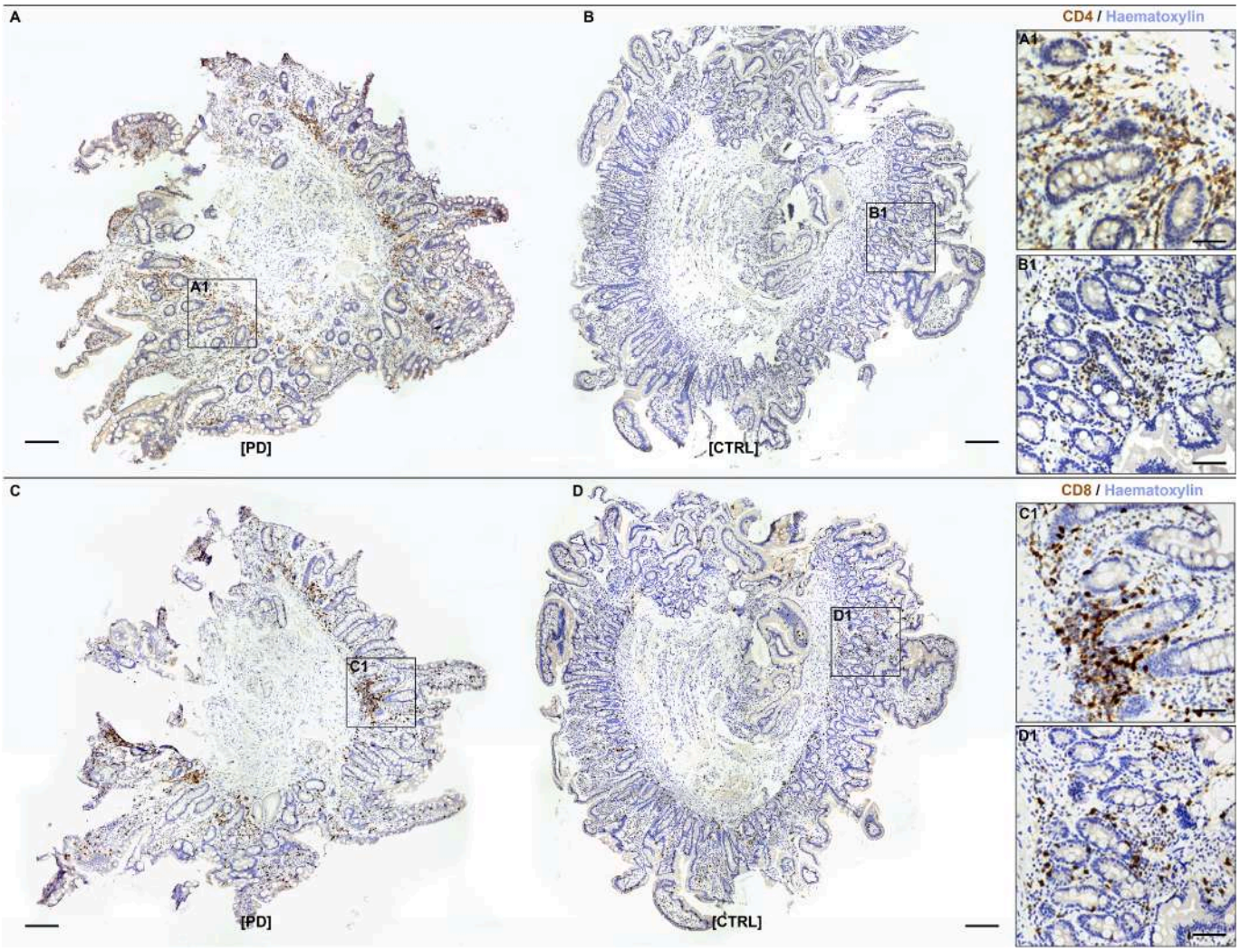


Fig. 2. CD4 and CD8 (brown) immunohistochemistry of duodenal biopsies in Parkinson's Disease patients (A,C) and Healthy Controls (B,D). Magnifications (A1, B1, C1, D1) display cellular reactivities of t-helper cells (CD4+) and natural killer t-cells (CD8+). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.5. Immune cell populations and inflammatory markers

T-lymphocytes were marked with anti-CD3 immunohistochemistry, while B-lymphocytes were detected with an anti-CD20 antibody. Macrophages and monocytes express CD68 antigen, a well-established phagocytic marker, indicating increased lysosomal activity. Furthermore, the expression of MHC-II molecules (HLA-DR) was evaluated. HLA-DR is a cell surface receptor and constitutes a T-cell receptor (TCR) ligand involved in presenting foreign antigens.

2.6. Statistical analyses

Statistical analyses and visualizations were performed using GraphPad Prism v.9. Clinical characteristics, and duodenal inflammatory markers were compared among healthy individuals, early-stage PD, and advance stage PD using the non parametric Kruskal-Wallis ANOVA. Post-hoc comparisons were conducted using the Mann–Whitney *U* test. The sensitivitity of duodenal Inflammatory markers to motor deficit severity (low severity H&Y $\leq$ 2 vs. high severity H&Y $>$ 2) and to the presence of mild cognitive impairment (MCI) versus PD with normal cognition (NC) were analyzed with Mann–Whitney U test. Pearson's correlation analysis has been employed to assess possible correlations between αSyn expression in duodenum and clinical characteristics, including motor and non-motor scales, cognitive assessments and main non-motor symptoms of PD. A Bonferroni-corrected threshold of $P = 0.05$ was used to determine significance.

3. Results

3.1. Demographics

There were no differences among the three participant subgroups. In particular, we observed no significant difference for age as a variable among PD patients and healthy controls (Supplementary Fig. 3), as well as no significant differences based on gender in PD patients (Supplementary Table 2). As expected, we found a significant difference for disease duration (median 3 yrs., range 1–6 yrs. vs median 13 yrs., range 5–22) and for the MDS-UPDRS IV subscore for motor complications (median 0, range 0–2 vs median 8, range 3–13 between early and advanced PD).

Demographic and clinical data are reported in Table 1.

3.2. Immune landscape and gliosis markers

All data are reported in Figs. 1–6, Tables 2–4 and the Supplementary Materials.

