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Chronic constipation in Parkinson's disease: clinical features and molecular insights on the intestinal epithelial barrier

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Abstract

Background Chronic constipation (CC) is a severe symptom in Parkinson's disease (PD), with an unclear pathogenesis. Abnormalities of the enteric nervous system (ENS) and/or intestinal epithelial barrier (IEB) may be pathophysiologically relevant in PD patients with CC. We investigated possible molecular changes of the IEB in PD/CCs compared with CCs and controls.

Methods Twelve PD/CCs (2 female, age range 51-80 years), 20 CCs (15 female, age range 27-78 years), and 23 controls (11 female, age range 32-74 years) were enrolled. Ten PD/CCs and 10 CCs were functionally characterized by anorectal manometry (AM) and transit time (TT). Colon biopsies were obtained and assessed for gene and protein expression, and localization of IEB tight junction markers claudin-4 (CLDN4), occludin-1 (OCCL-1), and zonula occludens-1 (ZO-1) by RT-qPCR, immunoblot and immunofluorescence labeling.

Results PD/CCs were clustered in 2 functional categories: patients with delayed TT and altered AM (60%), and a second group showing only modifications in AM pattern (40%). Gene expression of CLDN4, OCCL-1 and ZO-1 was higher in PD/CCs than controls ($P<0.05$). Conversely, PD/CCs showed a trend to decrease ($P>0.05$) in CLDN4 and OCCL-1 protein levels than controls, whereas ZO-1 protein was comparable. In PD/CCs compared with controls, decreasing tendency of vasoactive intestinal polypeptide mRNA, protein and immunoreactive fiber density were observed, although the difference was not statistically significant.

Conclusion Transit and anorectal dysfunctions in PD/CCs are associated with difference in ZO-1, OCCL-1 and CLDN4 expression, thus supporting the role of an altered IEB as a contributory mechanism to possible neuronal abnormalities.

Keywords Parkinson's disease, chronic constipation, anorectal manometry, enteric nervous system, intestinal epithelial barrier

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Introduction

Chronic constipation (CC) in Parkinson's Disease (PD) is a severe condition that impacts significantly the patients' quality of life. With an overall prevalence of up to 80%, PD/CC represents one of the most common manifestations of autonomic dysfunction in PD; it is observed up to 4 times more frequently than in non-PD subjects [1,2]. Moreover, PD/CC is often resistant to laxative treatment and worsens with L-Dopa, the mainstay treatment of motor symptoms in PD patients, as a result of its inhibitory effect on gastrointestinal (GI) motility [3]. Consequently, the therapeutic approach to PD/CC is unsatisfactory for both patients and clinicians.

From a pathophysiological standpoint, PC/CC can be the result of a number of converging mechanisms involving a delayed intestinal (mainly colonic) transit and impaired anorectal function. The slow colonic transit in PD/CC can be due to various factors, including the presence of Lewy Bodies (LB: i.e., aggregates of phosphorylated alpha-synuclein, the molecular hallmark of PD) in neurons of the enteric nervous system (ENS). Various subtypes of enteric neurons can be targeted by PD, especially those controlling contractile motility patterns, e.g., inhibitory VIPergic myenteric neurons [4]. In addition, incoordination of the defecatory muscles (puborectalis and external anal sphincters) and anorectal sphincter leads to outlet obstruction, with excessive straining and a sense of incomplete evacuation. Outlet obstruction affects up to 60% of PD/CCs [5,6]. To date, however, the molecular mechanisms responsible for the onset of PD/CC remain largely unclear. The relationship between PD, ENS and the central nervous system (CNS) has led Braak *et al* to postulate the hypothesis carrying his name (i.e., "Braak's hypothesis") according to which the ENS is the gateway to external *noxae* and the site of origin of phosphorylated alpha-synuclein, which spreads to the CNS via intrinsic and extrinsic circuits [7]. Recently, the ability to obtain mucosal endoscopic biopsies of the colon, including the submucosal ganglionated plexuses, fueled a number of studies looking at the ENS as a "window" to show molecular changes in PD/CC [8,9]. Consistent with Braak's hypothesis, there would be a continuum from the ENS to the CNS that might cause the abnormalities associated with PD/CC. However, the mechanisms explaining how *noxae* can enter the human body through the gut remain unknown. One possibility is that alterations in the integrity of the intestinal epithelial barrier (IEB) may result in a cascade of events, involving inflammatory processes that are capable of causing ENS abnormalities in PD/CC [10].

