Modern Pathology (2007) 20, 367–374

www.modernpathology.org

Colonic neuropathological aspects in patients with intractable constipation due to obstructed defecation

Gabrio Bassotti[1], Vincenzo Villanacci[2], Riccardo Nascimbeni[3], Corrado R Asteria[4], Simona Fisogni[2], Gabriella Nesi[5], Laura Legrenzi[2], Marina Mariano[1], Francesco Tonelli[4], Antonio Morelli[1] and Bruno Salerni[3]

[1]*Gastroenterology & Hepatology Section, Department of Clinical and Experimental Medicine, University of Perugia, Perugia, Italy;* [2]*2nd Pathology Unit, Department of Pathology, University of Brescia, Brescia, Italy;* [3]*2nd Surgical Chair, Department of Surgery, University of Brescia, Brescia, Italy;* [4]*Surgical Unit, Department of Clinical Pathophysiology, University of Firenze, Firenze, Italy and* [5]*Pathology Unit, Department of Human Pathology & Oncology, University of Firenze, Firenze, Italy*

One of the most frequent subtypes of constipation is represented by obstructed defecation, and it has recently been reported that these patients may have colonic motor abnormalities in addition to alterations of the anorectal area. However, it is unknown whether these patients display abnormalities of the enteric nervous system, as reported in other groups of constipated subjects. For this reason, we evaluated the neuropathologic aspects of the enteric nervous system in a homogeneous group of patients with obstructed defecation. Colonic specimens from 11 patients (nine women, age range 39–66 years) undergoing surgery for symptoms refractory to any therapeutic measure, including biofeedback training, were obtained and examined by means of conventional histological methods and immunohistochemistry (NSE, S100, c-Kit, formamide-mAb, Bcl-2, CD34, alfa-actin). Analysis of the specimens showed that the enteric neurons were significantly decreased only in the submucosal plexus of patients ($P<0.0001$ *vs* controls), whereas the enteric glial cells of constipated patients were reduced in both the myenteric ($P=0.018$ *vs* controls) and the submucosal plexus ($P=0.004$ *vs* controls). No difference between patients and controls were found concerning c-Kit and CD34 expression, and the number of apoptotic neurons. These findings support the concept that at least a subgroup of patients with obstructed defecation and severe, intractable symptoms display abnormalities of the enteric nervous system, mostly related to the enteric glial cells. These findings might explain some of the pathophysiological abnormalities, and help to better understand this condition.
Modern Pathology (2007) **20,** 367–374. doi:10.1038/modpathol.3800748; published online 2 February 2007

Keywords: constipation; enteric glial cells; enteric nervous system; outlet obstruction

Obstructed defecation is one of the two main subtypes of chronic constipation (the other being represented by the slow transit variety),[1] and it is estimated that it occurs in about 7% of the adult population.[2] Even though it appears now quite clear that this is a heterogeneous entity,[3,4] traditionally obstructed defecation has been considered as due to rectoanal dysfunction, including failure to relax or paradoxical contraction of the pelvic floor while attempting to defecate,[5–7] lack of rectal motor activity,[8] and abnormal rectal sensitivity.[9–11]

However, there is also evidence that pathophysiological abnormalities in obstructed defecation may be not be confined to the very distal colonic area. Data from radiologic and scintigraphic studies during and after evacuation have shown that the act of defecation can empty a large part of the large bowel,[12,13] and manometric recordings reported that defecation follows a peculiar pattern of propagated high-amplitude colonic contractions[14,15] according to a complex sequence that starts up to 1 h before stools expulsion.[16] Studies carried out by these manometric techniques have then demonstrated that in some patients with obstructed defecation motor abnormalities are also present higher in the colon, in both the basal state[17] and after chemical or

Correspondence: Professor G Bassotti, MD, PhD, Clinica di Gastroenterologia ed Epatologia, Ospedale Santa Maria della Misericordia, Piazza Menghini, 06156 Perugia, Italy.
E-mail: gabassot@tin.it
Received 4 October 2006; revised 30 November 2006; accepted 1 December 2006; published online 2 February 2007

mechanical stimulation.[18] Thus, alterations of the neuroenteric circuitry have been postulated as a pathophysiological ground for these patients[17,18] although to date no study has demonstrated such abnormalities.

Purpose of the present study was to investigate the neuropathological aspects of the colon in a group of patients with obstructed defecation unresponsive to treatment, to see whether such patients may display abnormalities that could justify an altered motor behaviour of the large bowel.

Patients

Eleven patients (nine women, age range 39–66 years) fulfilling the criteria for obstructed defecation and undergoing surgery (colectomy with ileorectostomy) for constipation refractory to any therapeutic intervention (see below) entered the study.