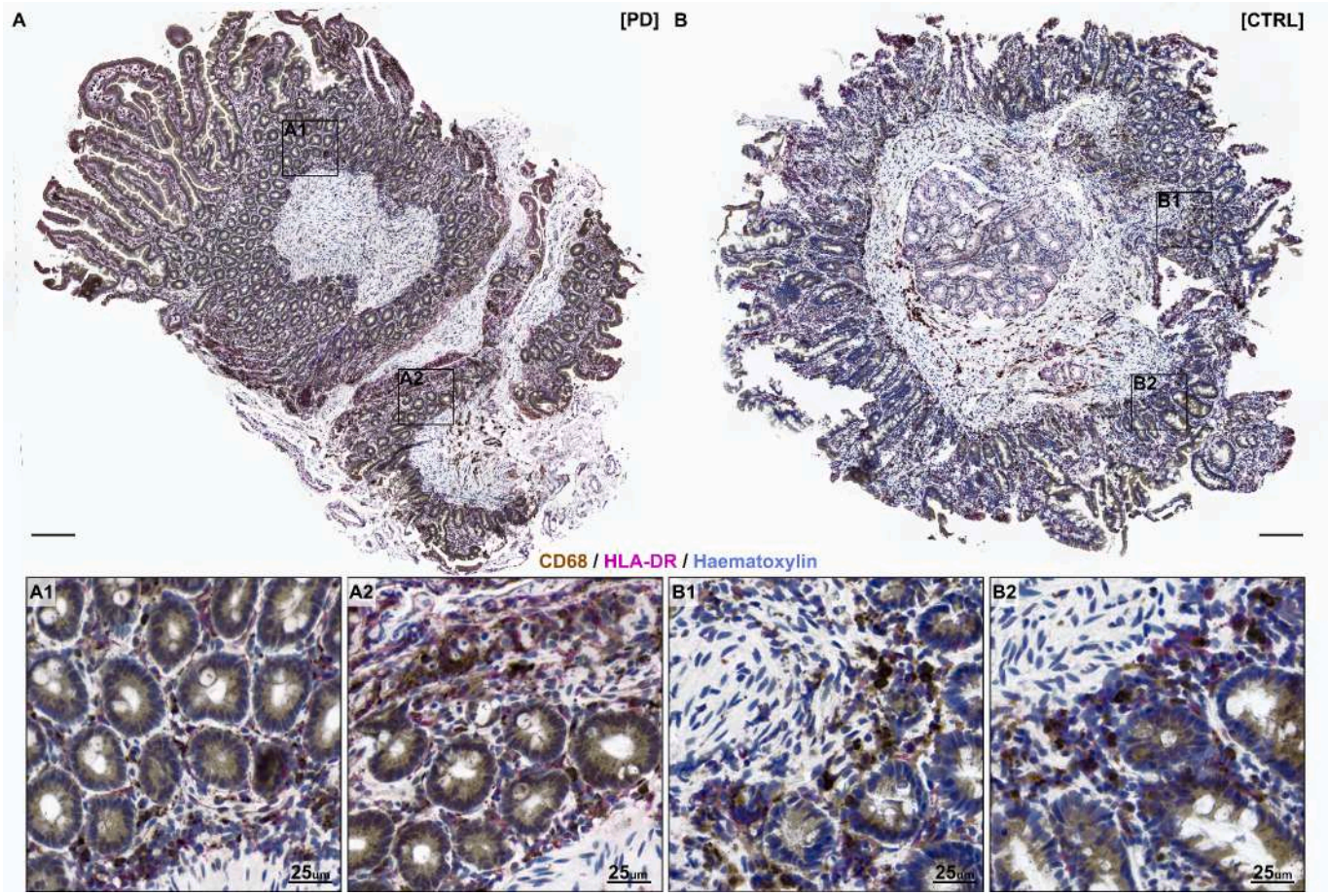


Fig. 3. CD68 (brown) and HLA-DR (magenta) immunohistochemistry of duodenal biopsies in Parkinson's Disease patients (A) and Healthy Controls (B). Magnifications (A1–2, B1–2) display cells with increased phagocytic activity (CD68) and antigen-presenting cells (HLA-DR). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2.1. Enteric T-lymphocytes (CD3+)

We detected a statistically significant difference when comparing CD3+ cell densities (CD3+ cells / mm^2) between HCs and early and advanced PD (median 215.5 vs 614.5 and 444 in early and advanced PD, respectively; p 0.00124). However, there was no significant difference between early and advanced PD, only a trend towards lower CD3+ densities with longer disease duration (Fig. 1, Table 2).

3.2.2. Enteric T-helper lymphocytes (CD4+)

We detected a statistically significant difference when comparing CD4+ cell densities (CD4+ cells / mm^2) between HCs and early and advanced PD (median 45.5 vs 166.5 and 184.5 in early and advanced PD, respectively; p 0.0031). However, there was no significant difference between early and advanced PD (Fig. 2, Table 2).

3.2.3. Enteric natural killer lymphocytes (CD8+)

We detected a statistically significant difference when comparing CD8+ cell densities (CD4+ cells / mm^2) between HCs and early and advanced PD (median 200 vs 388.5 and 303.5 in early and advanced PD, respectively; p 0.028). However, there was again no significant difference between early and advanced PD (Fig. 2, Table 2).

3.2.4. CD4+/CD8+ ratio

When comparing the ratio between CD4+ and CD8+ cells, we detected statistically significant differences between HCs and advanced PD patients, but not early PD patients (median 0.3 vs 0.5 and 0.6 in early and advanced PD, respectively; p 0.049) (Fig. 2, Table 2).

3.2.5. Enteric B-lymphocytes (CD20+)

There was a statistically significant difference in CD20+ cell densities (CD20+ cells / mm^2) between HCs and early and advanced PD (median 61.5 vs 240 and 157 in early and advanced PD, respectively; p 0.00053). As for the CD3+ cells, we found no statistically significant differences between PD groups despite an overall trend showing a reduction in CD20+ cell densities in advanced PDs (Fig. 3, Table 2).

3.2.6. CD68+

No significant difference was observed when comparing CD68+ cell densities among the three subgroups (median 230.5 in HCs vs 258.5 and 260 in early and advanced PD, respectively; p 0.5559) (Fig. 3, Table 2).

3.2.7. Enteric antigent presenting cells response:HLA-DR +

We detected a statistically significant difference when comparing the HLA-DR + % mean area between HCs and early and advanced PD (median 1.8 in HCs vs 3.2 and 3 in early and advanced PD, respectively; p 0.00251) (Fig. 3, Table 2).