Recent studies have addressed the role played by the IEB and related dysfunction in a broad spectrum of diseases, ranging from diabetes mellitus to inflammatory and functional disorders [11]. At the mucosal level, tight junctions (TJs) are the first structural units involved in the regulation of IEB. These structures consist mainly of membrane proteins (occludins [OCCLs], claudins [CLDNs]), junctional complex proteins (zonula occludens [ZO]-1, -2, and -3), and cytoskeleton proteins [12]. Notably, early studies showed that, among the various subclasses of enteric submucosal neurons, secretomotor-neurons exert immunomodulatory and regulatory effects on IEB permeability through the release of vasoactive intestinal polypeptide (VIP) [13,14]. A defective VIP-containing system in the submucosal neurons, as we showed in a previous study [9], would affect IEB, thereby increasing the exposure of potentially harmful molecules to the ENS. The combination of IEB and ENS abnormalities can be viewed as an integrated pathogenetic mechanism underlying the pathophysiology of PD/CC.

The present study aimed to assess whether an altered IEB and concomitant alterations of VIP-containing submucosal neurons may contribute to PD/CC symptoms. To this end, a translational approach was applied, by analyzing colonic mucosal biopsies routinely obtained during colonoscopy in relationship to motility functional studies. The characterization of IEB was assessed by measuring the expression of key proteins of TJs, such as OCCL-1, ZO-1, and CLDN4 in the intestinal epithelium of PD/CC patients, compared to non-Parkinsonian CC patients and asymptomatic controls (Ctrls). The expression of VIP mRNA and protein was also evaluated in the same groups of patients.

Patients and methods

Study cohort recruitment and description

A total of 55 subjects were enrolled for the study at the St. Orsola-Malpighi Hospital, Bologna, Italy: 12 PD/CCs (2 female, age range 51-80 years), 20 CCs (15 female, age range 27-78 years), and 23 Ctrls (11 female, age range 32-74 years). The investigation was carried out in accordance with the Declaration of Helsinki guidelines and with the approval of the Ethic Committee of St. Orsola-Malpighi Hospital (Prot. CCPD Basic 2017 – N: 19/2017/O/Tess). Signed informed consent was acquired from each patient prior to their enrollment in the study.

In PD/CCs, the PD diagnosis was established according to the United Kingdom Parkinson's Disease Society Brain Bank clinical diagnostic criteria [15]. Patients with a score less than 19 in the mini-mental state examination (MMSE) test were excluded from the study, in view of their marked cognitive impairment [16]. Demographic and clinical data collected for PD patients comprised date of diagnosis, MMSE test score, disease staging and severity, assessed by the Unified Parkinson's disease rating scale (UPDRS) [17], and the daily dosage of L-Dopa. In CC and PD/CC patients, CC was diagnosed according to the Rome IV criteria [18,19]. Ctrls were healthy subjects, asymptomatic for PD, CC and GI disorders, who underwent colonoscopy for colorectal cancer screening.

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Clinical assessment of GI functions

Anorectal manometry (AM) and colonic transit time (TT) were assessed in CCs and PD/CCs using standardized procedures. In brief, for TT evaluation, patients swallowed a capsule containing 24 radiopaque markers (Sitzmark-Konsyl Pharmaceutical Inc.). Abdominal radiographs from patients in the erect posture were acquired 5 days after markers intake. During the test period, patients were instructed to maintain their habitual diet and to avoid laxatives, enema and suppositories [19]. According to manufacturer's indications, the TT was described as "normal" if at least 19 markers (80%) were expelled at day 5.

The AM was performed using the stationed pull-through technique, with a 4-channel water-perfused catheter (Mui Scientific) linked to an electronic manometer (Sandhill Scientific, Inc.). The catheter was inserted via the anal canal and positioned in the rectum, with the patient in a left-lateral decubitus position. Functional parameters included the resting and squeeze pressures, the ability of the anal sphincter to relax with straining, the recto-anal inhibitory reflex (RAIR) and the rectal sensation [20].

Colonic mucosal biopsy specimens

The colonoscopy was recommended by standard of care for all enrolled subjects. Eight biopsies were collected from the descending colon during endoscopy and processed as previously described [9]. Specimens included both mucosa and submucosal layers of the colon. Biopsies for protein immunoblotting analysis were immediately snap-frozen in liquid nitrogen and stored at -80°C. Other specimens were subsequently processed for immunofluorescence labeling, according to standard protocols [9]. Biopsies for gene expression were preserved at -80°C in RNAlater (Sigma-Aldrich) until the real-time quantitative polymerase chain reaction (RT-qPCR) analysis.

