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Nerve growth factor content is increased in the rectal mucosa of children with diarrhea-predominant irritable bowel syndrome

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Abstract

Background *The enteric nervous system is a complex network that includes, in the digestive mucosa, neuronal bodies and fibers interacting with the immune system and mucosal mast cells (MC). These interactions involve the secretion of messengers, such as the neurotrophin nerve growth factor (NGF), which influence colonic motility and sensitivity, both affected in irritable bowel syndrome (IBS). This study was designed to test the hypothesis that, in children with IBS, colonic mucosal innervation, NGF content, and MC infiltration are altered. We aimed to measure MC infiltration, number of neuronal bodies, distance from MC to nerve fibers, inflammation, and NGF content in rectal mucosa of pediatric patients with IBS as compared with controls.* ***Methods*** *Rectal biopsies from children (median age: 14 years) with diarrhea-predominant IBS (n = 11) and controls (n = 14) were studied. MC and neuronal mucosal structures were identified by tryptase, CD117 and PGP9.5 immunoreactivity. Inflammatory cells (neutrophils, eosinophils, and lymphocytes) were counted. NGF was quantified in situ by ELISA.* ***Key Results*** *No mucosal inflammation was detected in IBS. MC infiltration and number of neuronal bodies were not significantly different between IBS and controls. The distance between MC and nerve fibers was not different in IBS compared with controls (5.2 ± 0.3 vs 5.0 ± 0.3 μm). Number of MC in close proximity to nerve fibers (<5 μm) was not different in the two groups. However, in IBS, NGF content was higher than controls (0.93 ± 0.3 vs 0.62 ± 0.3 pg mg^{-1} protein, P < 0.05) and significantly correlated with MC number.* ***Conclusions & Inferences*** *Regardless of inflammation, NGF content is increased in rectal mucosa of diarrhea-predominant IBS children.*

Keywords *abdominal pain, children, irritable bowel syndrome, mast cells, nerve growth factor, neurotrophins, visceral sensitivity.*

Abbreviations: DAPI, 4′, 6-diamidino-2-phenylindole; IBS, irritable bowel syndrome; NGF, nerve growth factor; QPGS, questionnaire on pediatric gastrointestinal symptoms in children;

INTRODUCTION

Irritable bowel syndrome (IBS), defined as recurrent abdominal pain associated with diarrhea or constipation unexplained by structural anomalies, affects up to 15% of children who, in the most severe cases, can have more important repercussions on their quality of life than those with organic digestive diseases.[1,2] IBS has been related to visceral hypersensitivity defined as an exaggerated perceptual response to visceral distensions.[3–6] Theoretically, visceral hypersensitivity could be the result of central anomalies (i.e., at the level of the central nervous system) and/or of a peripheral sensitization (i.e., at the level of the gastrointestinal tract and its afferent sensory innervation).

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Animal models of visceral hypersensitivity have permitted investigations of cellular and molecular abnormalities in the gastrointestinal mucosa.[7,8] In humans, studies have similarly found several modifications in the rectal and colonic mucosa. For instance, low-grade inflammation has been reported in the digestive mucosa of patients with IBS,[9,10] and abnormal mast cell numbers (increase[11,12] or decrease[13]) and close proximity to mucosal enteric nerve fibers[11] have been described in descending colon of adult patients with IBS.

Members of the neurotrophins family include nerve growth factor (NGF). NGF is expressed in the colonic mucosa and has been shown to influence visceral sensitivity in animals.[14–16] In animal models of IBS, NGF has been reported to play a pivotal role in the induction and maintenance of visceral hypersensitivity with[17,18] or without[15] increased mast cell infiltration.

Because IBS is characterized by visceral hypersensitivity and abnormal intestinal motility, and because no data are available on NGF in humans suffering from IBS, we tested the hypothesis that in children with IBS, colonic mucosal innervation and NGF content are altered as compared with controls. More specifically, we measured mast cells number, distance between mast cells and mucosal nerve fibers, number of neuronal bodies, mucosal inflammation, and NGF content in endoscopic rectal specimen of children with IBS and controls.

METHODS

Subjects

Colonic tissue samples were prospectively obtained from children aged 8–18 years for whom a colonoscopy was required in their evaluation. Potential subjects were excluded from the study if they had an acute intestinal infection (acute gastroenteritis) during the 4 weeks preceding the exam. On the day of the procedure, all children filled out the Questionnaire on Pediatric Gastrointestinal Symptoms in Children (QPGS), a validated questionnaire, which evaluates gastrointestinal symptoms and was adapted to the Rome III diagnostic criteria of IBS in children.[1,19]

Rectal biopsies were obtained from macroscopically normal appearing mucosa and each sample was immediately placed into prepared Eppendorf tubes, weighed, and processed for histology, immunohistology, and snap frozen for NGF content measurement.