3.2.8. Enteric expression of glial fibrillary acidic protein (GFAP)

We disclosed a statistically significant difference when comparing GFAP mean density (N/ mm^2) between HCs and both early and advanced PD (median 44.5 in HCs vs 80 and 91 in early and advanced PD, respectively; p 0.00018). Moreover, GFAP mean dimensions (μm^2) were significantly higher in the advanced PD cohort when compared with both HCs and early PDs (median 28.4 in advanced PDs vs 25.7 and 26.3 in HCs and early PDs, respectively; p 0.00041) (Figs. 4–6, Table 2).

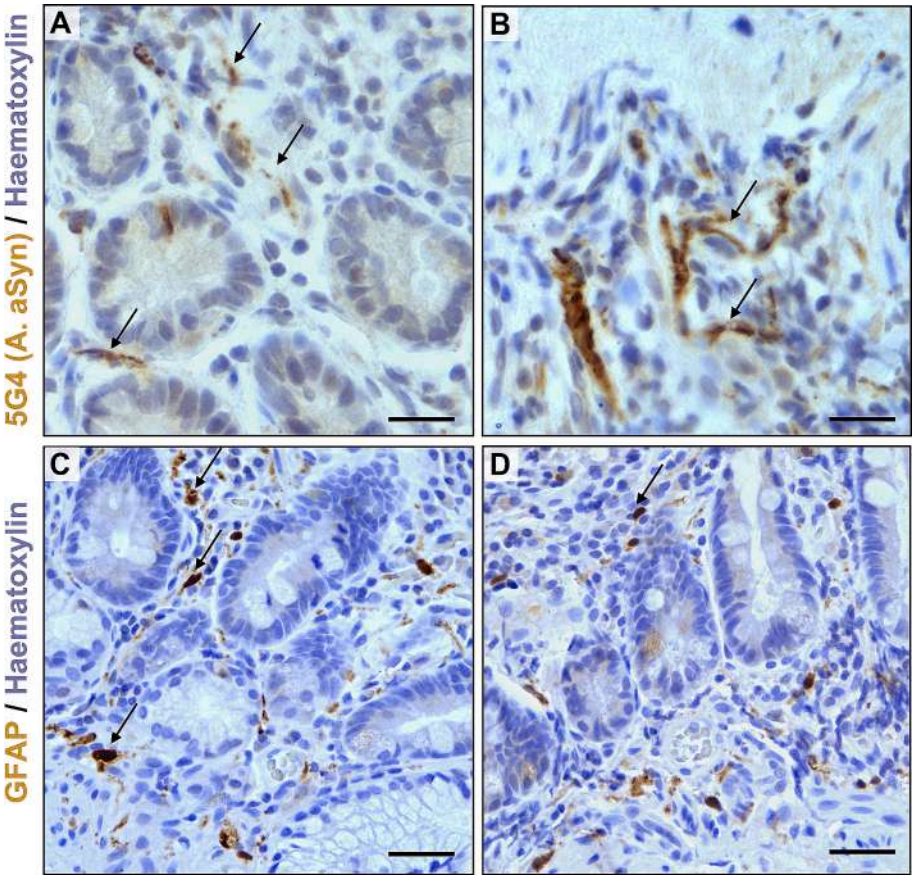


Fig. 4. Aggregated alpha-synuclein, clone 5G4, (A-B) and glial fibriallary acid (GFAP) (C-D) immunohistochemistry in Parkinson's Disease patients displaying specific immunoreactivity for pathological alpha-synuclein threads, and enteric glial cells, respectively.

3.2.9. Enteric α-synuclein

Mean % values of a-synuclein significantly differed between HCs and early and advanced PD (median 0.2 in HCs vs 1.2 and 1.5 in early and advanced PD, respectively; $p < 0.000001$) (Figs. 4–6, Table 2).

3.3. Inflammatory markers in 1st versus 2nd PEG-J repositioning

Among advanced PD patients, we selected 6 patients who underwent PEG-J repositioning once and 8 patients who underwent a second repositioning of PEG-J. We found no statistically significant differences in inflammatory markers between 1st and 2nd PEG-J positioning in advanced PD patients.

3.4. Correlation between inflammatory and gliosis markers and clinical data

There was no statistically significant correlation between inflammatory and glial markers and clinical severity (quantified using the Hoehn and Yahr staging system).

CD20+ mean densities disclosed significantly different values between normal subjects and subjects with evidence of mild cognitive impairment at the neuropsychological evaluation (median 231 in NCs vs 238 MCIs, p 0.0049).

GFAP mean dimensions significantly correlated with MDS-UPDRS III subscore (p 0.009) whereas it did not disclose a significant correlation with other clinical outcomes (Fig. 6).

The complete set of clinical correlation data are reported in Tables 3 and 4.

4. Discussion

The mechanisms underlying the primary location of α-syn misfolding and aggregation and its subsequent spreading are still a matter of debate. The widely known Braak's hypothesis, suggesting a bottom-top route (starting in the ENS and eventually moving towards the brain), has long been opposed to the idea that protein misfolding begins in the brain and spreads towards the periphery. However, recent studies shed light on a newer vision of PD, increasingly considering it as a systemic disease regardless of the brain- or body-first hypothesis (Braak et al., 2006; Dinan and Cryan, 2017; Emmi et al., 2023; Natale et al., 2021; Rietdijk et al., 2017; Yang et al., 2022).

Moreover, the role of inflammation both in the central and peripheral nervous system and possible connections between these two entities, have become increasingly investigated in several studies (Williams et al., 2021; Grillo et al., 2023). Different components of the central nervous system (including microglia, astrocytes, T cells, and recently the lymphatic system) have disclosed inflammatory changes. These findings are mirrored from the increase in proinflammatory cytokines and proliferative capacities in the peripheral blood of PD patients, and are supported from data derived from animal models that consistently confirm the role played by the peripheral immune system in this context (Williams et al., 2021; Lauritsen and Romero-Ramos, 2023; Bellini et al., 2023).