Gene expression

One of the biopsies for each patient was thawed, mechanically disrupted and processed using QIAshredder (Qiagen), according to the manufacturer's instructions. Total RNA was extracted using the RNeasy Mini kit (Qiagen) and genomic DNA-purified via DNase enzyme (Thermo Scientific). Purified RNA was quantified and quality was checked by NanoDrop 2000 spectrophotometer (Thermo Scientific), then 200 ng of RNA was reverse transcribed by TaqMan® Reverse Transcription Reagents (Life Technologies) according to the manufacturer's protocol. qPCR for relative expression assessment for each target gene was carried out using Duplex TaqMan® Gene Expression Assays (Life Technologies) in TaqMan® Fast Advanced master mix (Life Technologies) on an Applied Biosystem 7500 Fast real-time PCR system (Life Technologies). Applied Biosystem qPCR probes were: human_18S_#4448491 Hs99999901_s1 2900rxs VIC, human Claudin 4 #4351370 Hs_00976831_s1 FAM, human VIP #4331182 Hs00175021_m1 FAM, human ZO-1 #4331182 Hs01551861_m1 FAM, human OCLN #4331182 Hs05465837_g1 250 rxs FAM. All samples were assessed in duplicate and each gene expression assay was conducted in triplicate. The $2^{-\Delta\Delta CT}$ method was applied for data analysis, using 18s gene 6 as a reference. The mean of the Ctrls for each gene was set as calibrator at the unit value.

Protein expression

Frozen biopsies were thawed, resuspended in lysis buffer, composed by tissue protein extraction reagent (T-PER, Thermo Scientific™) to which protease and phosphatase inhibitors were added, and mechanically disrupted for lysis. Lysate resuspensions were centrifuged and total protein concentration quantified by Nanodrop™ (Thermo Scientific). Subsequently, all lysates were diluted in the lysis buffer at the same total protein concentration, mixed with the standard 6x Laemmli loading buffer pH 6.8 and the mixtures boiled 10 min to denature and reduce proteins. From each sample, an equal mass of protein (25 μg) was loaded on acrylamide electrophoresis gels (GOPAGE™, TGN Precast 16 Gel 4-15%, SMOBIO) and separated by SDS-PAGE. Separated samples were blotted onto nitrocellulose membranes (GE Healthcare) by wet transfer method (400mA constant for 1:45 h, at low temperature condition).

Subsequently, the membranes were blocked in 3% non-fat dry milk (Blotting-Grade Blocker, Biorad) in Tris-buffered saline 0.1% Tween 20 solution (TBST) and incubated at 4°C overnight with primary antibodies: rabbit anti-VIP (ab227850, Abcam), mouse anti-CLDN4 (clone 3E2C1, ref. 329400, Thermo Fisher Scientific), rabbit anti-vinculin (ab129002, Abcam). Membranes were washed in TBST and incubated with peroxidase-conjugated secondary antibody (anti-mouse or anti-rabbit, Sigma-Aldrich) for 1:30 h at room temperature (RT). Chemiluminescence signals were developed incubating membranes with ECL blotting substrate (Pierce TM ecl Western, Thermo Fisher Scientific) and acquired with ChemiDoc™ XRS+ (Biorad). Quantification of bands was performed with Image Lab Software (Biorad). Vinculin was used as internal loading control for normalization.

Morphological evaluation

Biopsies for morphological analysis of IEB proteins ZO-1 and OCCL-1 were placed in a sylgard-coated Petri dish, in HBSS (Sigma-Aldrich) at 4°C, oriented (mucosa faced up) under the stereomicroscope (Leica S6E, Leica Microsystems) and fixed in 4% paraformaldehyde (Sigma-Aldrich) for 1 h. After washing in phosphate-buffered saline (PBS, pH 7.2, Sigma-Aldrich), longitudinal cryosections were obtained for immunofluorescence labelling.

Tissues were stored in PBS containing 30% sucrose and 0.1% sodium azide (pH 7.4) at 4°C and subsequently in a mixture of PBS-sucrose-azide and OCT (Tissue Tek®, Sakura Finetek) overnight and then incorporated into 100% OCT. Tissues were frozen in dimethylbutane cooled in liquid nitrogen and mounted in Tissue Tek® Mounting Medium. Blocks were sectioned with cryostat to obtain longitudinal and transversal sections of 14-16 μm placed on glass slides. Each biopsy section was incubated for 3 h at RT in a solution containing 2% Triton X-100 and 20% donkey serum (Colorado Serum Co.) in PBS, to minimize non-specific bindings of antibodies. Tissues were subsequently incubated with the primary antibodies: rabbit anti-ZO-1 (ab216880, Abcam) or rabbit anti-OCCL (ab168986, Abcam). The specimens were washed and incubated with fluorochrome-conjugated secondary antibody donkey anti-rabbit Alexa Fluor® 488 (ab150073, Abcam). Prior to analysis, sections were mounted with Fluoromount-G™ Mounting Medium (Invitrogen) and a cover glass. Imaging was performed with a fluorescence microscope with specific filters and a camera to acquire high-definition micrographs. Morphological evaluation of IEB proteins was performed blindly by 2 experienced investigators, by evaluating images of 3 random fields acquired at high magnification and at the same exposure time. In all samples, for each marker evaluated, a score indicating the degrees of "structural normality" was attributed as follows: normal=4; slightly modified=3; modified=2; greatly reduced=1; no expression=0. Qualitative immunohistochemical assessment of VIP on submucosal whole mounts was performed as previously described [9].