Inclusion criteria were as follows: (1) severe constipation present for more than 12 months and unresponsive to standard medical treatment, including dietary fiber, laxatives (including polyethylene glycol), enemas and suppositories; (2) fulfilment of Rome II criteria for constipation,[19] that is two or more of six symptoms present for at least 12 weeks of the preceding 12 months: straining, lumpy or hard stools, sensation of incomplete evacuation, sensation of anorectal obstruction/blockage, or manual manoeuvres to facilitate defecation on more than one fourth of bowel movements, or less that three evacuations per week; (3) paradoxical contraction or failure to relax pelvic floor muscles during attempts to defecate, as shown by anorectal manometry and defecography; (4) inability to defecate a 50-ml, water-filled balloon within 5 min on repeated attempts; (5) failure to respond to biofeedback treatment, which has been shown as effective in a high percentage of cases.[7,20] It is worth noting that according to these criteria the patients also met the recently published Rome III criteria for functional defecation disorder.[21]

Exclusion criteria were as follows: (1) slow transit constipation, defined by evacuation of less than 80% of radiopaque markers 5 days after ingestion; however, patients with transit delay (two, with 85 and 88% of markers still present within the viscus) were included if the markers were concentrated in the rectosigmoid area; (2) barium enema consistent with abnormally dilated rectum or colon; (3) colonoscopy showing structural abnormalities of the large bowel; (4) abnormal biochemistry or thyroid function tests.

Controls

Ten patients (nine women, one man, age range 43–75 years) undergoing left hemicolectomy for non-obstructing colorectal cancer were used as controls, since there is evidence that the distribution of interstitial cells of Cajal is relatively uniform throughout the human colon.[22] No data are available on the regional density of the enteric neurons and glial cells in man, although in preliminary observations we did not detect significant regional differences between the various colonic segments, except in the rectum (G Bassotti and V Villanacci, personal observations). The control specimens were taken at least 5 cm from the resection margin in tumour free areas.

Ethical considerations

After explanation about the aims of the study, informed consent was obtained from both patients and controls, and the investigation was carried out according to local ethical rules, following the recommendations of the Declaration of Helsinki (Edinburgh revision, 2000).

Methods

Tissue samples were processed as described previously.[23–26] After removal, the surgical specimens were immediately fixed in 10% neutral-buffered formalin for 24 h, then 12–20 full-thickness samples from the whole ablated colon were taken and transversal sections obtained. For conventional histology 5 μm paraffin sections were stained with Hematoxylin–Eosin, Pas and Trichrome stain.

Immunohistochemistry

At least 20 slides for each patient were processed for immunohistochemistry. To evaluate markers of the enteric nervous system monoclonal antibodies toward neuron-specific enolase (NSE, NCL-NSE2, dilution 1:50, Novocastra Laboratories, Newcastle upon Tyne, UK) acting as a marker of gangliar cells, and the glial marker protein S100 (S-100, dilution 1:50, Dako, Carpinteria, CA, USA) were used. As interstitial cells of Cajal express Kit,[27] an anti-Kit antibody (rabbit polyclonal antibody, IgG, dilution 1:50, Dako, Carpinteria, CA, USA) was used to detect these cells, as reported previously.[28] Moreover, CD34 staining (CD34 Clone QBEnd/10, dilution 1:30, Neo markers, Union City, CA, USA) was used to evaluate the population of fibroblast-like cells which are intimately associated with the interstitial cells of Cajal.[29] Two methods were used as markers for apoptosis in the enteric nervous system: (a) the expression of Bcl-2 protein (BCL2 Oncoprotein clone 124, dilution 1:10, DBS, Pleasantown, Australia), a proto-oncogene responsible for specific suppression of apoptosis in several important situations,[30] and well displayed in human enteric neurons,[31,32] and (b) the monoclonal antibody to single-stranded DNA, using the formamide monoclonal antibody (formamide-MAb) method

(Mab F7–26 BMS 156, Bender MedSystem, Vienna, Austria), which detects apoptotic cells in tissue processed with routine histological techniques and allows discrimination of apoptosis from necrosis.[33] This method has also been proved to yield the best results to identify enteric neuronal apoptosis when compared to other methods.[34]

NSE, S-100, CD34, and Bcl-2 immunostaining was carried out using a peroxidase-based visualization kit (Dako LSAB®), following the manufacturer's recommendations. Diaminobenzidine tetrahydrochloride was used as chromogen. The slides were then counterstained with Mayer's hematoxylin for 5 s, dehydrated and mounted in Clarion (Biomeda, Foster City, CA, USA). To account for nonspecific staining, peptides that blocked polyclonal antibody bindings (passage with normal goat serum) were used, or sections were incubated in the absence of primary antibody. In these cases, no immunostaining was detected. For Bcl-2, the expression in mucosal lymphoid cells served as internal control.