In our study, we observed a significant inflammatory background in the gastrointestinal samples of PD patients, with evidence of a chronic inflammatory response and concomitant enteric gliosis, as demonstrated by increased T and B lymphocytes and glial cells densities in the mucosa with evidence of hypertrophic features in astrocytes. The combination of these findings with thread-like aggregated α-syn deposition in the nerve

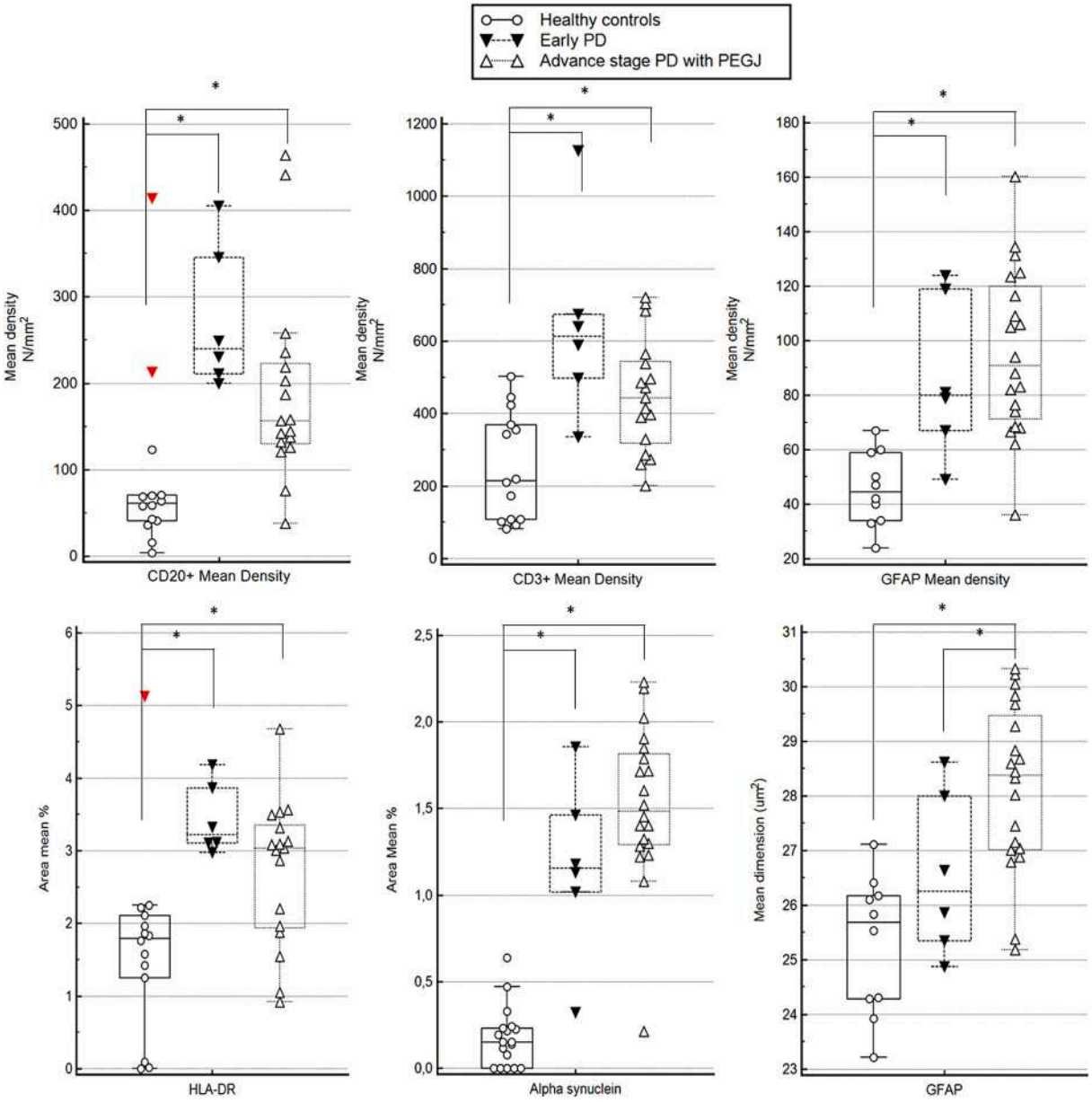


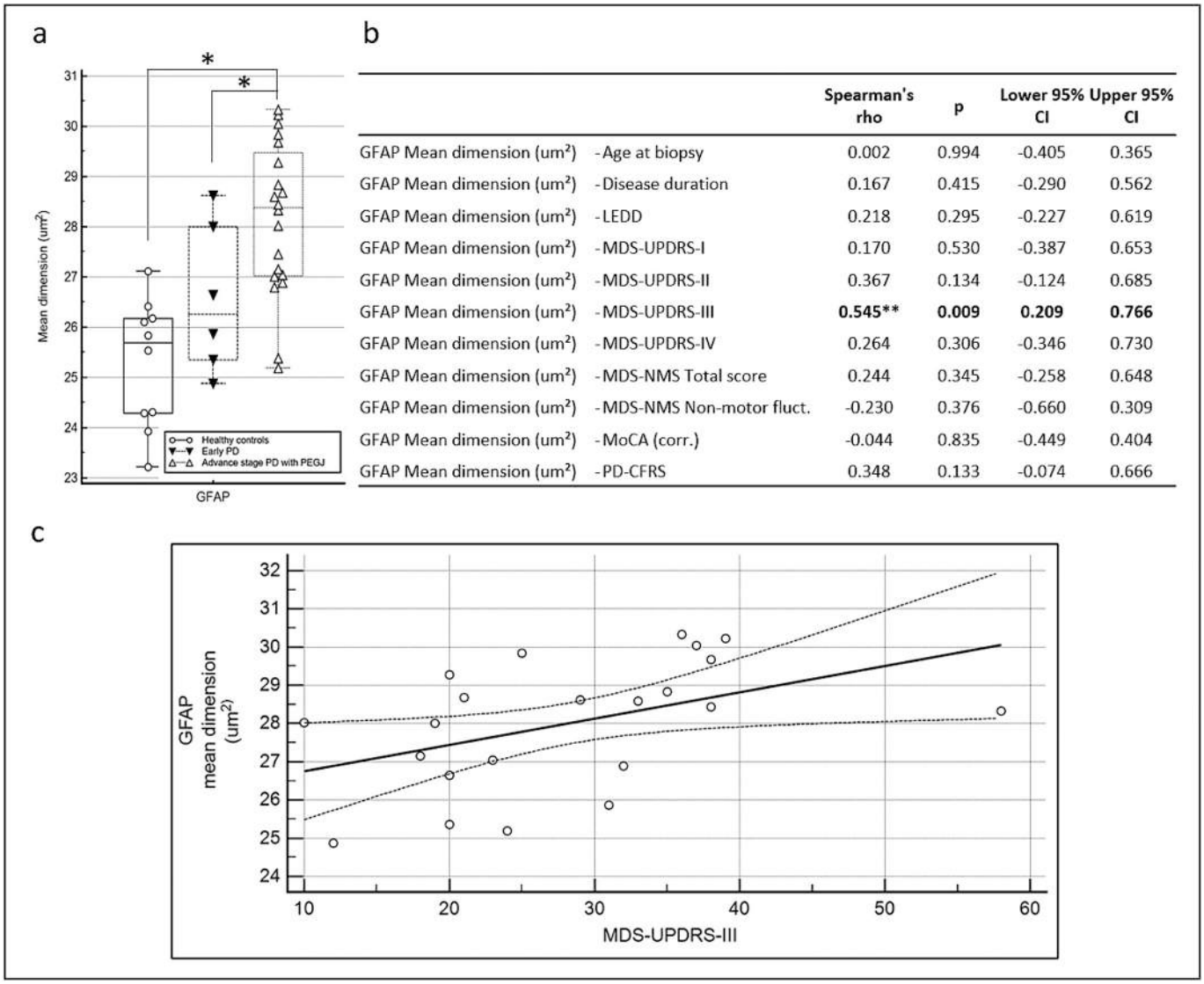
Fig. 5. Mann–Whitney *U* test of CD20+ density, CD3+ density, GFAP+ density, HLA-DR+ immunoreactive area, 5G4 Alpha-Synuclein immunoreactive area and GFAP cell size in early and advanced Parkinson's Disease patients and in healthy controls.