Statistical analysis

Statistical analysis and graphs were obtained using GraphPad Prism Software (version 8 for Windows). A Kolmogorov-Smirnov non-parametric test was performed, and data were expressed as the mean ± standard deviation (SD). One-way ANOVA and Tukey's multiple comparison test were applied to compare group means and identify significant differences. Statistical significance was set at $P<0.05$.

Results

TT and AM

Ten PD/CC and 10 CC underwent TT and AM (Table 1). Among PD/CCs, 60% (6/10) showed a delayed colonic TT, as all of them displayed at least 1 or more anomalies at AM, such as augmented basal anal sphincter pressure (RP, 6/10), impaired anal contractility (i.e., squeezing pressure, SQ, 2/10).

During straining attempts, paradoxical sphincter contraction was detected in 4/10 PD/CC. Moreover, 2 PD/CC showed impaired rectal sensitivity with decreased ampullary threshold (Table 1). The RAIR was visible in all PD/CC.

Based on TT and AM findings, 2 subgroups of PD/CC patients were identified: the first characterized by delayed TT and altered AM (60%), the second exclusively showing changes in AM pattern (40%) (Fig. 1A).

CC were grouped into 3 clusters: 1) delayed TT and abnormal AM (40%); 2) delayed TT only (30%); and 3) altered AM pattern only (20%) (Fig. 1B). One patient showed delayed TT, but AM was not performed. Collectively, 80% of CC (8/10) showed a delayed TT, while 67% (6/9) were found to have 1 or more AM abnormalities: increased (3/6) or decreased RP (1/6), defective SQ (5/6), paradoxical sphincter contraction (SP, 3/6) and rectal sensory dysfunction (5/6). The mean number of intracolonic residual pellets was comparable in PD/CC and CC (6.8±7.1 vs. 13.3±7.2, $P=0.06$).

VIP expression

VIP mRNA levels were significantly higher in CC than in Ctrls (3.98±3.93 vs. 1.52±1.55, $P=0.04$) and PD/CC (0.86±1.056), whereas a trend towards lower values was observed in PD/CC compared to Ctrls ($P=0.8$) (Fig. 2A).

The lower VIP levels in PD/CC than in Ctrls were confirmed at the protein level by immunoblot, showing a difference of 15% (0.86±0.43 vs. 1.01±0.56, $P=0.7$) (Fig. 2B,C). In addition, submucosal plexus specimens from PD/CC showed fewer VIP-immunoreactive fibers compared to Ctrls (Fig. 2D), while immunoblot in CCs showed 24% lower VIP protein levels (Fig. 2B,C) compared to Ctrls (0.77±0.38 vs. 1.01±0.56, $P=0.3$).

Evaluation of the IEB

Transcript levels and protein expression of the TJs components of the IEB were assessed in PD/CC, CC and Ctrls. CLDN4 mRNA levels were significantly higher in PD/CC (1.26±0.26) than in Ctrls (1.00±0.19, $P=0.03$) and CC (0.95±0.33, $P=0.02$) (Fig. 3A).

Immunoblot showed a trend towards lower values of CLDN4 protein in PD/CC (0.88±0.33) than in Ctrls (0.96±0.25, $P=0.9$) and CC (1.02±0.51, $P=0.7$) (Fig. 3B,C). Moreover, the OCCL-1 transcript was significantly higher in PD/CC (1.36±0.36) compared with Ctrls (1.05±0.33, $P=0.03$) and CC (0.81±0.18, $P<0.001$) (Fig. 3D). In all groups, ZO-1 mRNA relative levels were consistent with those of OCCL-1, showing significantly lower values in PD/CC (1.35±0.38) compared to Ctrls (1.02±0.27, $P=0.03$) and CC (0.85±0.24, $P<0.001$) (Fig. 3F). A trend towards lower levels of both OCCL-1 and ZO-1 was observed in CC compared to Ctrls (OCCL-1, $P=0.07$; ZO-1, $P=0.1$ Fig. 3D,F).

Immunofluorescence of the colonic specimens revealed lower OCCL-1 immunoreactivity (IR) in PD/CC than in CC and Ctrls, as indicated by a lower structural normality (score 1.5 vs. 4) (Fig. 3E).