Expression of Kit

Consecutive formalin-fixed, paraffin sections were dewaxed and rehydrated through decreasing alcohol series up to distilled water. Sections were then subjected to heat-induced epitope retrieval by immersion in a heat-resistant container filled with citrate buffer solution (pH 6.0) placed in a pressure cooker and microwaved for 20 min. Endogenous peroxidase activity was suppressed by incubation with 3% solution of H_2O_2 for 5 min. Kit immunostaining was carried out using a peroxidase-based visualization kit (Dako EnVision™), following the manufacturer's recommendations. Kit-positive mast cells served as internal control.

Anti Single-Stranded DNA Immunohistochemistry

Sections 2–3 μm thick were warmed overnight at 60°C, then dewaxed and rehydrated through decreasing alcohol series up to distilled water. Thereafter, the sections were incubated for 5 min in PBS with the addition of 20% Tween 20, followed by a passage with proteinase K (Dako) for 20 min. The sections were then rinsed with distilled water and heated in 50% formamide prewarmed to 60°C for 20 min. After cooling, endogenous peroxidase activity was suppressed by incubation with 3% solution of H2O2 for 5 min. Normal serum diluted 1:50 was applied for 10 min to room temperature, followed by anti-DNA MAb for 30 min, according to the manufacturer's recommendations. After that, the sections were incubated at room temperature with secondary polymeric antibody for 20 min and ABC (Kit super sensitive non biotin detection system, Menarini, Firenze, Italy) for 30 min. Finally, a 5 min reaction in the dark with diaminobenzidine (Bio-Optica, Milano, Italy) was carried out, and the sections were then counterstained with Mayer's hematoxylin for 5 s, dehydrated and mounted in Clarion (Biomeda). Positivity was observed under the microscope as an intense brown reaction.

The presence of lymphocytes was assessed by means of a monoclonal mouse anti-human CD 3 antibody (Dako Cytomation, dilution 1:40).

The colonic smooth muscle was evaluated by means of an anti alfa-actin monoclonal antibody (dilution 1:100, Biogenex, CA, USA).

Data analysis

All slides were coded and analysed blind by two pathologists. For NSE, S100, and CD3, as well as for Bcl-2 and formamide-MAb positive cells, both the submucosal and the myenteric plexuses were taken into account by optical microscopy at $\times 20$ magnification (Olympus BX 40). For each patient, the number of immunopositive cells was calculated and expressed as the mean of cells on 10 well stained and well oriented microscopic fields for each region of interest. To be considered as positive, the intensity of cell immunostaining had to be from moderate to strong, as described previously.[35] The density of interstitial cells of Cajal was graded, according to a previously described method,[23–26,36] after the evaluation of 10 well-stained and well-oriented fields at $\times 20$ magnification. The three previously identified populations of interstitial cells of Cajal were taken into consideration:[37,38] IC-SM, along the submucosal surface of the circular muscle bundle, IC-MY, within the intermuscular space between circular and longitudinal muscle layers (myenteric region, which displays the highest yield of interstitial cells of Cajal in normal tissue[22,35,39]), and IC-IM, within the muscle fibers of the circular and longitudinal muscle layers. Not only nucleated cells but also Kit positive labelled elongated structures were considered for analysis.[23–26]

For CD34, the strength of the immunostaining (graded as either present or severely depleted/absent, according to recently reported criteria[40]) was calculated around the myenteric plexus, between the elements of the plexus, within the longitudinal and circular muscle elements. Care was taken not to include vessels in the evaluation; however, the effectiveness of CD34 staining was indicated by the staining of capillaries in subjects with severe depletion/absence in the other locations.[40]

Statistical analysis

Data were analysed by means of nonparametric tests. The Wilcoxon's signed rank test and the χ^2 test were employed, where appropriate. Values of $P<0.05$ were chosen for rejection of the null hypothesis. Data are presented as median (95% CI).

Results

Conventional Histology

In both patients and controls the mucosa, submucosa, smooth muscle and nerve plexus architecture appeared normal at Hematoxylin–Eosin, Trichrome and Pas staining. No inflammatory cells (nor any intranuclear or viral inclusions) were observed in or around muscular or nervous structures.

No patient exhibited hyperplastic changes (eg giant ganglia) of the submucosal plexus, thereby excluding a diagnosis of intestinal neuronal dysplasia.[41]

Immunohistochemistry

Table 1 summarizes the main pathological findings. No differences between groups were found concerning NSE expression in the myenteric plexus, whereas a significant decrease was found in the patients' submucosal plexus (Figure 1). S100 expression was significantly decreased in the patients' group, in both the myenteric (Figure 2c and d) and the submucosal plexus (Figure 2a and b). With respect to interstitial cells of Cajal, no significant differences between patients and controls were detected for the three populations.

No significant differences between patients and controls were also found in the expression of Bcl-2 in the myenteric plexus, whereas this resulted significantly decreased in the submucosal plexus in the patients' group.

The expression of CD34 around the myenteric plexus, between the elements of the plexus, within the longitudinal and circular muscle elements was depleted/absent in three patients and two controls (χ^2 test $P = 0.89$).