fibers of the mucosa was consistently observed in PD patients while absent in healthy controls, suggesting that this pattern might represent a pathological hallmark of the disease. In light of the well-recognized ability of α-syn to elicit an inflammatory response in the gastrointestinal system, we hypothesized that chronic inflammation may ulteriorly trigger and facilitate α-syn misfolding and aggregation, fueling a vicious cycle that ultimately supports the production of pathological a-syn in the ENS (Bellini et al., 2023).

Both the innate and adaptive immune response may be involved in generating the inflammatory environment in the ENS, which appears to be sustained prominently by the ability of T cells to recognize epitopes derived from misfolded α-syn. In line with this hypothesis, a recent study showed that immunocompromised mice have an 8-fold increase in phosphorylated α-syn pathology in the substantia nigra compared to wild-type mice and that reconstituting the T cell population decreased the accumulation of phosphorylated α-syn, leading to increased microglial activation (George et al., 2021). A previous animal (mouse) model showed that α-synuclein overexpression determines MHCII upregulation in CNS myeloid cells and that CD4-deficient mice seem to be protected from the dopaminergic cell loss (Williams et al., 2021) (REF). Moreover, it has been observed that different genetic backgrounds and mutations in several genes, including those associated with MHCII might drive or influence the response of the immune system in PD patients, suggesting that antigen-presenting cells play a crucial role in sustaining neuroinflammation (Kline et al., 2021) (REF).

The B cell population also appear to be involved in the inflammatory response in PD (Scott, 2022). B cells have a major role in antibody and cytokine production, mediate T cell responses and act as antigen-presenting cells. In our study, findings regarding B cell subtypes appear to differ according to disease duration and treatment (including PEG-J placement). Hence, while broad markers for T and B cell populations suggested functional alterations in our cohort, more accurate phenotyping is required to elucidate further the role played by these cells in the inflammatory response in the context of PD.

In addition to inflammation playing a potential role in protein misfolding and aggregation, recent evidence also supports a direct



		Spearman's rho	p	Lower 95% CI	Upper 95% CI
GFAP Mean dimension (um²)	-Age at biopsy	0.002	0.994	-0.405	0.365
GFAP Mean dimension (um²)	-Disease duration	0.167	0.415	-0.290	0.562
GFAP Mean dimension (um²)	-LEDD	0.218	0.295	-0.227	0.619
GFAP Mean dimension (um²)	-MDS-UPDRS-I	0.170	0.530	-0.387	0.653
GFAP Mean dimension (um²)	-MDS-UPDRS-II	0.367	0.134	-0.124	0.685
GFAP Mean dimension (um²)	-MDS-UPDRS-III	**0.545****	**0.009**	**0.209**	**0.766**
GFAP Mean dimension (um²)	-MDS-UPDRS-IV	0.264	0.306	-0.346	0.730
GFAP Mean dimension (um²)	-MDS-NMS Total score	0.244	0.345	-0.258	0.648
GFAP Mean dimension (um²)	-MDS-NMS Non-motor fluct.	-0.230	0.376	-0.660	0.309
GFAP Mean dimension (um²)	-MoCA (corr.)	-0.044	0.835	-0.449	0.404
GFAP Mean dimension (um²)	-PD-CFRS	0.348	0.133	-0.074	0.666

Fig. 6. Sensitivity of GFAP mean dimension to clinical outcomes of Parkinson's disease progression. A) Distribution of duodenal Glial Fibrillary Acidic Protein (GFAP) mean dimensions among healthy individuals, early-stage PD, and late-stage PD. B) Spearman rank correlation between GFAP dimensions and clinical outcomes in PD patients, showing a significant correlation only with MDS-UPDRS-III. C) Dot plot illustrating the relationship between GFAP mean dimensions and MDS-UPDRS-III.

Table 2
Duodenal inflammatory markers in Parkinson's Disease patients and healthy controls.

	Healthy controls (1)		Early PD (2)		Advance stage PD with PEGJ (3)		Kruskal Wallis Anova	Mann Whitney Post-hoc comparisons		
Duodenum	Median	2.5–97.5 P	Median	2.5–97.5 P	Median	2.5–97.5 P	P value	1 vs. 2	1 vs. 3	2 vs. 3
CD20+ Mean Density (N/mm²)	61.5	4.0–414.0	240	200.0–405.0	157	38.0–464.0	**0,00053**	x	x	
CD3+ Mean Density (N/mm²)	215.5	82.0–502.0	614.5	336.0–1126.0	444	201.0–721.0	**0,00124**	x	x	
CD4+ Mean Density (N/mm²)	45.5	6.0–246.0	166.5	68.0–291.0	184.5	10–0-351.0	**0,0031**	x	x	
CD8+ Mean Density (N/mm²)	200.0	73.0–523.0	338.5	189.0–596.0	303.5	91.0–423.0	**0,02873**	x	x	
CD4+/CD8+ Ratio	0.3	0.1–0.9	0.5	0.2–0.8	0.6	0.1–1.7	**0,04956**		x	
CD3+/CD8+ Ratio	1.1	0.4–2.0	1.7	1.5–2.3	1.4	0.8–3.8	**0,01176**	x	x	
CD68+ Mean Density (N/mm²)	230.5	76.0–455.0	258.5	196.0–399.0	260	179.0–619.0	0,5559			
GFAP Mean Density (N/mm²)	44.5	24.0–67.0	80	49.0–124.0	91	36.0–160.3	**0,00018**	x	x	
GFAP Mean dimension (um²)	25.7	23.2–27.1	26.3	24.9–28.6	28.4	25.2–30.3	**0,00041**		x	x
HLA-DR Area Mean %	1.8	0.0–5.1	3.2	3.0–4.2	3	0.9–4.7	**0,00251**	x	x	
Alpha synuclein- Mean %	0.2	0.0–0.6	1.2	0.3–1.9	1.5	0.2–2.2	**<0,000001**	x	x	