Conversely, ZO-1-IR displayed a normal morphology pattern (score=4) in the mucosal lining of both Ctrls and

Table 1 Colonic transit time evaluation and anorectal manometric features of PD/CC and CC patients

PD/CC							
Patient	Retained markers at day 5	TT	RP	SQ	SP	S	AM
P1	0	Normal	Increased	Normal	Paradoxical increase	Normal	Altered
P2	20	Delayed	Normal	Defective	Normal	Hypersensitive	Altered
P3	0	Normal	Increased	Normal	Normal	Normal	Altered
P4	8	Delayed	Normal	Normal	Paradoxical increase	Normal	Altered
P5	12	Delayed	Increased	Normal	Normal	Normal	Altered
P6	15	Delayed	Increased	Normal	Normal	Normal	Altered
P7	6	Delayed	Normal	Defective	Normal	Normal	Altered
P8	8	Delayed	Increased	Normal	Normal	Normal	Altered
P9	0	Normal	Normal	Normal	Paradoxical increase	Normal	Altered
P10	0	Normal	Increased	Normal	Paradoxical increase	Hypersensitive	Altered
CC							
Patient	Retained markers at day 5	TT	RP	SQ	SP	S	AM
P1	19	Delayed	Normal	Defective	Normal	Hypersensitive	Altered
P2	10	Delayed	Normal	Normal	Normal	Normal	Normal
P3	20	Delayed	Increased	Normal	Paradoxical increase	Normal	Altered
P4	20	Delayed	Normal	Defective	Normal	Hypersensitive	Altered
P5	19	Delayed	Increased	Normal	Normal	Normal	Normal
P6	19	Delayed	-	-	-	-	-
P7	5	Normal	Decreased	Defective	Normal	Hypersensitive	Altered
P8	9	Delayed	Normal	Defective	Paradoxical increase	Hypersensitive	Altered
P9	0	Normal	Increased	Normal	Paradoxical increase	Normal	Altered
P10	12	Delayed	Normal	Defective	Normal	Hypersensitive	Normal

PD/CC, Parkinson's disease patients with chronic constipation; TT, colonic transit time, AM, anorectal manometry; RP, resting pressure; SQ, squeezing pressure; SP, strain pattern; S, sensitivity

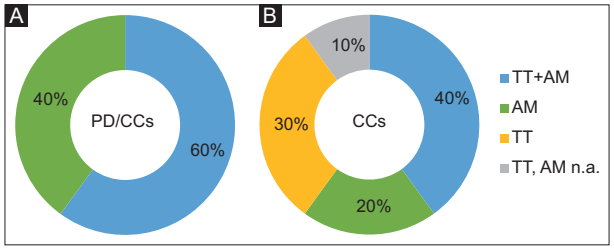

Figure 1 Subgroups of PD/CC (n=10) and CC (n=10) patients divided on the basis of functional findings with TT and AM tests. (A) Percentages of PD/CCs with both delayed TT and altered AM (blue) or altered AM only (green). None of the patients had only delayed TT (yellow). (B) Percentages of CC (n=10) patients showing delayed TT and altered AM (40%, blue), delayed TT only (30%, yellow), altered AM only (20%, green), delayed TT and missing AM test (10%, grey).
PD/CC, Parkinson's disease patients with chronic constipation; AM, anorectal manometry; TT, colonic transit time; n.a., not available

PD/CC, whereas it was markedly modified (score=2) in CC (Fig. 3G).

Discussion

The present study was conducted to better understand the mechanisms underlying the development of PD/CC, focusing on the role of an impaired IEB responsible for the passage of harmful substances through the mucosa, probably affecting the submucosal ganglia of the ENS.

The age and sex of the enrolled populations were consistent with the epidemiological data regarding CC and PD. Specifically, in our study the CC group was predominantly (75%) composed of female patients, with a female-to-male ratio of approximately 3:1 [21].

Conversely, PD typically affects elderly males (60-70% of male preponderance above 65 years) [22]. In line with previous data from our laboratory, the 60% of PD/CC patients had delayed TT and altered AM [9], whereas 40% showed only an altered AM pattern. CLDN4, OCCL-1 and ZO-1 levels were higher in PD/CC than in Ctrls. Conversely, PD/CC showed lower CLDN4 and OCCL-1 protein levels