All patients were reassessed 3 months after the procedure to determine their final diagnosis. The final diagnosis of each participant was determined by consulting the patient medical record and when necessary in collaboration with the patient's physician after examination of the QPGS. Participants were classified in one of the two following groups:

1 Patients with IBS according to Rome III criteria and

2 Subjects with a non-functional (organic) gastrointestinal disorder.

According to the responses provided in the QPGS (in which abdominal pain and bowel habits were evaluated) the subjects with non-functional gastrointestinal disorders who did not report any painful complaints or abnormal bowel habits were considered as controls.

The protocol was approved by the institutional ethics committee and appropriate consent was obtained from all participants; consent was signed by the parents or legal guardian and by the child himself/herself if 14 years or older prior to the procedure.

Table 1 reports the demographics and the clinical characteristics of the 25 included subjects. Eleven children with IBS according to Rome III criteria were included in the study. Fourteen subjects who underwent a colonoscopy which was normal or displayed one juvenile polyp and who did not report any painful complaints or bowel habits anomaly on QPGS were used as controls. Although female patients were more numerous in the IBS group, there was no difference regarding the gender ratio between patients and control subjects (χ^2-test; $P > 0.05$).

Immunohistochemistry and immunofluorescence

One biopsy from each subject was fixed for 2–3 h in 2% paraformaldehyde/0.2% picric acid and transferred to 70% ethanol at 4 °C. Biopsies were carefully oriented and transverse sections (4 μm) were obtained and immunohistochemistry techniques were performed on an automate (NextES IHC, Ventana). Indirect immunoperoxidase staining was performed with the following primary antibodies: prediluted rabbit polyclonal anti-CD3 (Dako, Burlington, ON, Canada), rabbit anti-CD117 (ID Labs Biotechnology Inc., London, ON, Canada; 1 : 100). The first primary antibody was followed by incubation with the appropriate biotinylated secondary antibodies and with streptavidin and 3,3-diaminobenzidine (DAB)-tetrachloride (ChemMate detection Kit, Dako). Slides were counterstained with hematoxylin. The number of CD3 and CD117 immunoreactive cells were evaluated blindly at the 40× magnification on non-overlapping fields.

Double immunofluorescence was performed simultaneously to label mucosal mast cells and mucosal neurons. Slides were washed for 10 min and incubated 1 h with PBS and appropriate 10% normal goat serum. The solution was removed and specimens were then incubated overnight with PBS, normal goat serum (4%), and the primary antibodies against PGP9.5 – a ubiquitous neuronal marker (rabbit IgG polyclonal, AbD Serotec, Oxford, UK, 1 : 1000) and tryptase – a mast cell marker (Mouse IgG1

Table 1 Demographics and diagnoses of the 25 subjects included in the study

Diagnosis	Number	Gender M/F	Age (years; median, range)
IBS	11	3/8	14 (11–18)
IBS-D	10	–	–
IBS-A	1	–	–
Controls	14	8/6	14 (8–18)
Polyps screening for familial polyposis with normal colonoscopy	5	–	–
Normal colonoscopy	7	–	–
Patients diagnosed with one juvenile polyp	2	–	–

IBS, irritable bowel syndrome; IBS-D, diarrhea-predominant IBS; IBS-A, alternating IBS.

monoclonal, Dako,1 : 2000). The preparations were washed three times for 10 min in PBS and then incubated for 2 h at 4 °C with appropriate secondary antibodies (Goat antirabbit IgG, Alexa Fluor 594 and Goat antimouse IgG, ALexa Fluor 488, 1 : 400). Cell nuclei were counterstained using 4′,6-diamidino-2-phenylindole (DAPI). Tissue sections were rinsed three times for 10 min in PBS, dried and mounted using Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA). Negative controls, in which primary antibodies were omitted, were included in each experiment. Immunoreactive cells were counted on non-overlapping high power fields (40×) photographed with a fluorescence microscope (Zeiss AX 10; Zeiss, Toronto, ON, Canada).

Inflammation and mast cells infiltration assessment

Lymphocytes, neutrophils, and eosinophils in the rectal mucosa of patients with IBS were quantified and compared with controls. Cells in the lamina propria were assessed as the total number of inflammatory cells with H&E staining. Cells were classified as lymphocytes, neutrophils, or eosinophils according to their typical morphology and staining properties. Fields containing lymphoid aggregates were excluded.

Lymphocytes were counted in the lamina propria, respectively, as CD3-positive cells. Intraepithelial lymphocytes were enumerated as CD3-positive cells per 100 epithelial cells. Number of mast cells was assessed by counting CD117 immunoreactive and tryptase immunoreactive cells. All counts were performed within three non-overlapping high power fields on three different sections at 40× magnification in each subject.