Note. Among inflammatory biomarkers this table include: 1)biomarkers associated with both innate and adaptive immune responses, 2) biomarkers associated with the mysfolded Alpha synuclein 3) biomarker, For adaptive immunity, the biomarkers include CD20+, which is indicative of B-Lymphocytes, and CD3+, which denotes T-Lymphocytes. Within the T-Lymphocytes, we have CD4+ cells, primarily T-helper cells, and CD8+ cells, primarily cytotoxic T cells. For the innate we have CD68+ (monocytes and macrophages). GFAP: Glial Fibrillary Acidic Protein HLA-DR: Human Leukocyte Antigen, molecules present in macrophages, B cells or Dendritic cells that are upregulated in response to signaling.

Table 3
Sensitivity of duodenal inflammatory markers to motor deficits severity in Parkinson's Disease.

	H&Y ≤ 2		H&Y > 2		Mann Whitney U test
	Median	2.5–97.5 P	Median	2.5–97.5 P	P value
CD20+ Mean Density (N/mm^2)	178.5	121.0–405.0	195	76.0–441.0	0.7576
CD3+ Mean Density (N/mm^2)	497.5	274.0–1126.0	401.5	201.0–721.0	0.3158
CD4+ Mean Density (N/mm^2)	187.5	10.0–351.0	204.0	60.0–257.0	0.8774
CD8+ Mean Density (N/mm^2)	292.0	183.0–596.0	303.5	91.0–408.0	0.6712
CD4+/ CD8+ Ratio	0.5	0.1–1.7	0.7	0.2–1.5	0.2314
CD3+/ CD8+ Ratio	1.7	0.9–2.4	1.4	0.8–3.8	0.4402
GFAP Mean Density (N/mm^2)	100	49.0–134.5	68.5	36.0–160.3	0.3135
GFAP Mean dimension (um^2)	28.3	24.9–30.2	28.3	25.2–30.3	0.9497
HLA-DR Area Mean %	3.2	1.5–4.7	3	0.9–3.6	0.1426
Alpha synuclein-Mean %	1.4	0.3–2.2	1.3	0.2–2.2	0.5287

Table 4
Sensitivity of duodenal inflammatory markers to cognitive decline in Parkinson's Disease.

	NC		MCI		Mann Whitney U test
	Median	2.5–97.5 P	Median	2.5–97.5 P	P value
CD20+ Mean Density (N/mm^2)	231	38.0–464.0	138	121.0–187.0	**0.0049**
CD3+ Mean Density (N/mm^2)	565	260.0–1126.0	397	274.0–721.0	0.088
CD4+ Mean Density (N/mm^2)	180.0	60.0–291.0	229.0	10.0–351.0	0.7214
CD8+ Mean Density (N/mm^2)	366.0	280.0–596.0	21.0	91.0–423.0	**0.0357**
CD4+/ CD8+ Ratio	0.5	0.2–0.8	0.8	0.1–1.7	0.1535
CD3+/ CD8+ Ratio	1.7	0.8–2.3	1.3	0.9–3.8	0.9053
GFAP Mean Density (N/mm^2)	94	36.0–160.3	94.5	62.0–134.5	0.6514
GFAP Mean dimension (um^2)	28	25.4–30.1	28.7	25.2–30.3	0.1752
HLA-DR Area Mean %	3.1	1.1–4.2	3.1	1.5–4.7	0.9684
Alpha synuclein-Mean %	1.4	0.3–1.9	1.8	0.2–2.2	0.1376

involvement of α-syn in mediating antiviral and inflammatory responses (Tulisiak et al., 2019). It has been observed that α-syn production is upregulated during viral infections to mediate interferon signaling and contribute to the inhibition of viral replication (Beatman et al., 2016; Monogue et al., 2022). While this may represent a protective factor during infection, recent in vitro studies (Wu et al., 2022) disclosed that viral-induced overexpression of α-syn may lead to protein misfolding, aggregation and Lewy-body pathology. It has been observed that exposure to specific viral proteins, such as the nucleocapsid protein of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), may trigger α-syn misfolding and Lewy Body pathology due to the innate α-syn upregulation occurring upon infection (Emmi et al., 2022), with recent evidence confirming a higher incidence of neurodegenerative diseases following viral infection (Levine et al., 2023).

In conclusion, our study investigated the possible role of the ENS and inflammation in the gastrointestinal system in the pathogenesis of PD through the identification of specific histopathological and immunological markers in duodenal tissue samples. The presence of α-synuclein deposition in peripheral nerves within the gastrointestinal tract, coupled with reactive enteric gliosis and a marked increase in lymphocyte populations alongside elevated MHC-II molecules indicating inflammation, is characteristic of PD patients and is not observed in HCs. These observations suggest that these features could serve as a defining pathological hallmark of PD. Additionally, the degree of enteric gliosis holds promise as a potential progression marker. These insights expand our understanding of the mechanisms underlying the involvement of the ENS, indicating the gastrointestinal system as a plausible therapeutic target, particularly in the early stages of the illness. Furthermore, the role of B-lymphocytes in perpetuating ENS inflammation and subsequent α-synuclein misfolding and deposition may offer new therapeutic opportunities. Further studies in larger patient cohorts are warranted and will help in understanding the link between inflammation and α-synuclein pathology.

5. Study limitations

Limitations of this study include the relatively small sample size and few patients with early-stage PD, with results possibly being affected by sample bias. Incontrast, the strength of this study includes the prospective and systematic assessment of the patients and theextensive recording of clinical data, which made thecorrelations more reliable.

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Author roles

AE, MC (Marta Campagnolo), AA and AP contributed to conception and design of the study. FPR and ES performed the GI biopsies. AE, MS and AT performed the immunohistochemical stainings. AE, AP, AT evaluated the specimens and performed the morphometrical evaluations. AE, MC, MC2 (Miryam Carecchio), ES, FPR, LW, AP and AA contributed to acquisition and/or analysis of clinical and pathological data. AE, MC, AA, and PP contributed to drafting the text and preparing the figures. AE, AP and AA supervised the study. All authors reviewed the manuscript for intellectual content.