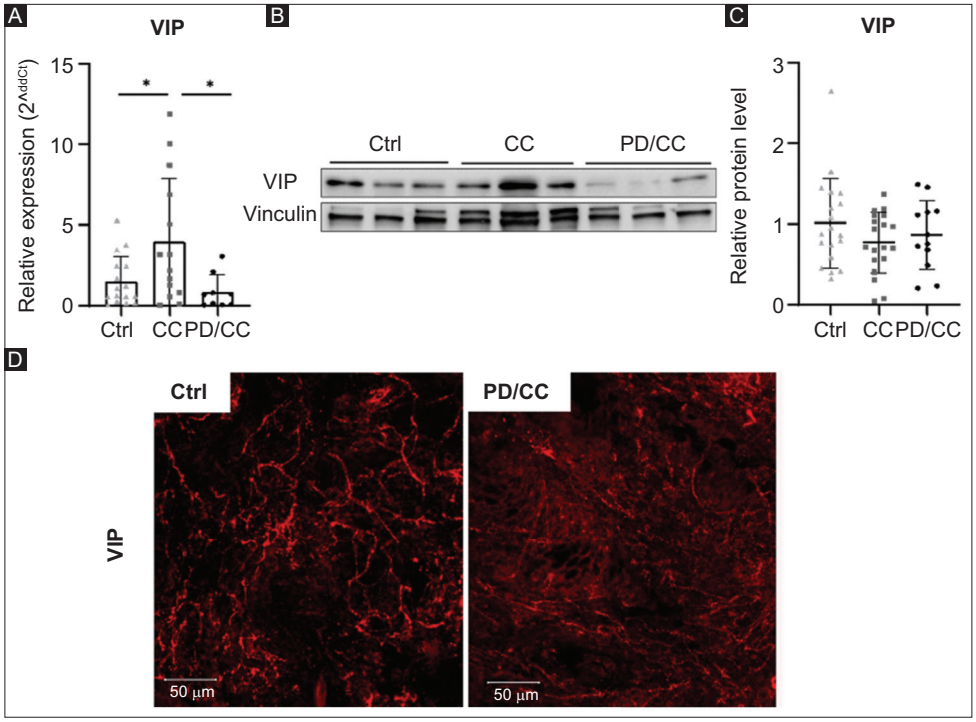

Figure 2 VIP transcript levels and protein expression in PD/CCs, CC patients and controls (Ctrls). (A) VIP mRNA levels were significantly higher in CCs vs. Ctrls and PD/CCs (*P<0.05), whereas lower levels (P>0.05) were observed in PD/CCs. The relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ methods, using 18s as reference gene. Data were expressed as mean ± SD and compared to the mean expression of VIP in the Ctrl group. (B) Representative immunoblot of the VIP protein and vinculin (reference). (C) Relative protein levels of VIP. Lower levels were observed in CCs (-24%) and PD/CCs (-15%) vs. Ctrls (P>0.05). (D) Representative micrographs of the immunofluorescence labelling showing VIP-containing fibers in the submucosa layer from colonic biopsies. Compared to Ctrls, PD/CC patients showed fewer VIP-immunoreactive fibers
VIP, vasoactive intestinal polypeptide; PD/CC, Parkinson's disease patients with chronic constipation

compared with Ctrls, whereas ZO-1 was comparable in the 2 groups. Thus, transit and anorectal dysfunction and ZO-1, OCCL-1 and CLDN4 abnormalities were observed in our patients, thus supporting the role of an altered IEB as a contributory mechanism to neuronal abnormalities in PD/CC patients. In addition to motor disorders, PD is characterized by dysautonomic manifestations affecting several organs, including the GI tract, which is innervated by the ENS and by extrinsic components such as parasympathetic and sympathetic nerves [23]. GI manifestations in PD patients include dysphagia, gastroparesis (which strongly impacts on the absorption of PD treatment) and CC. Up to 50-80% of PD patients suffer from CC, which is often resistant to common laxatives and is associated with severe manifestations, such as megacolon and intestinal pseudo-obstruction [24].

L-Dopa, which is the mainstay therapy for PD patients, contributes to a worsening of CC via its known inhibitory effect on GI motility [3]. CC is also a characteristic condition of the prodromal phase of PD [25]. Nonetheless, PD/CC pathophysiology is still poorly defined, therefore limiting both therapeutic and drug developing strategies. Indeed, for other forms of severe constipation, such as opioid-induced constipation, an understanding of the underlying mechanism of the pathophysiology allowed the development of innovative drugs (i.e., PAMORAs) for treating CC in compromised patients [26,27]. A previous study of ours [9] focused on the evaluation of motor neuroenteric and molecular aspects in a cohort of patients with PD/CC. In that study we demonstrated the downregulation of VIP-submucosal neurons and the reduction of VIP 11 mRNA and VIP-receptors in both PD/CC and, though to a lesser extent, CC, compared to Ctrls. There were no differences in the number of neurons/ganglia in patients with PD/CC compared to CC and Ctrls. VIP reduction in colonic tissue of PD/CC is further supported by the present study, based on biopsies collected from a new PD/CC cohort. The demonstration of altered VIP protein levels is suggestive of an involvement of this polypeptide in the pathogenetic mechanisms of PD/CC. In CC, we detected a discrepancy between mRNA levels and VIP peptide, which may have been due to a possible compensatory mechanism of an ongoing defective peptide system in submucosal neurons. VIP-containing neurons in the submucosal plexus are known to exert secretomotor functions in the intestine, as VIP is a key modulator of intestinal secretion. Therefore, a reduced production of VIP is likely to result in