The number of apoptotic enteric neurons was not significantly different in patients and controls, in both the myenteric and the submucosal plexus.

No lymphocytic infiltration (assessed by CD3) was observed in either the submucosal or the myenteric plexus of the patients and controls.

All patients and controls showed strong intensity for alfa-actin immunostaining, so that colonic smooth muscle was judged to display normal characteristics.

Table 1 Immunohistochemical findings (number of positive cells) in controls and patients with obstructed defecation

Marker	*Controls*	*Patients*	P
NSE			
Myenteric plexus	62 (52–76)	42 (20–97)	0.29
Submucosal plexus	50 (45–84)	27 (17–38)	<0.0001
S100			
Myenteric plexus	204 (185–271)	178 (172–191)	0.018
Submucosal plexus	115 ((92–182)	84 (54–100)	0.004
Anti-Kit			
IC-SM	28 (22–34)	20 (12–38)	0.23
IC-MY	204 (138–257)	213 (100–260)	0.50
IC-IM	40 (27–46)	44 (20–70)	0.38
Bcl-2			
Myenteric plexus	146 (115–239)	169 (114–201)	0.75
Submucosal plexus	56 (43–71)	40 (31–46)	0.006
Formamide-mAb			
Myenteric plexus	11 (7–14)	10 (11–16)	0.67
Submucosal plexus	12 (10–14)	14 (13–16)	0.12

Data are presented as median (95% CI).

Discussion

This study gives evidence, for the first time, that in some patients with obstructed defecation and intractable symptoms the colonic enteric nervous system may be abnormal. It is interesting to note that these abnormalities are quite different from those reported in patients with severe slow transit constipation, requiring surgery for symptoms' relief.[24] In fact, in patients with obstructed defecation the more striking alterations consisted in a significant loss of the overall population of enteric glial cells, decreased in both the myenteric and the submucosal plexus compared to controls, whereas a reduction of enteric neurons was observed only in the submucosal plexus, and no difference in the interstitial cells of Cajal subpopulations was found between patients and controls.

We found particularly intriguing that the population of enteric glial cells was globally reduced in obstructed defecation patients, together with the decrease of the enteric neurons only in the submucosal plexus and no involvement of the interstitial cells of Cajal. Some pathophysiological meanings can be inferred from these findings, and will be discussed below.

It is worth noting that enteric glial cells represent the most abundant cell population in the enteric nervous system,[42,43] and although they have always traditionally been considered as mere support structures for enteric ganglia, evidence is being accumulated that this cell population may have more active functions in the economy of the enteric neurophysiology.[44] In fact, experimental human and animal studies have shown that enteric glial cells may also be involved in intestinal inflammation,[45,46] enteric neurotransmission[47–49] and, importantly, are essential for the homeostasis of enteric neurons.[50] With respect to this last aspect, a recent study showed that enteric glia disruption may alter the neurochemical coding of enteric neurons in an experimental animal model.[51] All together, these data strongly indicate that enteric glial cells can orchestrate gastrointestinal motor activity interacting with several enteric functions, and especially controlling neurochemical phenotypes in the enteric nervous system.[52]