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All other authors have no other financial disclosures to declare.

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Marta Campagnolo: Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. **Luca Weis:** Formal analysis. **Michele Sandre:** Methodology, Data curation. **Aleksandar Tushevski:** Methodology, Investigation. **Francesco Paolo Russo:** Investigation. **Edoardo Savarino:** Resources, Investigation. **Miryam Carecchio:** Resources, Funding acquisition, Conceptualization. **Elena Stocco:** Data curation, Resources. **Veronica Macchi:** Resources, Project administration. **Raffaele De Caro:** Supervision, Resources, Project administration. **Piero Parchi:** Writing – review & editing, Supervision, Conceptualization. **Luigi Bubacco:** Writing – review & editing, Supervision, Resources. **Andrea Porzionato:** Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation. **Angelo Antonini:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization. **Aron Emmi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no disclosures or conflicts of interest related to the manuscript's content.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2024.106609.

References

Antonini, A., Barone, P., Marconi, R., et al., 2012. The progression of non-motor symptoms in Parkinson's disease and their contribution to motor disability and quality of life. J. Neurol. 259 (12), 2621–2631. https://doi.org/10.1007/s00415-012-6557-8.

Antonini, A., Abbruzzese, G., Ferini-Strambi, L., Tilley, B., Huang, J., Stebbins, G.T., Goetz, C.G., Barone, P., Bandettini di Poggio, M., Fabbrini, G., Di Stasio, F., Tinazzi, M., Bovi, T., Ramat, S., Meoni, S., Pezzoli, G., Canesi, M., Martinelli, P., Maria Scaglione, C.L., Del Sorbo, F., 2013. Validation of the Italian version of the movement disorder society—unified Parkinson's disease rating scale. Neurol. Sci. 34 (5), 683–687. https://doi.org/10.1007/s10072-012-1112-z.

Beatman, E.L., Massey, A., Shives, K.D., Burrack, K.S., Chamanian, M., Morrison, T.E., Beckham, J.D., 2016. Alpha-synuclein expression restricts RNA viral infections in the brain. J. Virol. 90 (6), 2767–2782. https://doi.org/10.1128/JVI.02949-15.

Bellini, G., Benvenuti, L., Ippolito, C., Frosini, D., Segnani, C., Rettura, F., Pancetti, A., Bertani, L., D'Antongiovanni, V., Palermo, G., Del Prete, E., Antonioli, L., Nardini, V., Morganti, R., Pellegrini, C., Bernardini, N., Ceravolo, R., Fornai, M., Bellini, M., 2023 Nov. Intestinal histomorphological and molecular alterations in patients with Parkinson's disease. Eur. J. Neurol. 30 (11), 3440–3450. https://doi.org/10.1111/ene.15607. Epub 2022 Nov 6. PMID: 36263629.

Braak, H., Alafuzoff, I., Arzberger, T., Kretzschmar, H., Del Tredici, K., 2006. Staging of Alzheimer disease-associated neurofibrillary pathology using paraffin sections and immunocytochemistry. Acta Neuropathol. 112 (4), 389–404. https://doi.org/10.1007/s00401-006-0127-z.

Chaudhuri, K.R., Schrag, A., Weintraub, D., Rizos, A., Rodriguez-Blazquez, C., Mamikonyan, E., Martinez-Martin, P., 2020. The movement disorder society nonmotor rating scale: initial validation study. Mov. Disord. 35 (1), 116–133. https://doi.org/10.1002/mds.27862.

Dinan, T.G., Cryan, J.F., 2017. The microbiome-gut-brain axis in health and disease. Gastroenterol. Clin. N. Am. 46 (1), 77–89. https://doi.org/10.1016/j.gtc.2016.09.007.

Emmi, A., Porzionato, A., Contran, M., De Rose, E., Macchi, V., De Caro, R., 2021. 3D reconstruction of the morpho-functional topography of the human vagal trigone. Front. Neuroanat. 15 https://doi.org/10.3389/fnana.2021.663399.

Emmi, A., Boura, I., Raeder, V., Mathew, D., Sulzer, D., Goldman, J.E., Leta, V., 2022. Covid-19, Nervous System Pathology, and Parkinson's Disease: Bench to Bedside, pp. 17–34. https://doi.org/10.1016/bs.irn.2022.06.006.

Emmi, A., Sandre, M., Russo, F.P., Tombesi, G., Garrì, F., Campagnolo, M., Carecchio, M., Biundo, R., Spolverato, G., Macchi, V., Savarino, E., Farinati, F., Parchi, P., Porzionato, A., Bubacco, L., De Caro, R., Kovacs, G.G., Antonini, A., 2023. Duodenal alpha-synuclein pathology and enteric gliosis in advanced Parkinson's disease. Mov. Disord. 38 (5), 885–894. https://doi.org/10.1002/mds.29358.

Fiorenzato, E., Moaveninejad, S., Weis, L., Biundo, R., Antonini, A., Porcaro, C., 2024. Brain dynamics complexity as a signature of cognitive decline in Parkinson's disease. Mov. Disord. 39 (2), 305–317. https://doi.org/10.1002/mds.29678.

Furness, J.B., Callaghan, B.P., Rivera, L.R., Cho, H.J., 2014. The enteric nervous system and gastrointestinal innervation: integrated local and central control. In: Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease, pp. 39–71.

Garon, M., Weis, L., Siquier, A., et al., 2024. Validation of the Italian version of the Parkinson's disease- cognitive functional rating scale. J. Neural Transm. (Vienna). https://doi.org/10.1007/s00702-024-02746-6. Published online January 27.

George, S., Tyson, T., Rey, N.L., Sheridan, R., Peelaerts, W., Becker, K., Schulz, E., Meyerdirk, L., Burmeister, A.R., von Linstow, C.U., Steiner, J.A., Galvis, M.L.E., Ma, J., Pospisilik, J.A., Labrie, V., Brundin, L., Brundin, P., 2021. T cells limit accumulation of aggregate pathology following intrastriatal injection of α-synuclein fibrils. J. Parkinsons Dis. 11 (2), 585–603. https://doi.org/10.3233/JPD-202351.