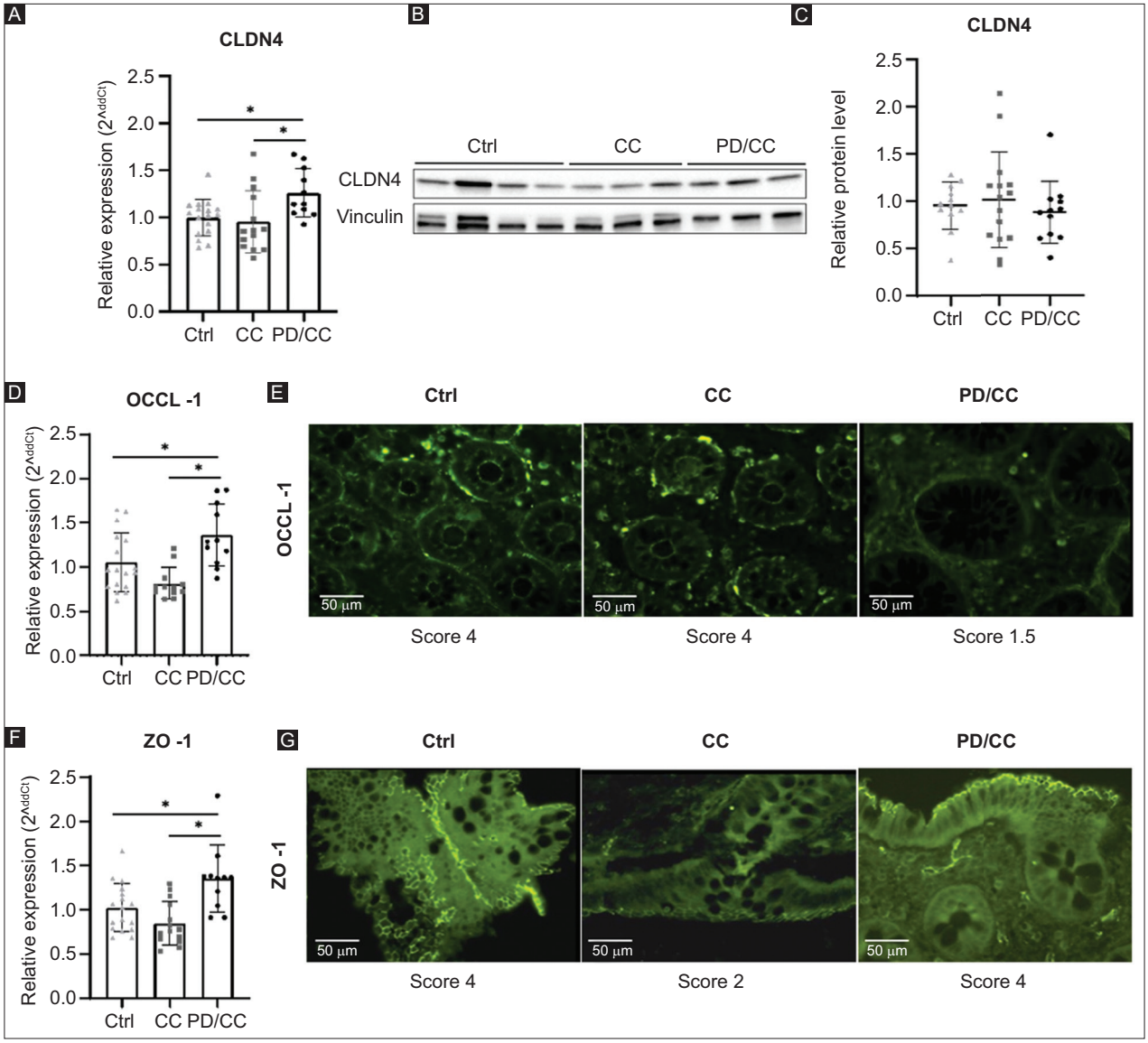

Figure 3 CLDN4, OCCL-1 and ZO-1 transcript levels and protein expression in PD/CCs, CCs and controls (Ctrls). For all targets, the relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method, using 18s as reference gene. Data were expressed as mean ± SD and compared to the mean expression of the gene in the Ctrl group. (A) CLDN4 mRNA levels were significantly higher in PD/CCs vs. Ctrls and CCs (*P<0.05). (B) Representative image of CLDN4 protein immunoblot and vinculin (reference). (C) Relative protein levels of CLDN4 tended to be lower in PD/CCs vs. Ctrls, CCs but there was no statistically significant difference. (D) OCCL-1 transcript was significantly higher in PD/CC vs. Ctrls and CCs (*P<0.05). (E) Representative micrographs of OCCL-1 immunolabeling in the colonic biopsies. In PD/CCs, OCCL-1 immunoreactivity (IR) showed lower structural normality (score=1.5) vs. CCs and Ctrls (score=4). (F) ZO-1 transcript was higher (*P<0.05) in PD/CCs vs. Ctrl and CCs. (G) Representative micrographs of ZO-1 immunolabeling in the colonic biopsies. (G) ZO-1 immunoreactivity (IR) displayed an intense pattern (score=4) in the mucosal lining of Ctrls and PD/CCs; in contrast, ZO-1 IR was markedly lower (score=2) in CCs
PD/CC, Parkinson's disease patients with chronic constipation

less fluid secretion, which could explain the development or aggravation of constipation in PD/CC patients. In fact, secretory abnormalities may affect the composition of fecal water content, thus leading to the formation of hard stools, which are difficult to transport through the intestinal lumen and expel.