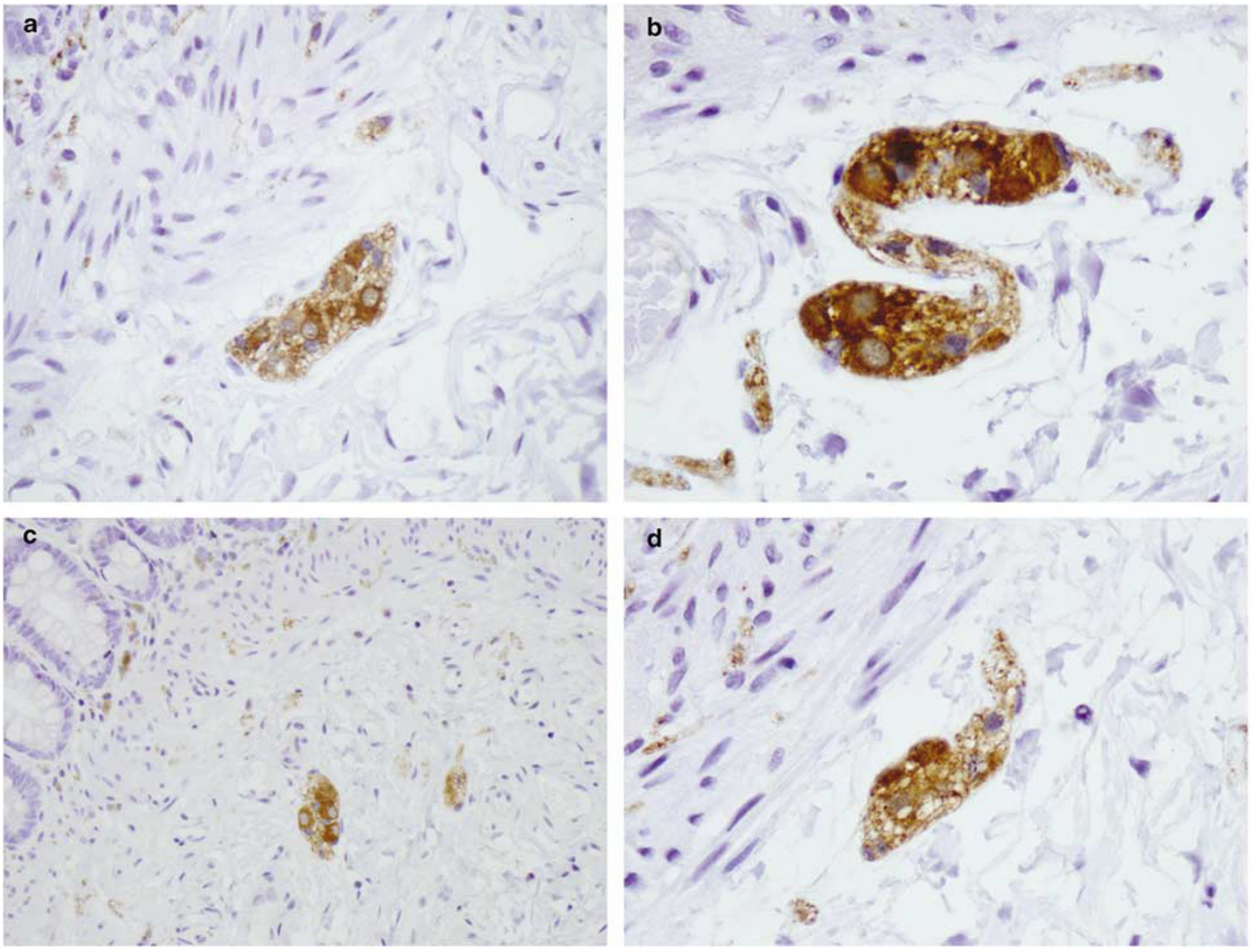

Figure 1 NSE expression in the submucosal plexus of a control (**a**, **b**) and of a OD patients (**c**, **d**): the latter show reduced number of enteric neurons. Original magnifications: ×40 (**a**, **d**), ×100 (**b**), ×20 (**c**).

The enteric neuronal loss (not due to an increase of apoptotic phenomena) in patients with obstructed defecation was limited to the submucosal plexus; since this plexus in humans mainly regulates mucosal functions[53] (although some neurons of the outer submucosal plexus also innervate the circular muscle[54]) it is likely that, together with the integrity of the interstitial cells of Cajal circuitry, the maintenance of the enteric neuronal populations within the myenteric plexus may account for the preservation of a discrete colonic motor activity, more effective than that usually recorded in patients with slow transit constipation.

These findings are interesting, and consistent with the recent hypothesis, based on abnormal colonic manometric findings, that at least one subpopulation of patients with obstructed defecation might result from defective colonic, rather than anorectal, function.[17] Of practical importance, our results could give an explanation for the lack of response to treatments, especially to biofeedback, in these patients. Biofeedback is to one of the most effective forms of treatment for obstructed defecation[7,20] as also shown by controlled studies,[55] and it is thought that it acts by a learned mechanism of relaxation of pelvic floor muscles.[56–58] This would imply that most of the pathophysiological mechanisms are concentrated in the pelvic floor area; however, since patients with obstructed defecation represent an heterogeneous entity, it is likely that in a subgroup the abnormalities are also present higher up in the colon, accounting for refractoriness to medical and behavioural therapeutic measures.

We are at present unable to explain whether these abnormalities are primary or secondary to long-standing constipation or treatments. We favour the first hypothesis, since severe idiopathic constipation is relatively frequent also in children,[59] and there is no firm evidence of treatment damage of the enteric nervous system.[25,60]

Of course, the results of this study are limited to a very selected and homogeneous group of patients with obstructed defecation, and no rectal tissue was available to see whether this area was also involved. Therefore, more investigations are needed in different subgroups of these patients to ascertain whether these abnormalities are confined to particular subjects and to the segments above the anorectal area.