Grillo, P., Sancesario, G.M., Bovenzi, R., Zenuni, H., Bissacco, J., Mascioli, D., Simonetta, C., Forti, P., Degoli, G.R., Pieri, M., Chiurchiù, V., Stefani, A., Mercuri, N. B., Schirinzi, T., 2023 Jul. Neutrophil-to-lymphocyte ratio and lymphocyte count reflect alterations in central neurodegeneration-associated proteins and clinical severity in Parkinson disease patients. Parkinsonism Relat. Disord. 112, 105480 https://doi.org/10.1016/j.parkreldis.2023.105480. Epub 2023 Jun 4. PMID: 37290213.

Iranzo, A., Mammana, A., Muñoz-Lopetegi, A., Dellavalle, S., Mayà, G., Rossi, M., Serradell, M., Baiardi, S., Arqueros, A., Quadalti, C., Perissinotti, A., Ruggeri, E., Cano, J.S., Gaig, C., Parchi, P., 2023 May 2. Misfolded α-synuclein assessment in the skin and CSF by RT-QuIC in isolated REM sleep behavior disorder. Neurology 100 (18), e1944–e1954. https://doi.org/10.1212/WNL.0000000000207147 (Epub 2023 Mar 17. PMID: 36931726; PMCID: PMC10159765).

Jost, S.T., Kaldenbach, M.A., Antonini, A., Martinez-Martin, P., Timmermann, L., Odin, P., Katzenschlager, R., Borgohain, R., Fasano, A., Stocchi, F., Hattori, N., Kukkle, P.L., Rodríguez-Violante, M., Falup-Pecurariu, C., Schade, S., Petry-Schmelzer, J.N., Metta, V., Weintraub, D., Deuschl, G., Espay, A.J., Tan, E.K., Bhidayasiri, R., Fung, V.S.C., Cardoso, F., Trenkwalder, C., Jenner, P., Ray Chaudhuri, K., Dafsari, H.S., 2023 Jul. International Parkinson and movement disorders society non-motor Parkinson disease study group. Levodopa dose equivalency in Parkinson's disease: updated systematic review and proposals. Mov. Disord. 38 (7), 1236–1252. https://doi.org/10.1002/mds.29410 (Epub 2023 May 5. PMID: 37147135).

Kline, E.M., Houser, M.C., Herrick, M.K., Seibler, P., Klein, C., West, A., Tansey, M.G., 2021 Jan. Genetic and environmental factors in Parkinson's disease converge on immune function and inflammation. Mov. Disord. 36 (1), 25–36. https://doi.org/10.1002/mds.28411. Epub 2020 Dec 14. PMID: 33314312; PMCID: PMC8285924.

Lauritsen, J., Romero-Ramos, M., 2023 Oct. The systemic immune response in Parkinson's disease: focus on the peripheral immune component. Trends Neurosci. 46 (10), 863–878. https://doi.org/10.1016/j.tins.2023.07.005. Epub 2023 Aug 17. PMID: 37598092.

Levine, K.S., Leonard, H.L., Blauwendraat, C., Iwaki, H., Johnson, N., Bandres-Ciga, S., Ferrucci, L., Faghri, F., Singleton, A.B., Nalls, M.A., 2023. Virus exposure and neurodegenerative disease risk across national biobanks. Neuron 111 (7), 1086–1093 e2. https://doi.org/10.1016/j.neuron.2022.12.029.

Monogue, B., Chen, Y., Sparks, H., Behbehani, R., Chai, A., Rajic, A.J., Massey, A., Kleinschmidt-Demasters, B.K., Vermeren, M., Kunath, T., Beckham, J.D., 2022. Alpha-synuclein supports type 1 interferon signalling in neurons and brain tissue. Brain 145 (10), 3622–3636. https://doi.org/10.1093/brain/awac192.

Montalbán-Rodríguez, A., Abalo, R., López-Gómez, L., 2024 Jan 20. From the gut to the brain: the role of enteric glial cells and their involvement in the pathogenesis of Parkinson's disease. Int. J. Mol. Sci. 25 (2), 1294. https://doi.org/10.3390/ijms25021294. PMID: 38279293; PMCID: PMC10816228.

Natale, G., Ryskalin, L., Morucci, G., Lazzeri, G., Frati, A., Fornai, F., 2021. The baseline structure of the enteric nervous system and its role in Parkinson's disease. Life 11 (8), 732. https://doi.org/10.3390/life11080732.

Rietdijk, C.D., Perez-Pardo, P., Garssen, J., van Wezel, R.J.A., Kraneveld, A.D., 2017. Exploring Braak's hypothesis of Parkinson's disease. Front. Neurol. 8 https://doi.org/10.3389/fneur.2017.00037.

Scott, K.M., 2022. B lymphocytes in Parkinson's disease. J. Parkinsons Dis. 12 (s1), S75–S81. https://doi.org/10.3233/JPD-223418.

Tulisiak, C.T., Mercado, G., Peelaerts, W., Brundin, L., Brundin, P., 2019. Can Infections Trigger Alpha-Synucleinopathies?, pp. 299–322. https://doi.org/10.1016/bs.pmbts.2019.06.002.

Wang, S., Ye, Q., Zeng, X., Qiao, S., 2019. Functions of macrophages in the maintenance of intestinal homeostasis. J Immunol Res 2019, 1–8. https://doi.org/10.1155/2019/1512969.

Warnecke, T., Schäfer, K.-H., Claus, I., Del Tredici, K., Jost, W.H., 2022. Gastrointestinal involvement in Parkinson's disease: pathophysiology, diagnosis, and management. Npj Parkinsons Dis. 8 (1), 31. https://doi.org/10.1038/s41531-022-00295-x.

Williams, G.P., Schonhoff, A.M., Jurkuvenaite, A., Gallups, N.J., Standaert, D.G., Harms, A.S., 2021 Aug 17. CD4 T cells mediate brain inflammation and neurodegeneration in a mouse model of Parkinson's disease. Brain 144 (7), 2047–2059. https://doi.org/10.1093/brain/awab103. PMID: 33704423; PMCID: PMC8370411.

Wu, Z., Zhang, X., Huang, Z., Ma, K., 2022. SARS-CoV-2 proteins interact with alpha synuclein and induce Lewy body-like pathology in vitro. Int. J. Mol. Sci. 23 (6), 3394. https://doi.org/10.3390/ijms23063394.

Yang, H., Li, S., Le, W., 2022. Intestinal permeability, dysbiosis, inflammation and enteric glia cells: the intestinal etiology of Parkinson's disease. Aging Dis. 13 (5).