In vitro studies by Neunlist *et al* provided the first evidence that the activation of the ENS elicited VIP release, thereby regulating ZO-1 expression in CaCo-2 intestinal epithelial cells [28]. It was suggested that changes to IEB components might also promote symptom generation in disorders of gut–brain interaction. In particular, decreased and increased CLDN4 expression, respectively, were detected in diarrhea- and constipation-predominant irritable bowel syndrome, respectively [11]. Moreover, the downregulation of CLDN4 has been observed in inflammatory bowel disease

patients with leaky-flux diarrhea [29]. Recently, CLDN4 was also described as pore-regulating, rather than barrier-forming, protein [30]. A pilot study by Clairembault *et al* on intestinal permeability applying Ussing chamber method on biopsies from 31 patients with PD showed no significant differences in either para- or transcellular permeability compared to Ctrls [31]. In the same study, OCCL, but not ZO-1, protein expression was found to be significantly lower in colon biopsies from PD compared with those from Ctrls, whereas the cellular distribution of both proteins was altered. Our data showed significantly higher levels of OCCL-1 and ZO-1, as well as CLDN4 transcripts, in PD/CC than in CC and Ctrls, supporting the involvement of IEB abnormalities in PD/CC. Interestingly, in the same specimens, the protein analysis showed lower levels of CLDN4 and OCCL-1 expression compared to CC and Ctrls, while ZO-1 was comparable in both groups. These findings suggest that transcriptional and post-translational changes of IEB markers are likely to be different in CC and PD/CC patients. In line with this possible interpretation, recent studies identified peculiar gut microbiota alterations [32], microRNA signatures [33] and high zonulin levels [34] in PD, all factors known to alter IEB integrity [35-37].

In the IEB assessment, our data showed some differences from those of Clairembault *et al* [31]. Possible explanations pertain to the different cohorts (Clairembault's study was conducted in PD patients without a clear diagnosis of CC), in addition to the type of analysis and techniques used. In contrast, our study enrolled a subgroup of the PD population with an established diagnosis of CC based on the Rome IV criteria, and if possible confirmed by functional tests. Moreover, TT and AM tests in our studies [9] highlighted the presence of distinct subgroups of PD/CC patients, i.e., those with a combination of delayed transit and anorectal dysfunction and those with apparently isolated anorectal abnormalities. As in the study by Giancola *et al* [9], the number of retained markers was not statistically different between PD/CC and CC patients. Using the means of our previous TT findings [9], a *post hoc* power calculation was performed [38], yielding a power (1-β) of 0.63. Collectively, the results of our studies showed a mean number of retained markers consistent with delayed GI transit in both CC and PD/CC.

Our findings support the need for further investigations using larger PD/CC cohorts and related subsets (delayed TT with or without anorectal impairment vs. anorectal dysfunction alone), which should be accompanied by thorough molecular characterization of tissue and circulating markers of IEB, the blood–brain barrier and other neuroglial markers with potential pathogenetic impact on PD/CC. We believe a multicenter, translational strategy is key for a better elucidation, with a view to developing targeted therapeutic interventions effective for PD/CC patients.

Summary Box

What is already known:

- Chronic constipation (CC) occurs in up to 80% of patients with Parkinson's disease (PD), is commonly refractory to laxatives, and is worsened by L-Dopa treatment
- The pathophysiological mechanisms underlying CC in PD (PD/CC) are still poorly understood and their characterization is key to an appropriate therapeutic management of PD/CC
- Transcription of the neuromediator vasoactive intestinal polypeptide (VIP) and the number of submucosal VIP-secretomotor neurons are reduced in the neuroenteric system of PD/CCs vs. healthy controls
- Secretomotor neurons regulate the function of the intestinal epithelial barrier (IEB) via the release of VIP

What the new findings are:

- Gastrointestinal transit time, anorectal manometry abnormalities and molecular changes of the IEB occur in PD/CCs
- Transcription of the IEB tight junction markers claudin-4, occludin-1 and zonula occludens-1 were greater in PD/CCs than in healthy controls
- Occludin-1 immunofluorescence staining showed structural changes in PD/CCs
- Altered IEB markers and neuroenteric (VIP) abnormalities are contributory factors in the complex pathophysiology underlying PD/CC patients

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