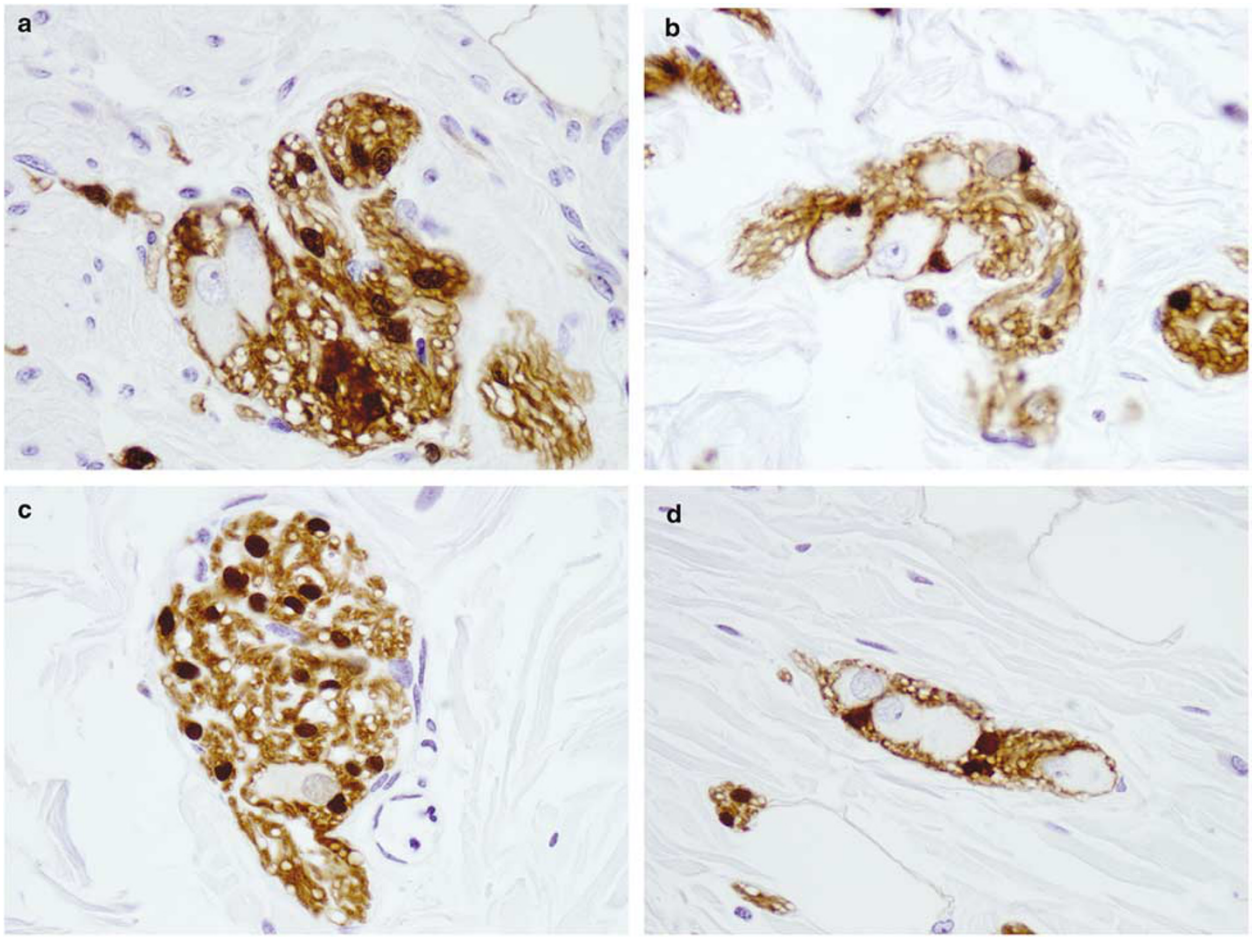

Figure 2 S100 expression in the submucosal plexus in a control (**a**) and a patient with obstructed defecation (**b**), and in the myenteric plexus in a control (**c**) and a patient with OD (**d**). Note the decrease of enteric glial cells in patients. Original magnification, ×100.

Finally, we feel that these findings may have some importance to establish whether alternative treatment approaches (for instance, it could be hypothesized the development of molecular targets for therapeutic interventions apt to restore or replace glial functions and restart appropriate neurochemical phenotypes in order to ultimately restore a proper function of the enteric nervous system[52]) may be pursued in future.

Conflict of interest

These authors have no conflict of interest.

References

1 Lembo A, Camilleri M. Chronic constipation. N Engl J Med 2003;349:1360–1368.
2 D'Hoore A, Penninkx F. Obstructed defecation. Colorectal Dis 2003;5:280–287.
3 Rao SS, Mudipalli RS, Stessman M, *et al.* Investigation of the utility of colorectal function tests and Rome II criteria in dyssynergic defecation (anismus). Neurogastroenterol Motil 2004;16:589–596.
4 Bharucha AE, Fletcher JG, Seide B, *et al.* Phenotypic variation in functional disorders of defecation. Gastroenterology 2005;128:1199–1210.
5 Preston DM, Lennard-Jones JE. Anismus in chronic constipation. Dig Dis Sci 1985;30:413–418.
6 Rao SS, Welcher KD, Leistikow BS. Obstructed defecation: a failure of rectoanal coordination. Am J Gastroenterol 1998;93:1042–1050.
7 Bassotti G, Chistolini F, Sietchiping-Nzepa F, *et al.* Biofeedback for pelvic floor dysfunction in constipation. BMJ 2004;328:393–396.
8 Faucheron JL, Dubreuil A. Rectal akinesia as a new cause of impaired defecation. Dis Colon Rectum 2000;43:1545–1549.
9 Gosselink MJ, Hop WC, Schouten WR. Rectal compliance in females with obstructed defecation. Dis Colon Rectum 2001;44:971–977.
10 Sloots CE, Felt-Bersma RJ. Rectal sensorimotor characteristics in female patients with idiopathic constipation with or without paradoxical sphincter contraction. Neurogastroenterol Motil 2003;15:187–193.
11 Karlbom U, Lundin E, Graf W, *et al.* Anorectal physiology in relation to clinical subgroups of patients

with severe constipation. Colorectal Dis 2004;6: 343–349.
12 Halls J. Bowel content shift during normal defaecation. Proc R Soc Med 19865;58:859–860.
13 Lubowski DZ, Meagher AP, Smart RC, *et al.* Scintigraphic assessment of colonic function during defaecation. Int J Colorectal Dis 1995;10:91–93.
14 Bassotti G, Gaburri M. Manometric investigation of high-amplitude propagated contractile activity of the human colon. Am J Physiol 1988;255:G660–G664.
15 Bassotti G, Iantorno G, Fiorella S, *et al.* Colonic motility in man: features in normal subjects and in patients with chronic idiopathic constipation. Am J Gastroenterol 1999;94:1760–1770.
16 Bampton PA, Dinning PG, Kennedy ML, *et al.* Spatial and temporal organization of pressare patterns throughout the unprepared colon during spontaneous defecation. Am J Gastroenterol 2000;95: 1027–1035.
17 Dinning PD, Bampton PA, Andre J, *et al.* Abnormal predefecatory colonic motor patterns define constipation in obstructed defecation. Gastroenterology 2004; 127:49–56.
18 Dinning PD, Bampton PA, Kennedy ML, *et al.* Impaired proximal colonic motor response to rectal mechanical and chemical stimulation in obstructed defecation. Dis Colon Rectum 2005;48:1777–1784.
19 Thompson WG, Longstreth GF, Drossman DA, *et al.* Functional bowel disorders and functional abdominal pain. Gut 1999;45:43–47.
20 Heymen S, Jones KR, Scarlett Y, *et al.* Biofeedback treatment of constipation: a critical review. Dis Colon Rectum 2003;46:1208–1217.
21 Bharucha AE, Wald A, Enck P, *et al.* Functional anorectal disorders. Gastroenterology 2006;130: 1510–1518.
22 Lyford GL, He CL, Soffer E, *et al.* Pan-colonic decrease in interstitial cells of Cajal in patients with slow transit constipation. Gut 2002;51:496–501.
23 Bassotti G, Battaglia E, Bellone G, *et al.* Interstitial cells of Cajal, enteric nerves, and glial cells in colonic diverticular disease. J Clin Pathol 2005;58:973–977.
24 Bassotti G, Villanacci V, Maurer CA, *et al.* The role of glial cells and apoptosis of enteric neurons in the neuropathology of intractable slow transit constipation. Gut 2006;55:41–46.
25 Villanacci V, Bassotti G, Cathomas G, *et al.* Is pseudomelanosis coli a marker of colonic neuropathy in severely constipated patients? Histopathology 2006; 49:132–137.
26 Bassotti G, Villanacci V, Fisogni S, *et al.* Apoptotic phenomena are not a major cause of enteric neuronal loss in constipated patients with dementia. Neuropathology 2006 (in press).
27 Williams DE, Eisenman J, Baird A, *et al.* Identification of a ligand for the *c-kit* proto-oncogene. Cell 1990; 63:167–174.
28 Horisawa M, Watanabe Y, Torihashi S. Distribution of c-Kit immunopositive cells in normal human colon and in Hirschsprung's disease. J Pediatr Surg 1998; 33:1209–1214.
29 Vanderwinden JM, Liu H, De Laet MH, *et al.* CD34+ cells in human intestine are fibroblasts adjacent to, but distinct from interstitial cells of Cajal. Lab Invest 1999; 79:59–65.
30 Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science 1998;281:1322–1326.
31 Wester T, Olsson Y, Olsen L. Expression of bcl-2 in enteric neurons in normal human bowel and in Hirschsprung disease. Arch Pathol Lab Med 1999;123: 1264–1268.
32 Park SH, Min H, Chi JG, *et al.* Immunohistochemical studies of pediatric intestinal pseudo-obstruction: bcl2, a valuable biomarker to detect immature enteric ganglion cells. Am J Surg Pathol 2005;29:1017–1024.
33 Frankfurt OS, Krishan A. Identification of apoptotic cells by formamide-induced DNA denaturation in condensed chromatin. J Histochem Cytochem 2001; 49:369–378.
34 Bassotti G, Villanacci V, Fisogni S, *et al.* Comparison of three methods to assess enteric neuronal apoptosis in patients with slow transit constipation. Apoptosis (in press).
35 Maurer CA, Friess H, Buhler SS, *et al.* Apoptosis inhibiting factor Bcl-XL might be the crucial member of the Bcl-2 gene family in colorectal cancer. Dig Dis Sci 1988;43:2641–2648.
36 Hagger R, Gharaie S, Finlayson C, *et al.* Regional and transmural density of interstitial cells of Cajal in human colon and rectum. Am J Physiol 1998;38: G1309–G1316.
37 Sanders KM. A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. Gastroenterology 1996;111: 492–515.
38 Ward SM, Sanders KM, Hirst GD. Role of interstitial cells of Cajal in neural control of gastrointestinal smooth muscles. Neurogastroenterol Motil 2004;16: 112–117.
39 Tong WD, Liu BH, Zhang LY, *et al.* Decreased interstitial cells of Cajal in the sigmoid colon of patients with slow transit constipation. Int J Colorect Dis 2004;19:467–473.
40 Streutker CJ, Huizinga JD, Campbell F, *et al.* Loss of CD117 (c-kit)- and CD34-positive ICC and associated CD34-positive fibroblasts defines a subpopulation of chronic intestinal pseudo-obstruction. Am J Surg Pathol 2003;27:228–235.
41 Stoss F, Meier-Ruge W. Experience with neuronal intestinal dysplasia (NID) in adults. Eur J Pediatr Surg 1994;4:298–302.
42 Gabella G. Ultrastructure of the nerve plexuses of the mammalian intestine: the enteric glial cells. Neuroscience 1981;6:425–436.
43 Gershon MD, Rothman TP. Enteric glia. Glia 1991; 4:195–204.
44 Rühl A. Glial cells in the gut. Neurogastroenterol Motil 2005;17:777–790.
45 Hirata I, Berrebi G, Austin LL, *et al.* Immunohistochemical characterization of intraepithelial and lamina propria lymphocytes in control ileum and colon in inflammatory bowel disease. Dig Dis Sci 1986;31: 593–603.
46 Cabarrocas J, Savidge TC, Liblau RS. Role of enteric glial cells in inflammatory bowel disease. Glia 2003; 41:81–93.
47 Nagahama M, Semba R, Tsuzuki M, *et al.* L-arginine immunoreactive enteric glial cells in the enteric nervous system of rat ileum. Biol Signals Recept 2001; 10:336–340.
48 Giaroni C, Zanetti E, Chiaravalli AM, *et al.* Evidence for a glutamatergic modulation of the cholinergic function in the human enteric nervous system via NMDA receptors. Eur J Pharmacol 2003;476:63–69.

49 Zhang W, Segura BJ, Lin T, *et al.* Intercellular calcium waves in cultured enteric glia from neonatal guinea pig. Glia 2003;42:252–262.
50 Bush TG. Enteric glial cells. An upstream target for induction of necrotizing enterocolitis and Crohn's disease? Bioessays 2002;24:130–140.
51 Aube AC, Cabarrocas J, Bauer J, *et al.* Changes in enteric neurone phenotype and intestinal functions in a transgenic mouse model of enteric glia disruption. Gut 2006;55:630–637.
52 Rühl A. Glial regulation of neuronal plasticity in the gut: implications for clinicians. Gut 2006;55:600–602.
53 Schemann M, Neunlist M. The human enteric nervous system. Neurogastroenterol Motil 2004;16:55–59.
54 Domoto T, Bishop AE, Oki M, *et al.* An *in vitro* study of the projections of enteric vasoactive intestinal polypeptide-immunoreactive neurons in the human colon. Gastroenterology 1990;98:819–827.
55 Chiarioni G, Whitehead WE, Pezza V, *et al.* Biofeedback is superior to laxatives for normal transit constipation due to pelvic floor dyssynergia. Gastroenterology 2006;130:657–664.
56 Bassotti G, Whitehead WE. Biofeedback, relaxation training, and cognitive behaviour modification as treatments for lower functional gastrointestinal disorders. Q J Med 1997;90:545–550.
57 Chiotakakou-Faliakou E, Kamm MA, Roy AJ, *et al.* Biofeedback provides long-term benefit for patients with intractable, slow and normal transit constipation. Gut 1998;42:517–521.
58 Whitehead WE, Heymen S, Schuster MM. Motility as a therapeutic modality: biofeedback treatment of gastrointestinal disorders. In: Schuster MM, Crowell MD, Koch KL (eds). Schuster Atlas of Gastrointestinal Motility, 2nd edn. BC Decker Inc.: Hamilton, 2002, pp 381–397.
59 Rubin G, Dale A. Chronic constipation in children. BMJ 2006;333:1051–1055.
60 Muller-Lissner S. What has happened to the cathartic colon? Gut 1996;39:486–488.