Evaluation of mucosal innervation

Neuronal cell bodies identified as PGP9.5 and DAPI immunoreactive cells were counted on three non-overlapping high power fields on three different sections at 40× magnification in each subject photographed with a fluorescence microscope (Zeiss AX 10; Zeiss).

Distance between mast cells and nerve fibers were measured using a microscope Zeiss AX-10 with AXIOVISION software. Number of mast cells located <5 and <10 μm from nerve fibers were counted for each subject. All counts were performed within three non-overlapping high power fields on three different sections at 40× magnification.

Measure of NGF in rectal mucosa

Nerve growth factor content in homogenized biopsy specimen from each individual was analyzed using an enzyme immunoassay kit according to the manufacturer's instructions (Fisher Scientific, Mississauga, ON). Each sample was homogenized, sonicated and acidified, and stored at −20 °C until assay. Briefly, the NGF immunoassay is designed for the sensitive and specific (less than 3% cross-reactivity with other neuro-

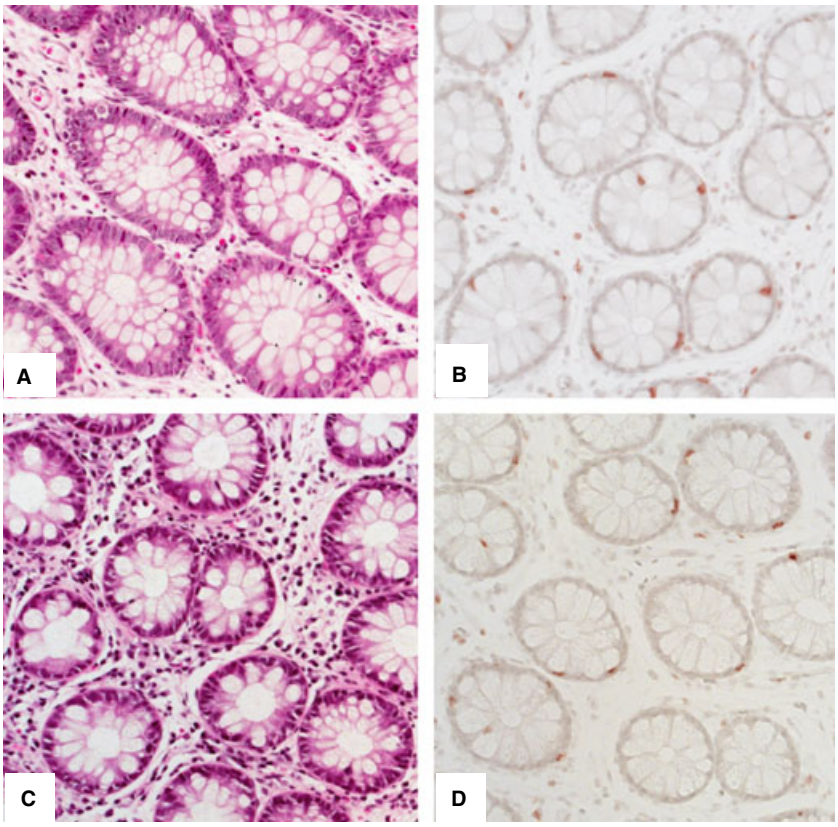

Figure 1 Hematoxylin & Eosin (A, C) and immunohistochemistry with anti-CD3 (B, D) of the rectal mucosa of an IBS patient (A, B) and of a control subject (C, D) showing no mucosal inflammation. Note the presence of CD3 immunoreactive lymphocytes in low number in the epithelium and in the lamina propria.

Table 2 Characterization of mast cell infiltration, mucosal inflammation, and mucosal neurons in patients with IBS and controls

	IBS	Controls	IBS *vs* controls
Mast cells (CD117 + cells)	34.8 (9.8–48)	36.2 (15–60)	NS
Mast cells (tryptase + cells)	10.2 (5.2–20.2)	10.5 (6.6–19.2)	NS
Intraepithelial lymphocytes	5.9 (4.1–14.6)	6.1 (1.0–10.2)	NS
Lymphocytes	48.5 (18–126)	44.2 (29–104)	NS
Neutrophils	0.0 (0–2)	0.0 (0–1)	NS
Eosinophils	0.0 (0–0)	0.0 (0–1)	NS
Neuronal cell bodies	5.2 (2.1–8.2)	5.9 (1.6–12.3)	NS

IBS, irritable bowel syndrome.
Data are expressed as median and range. Intraepithelial lymphocytes are expressed per 100 epithelial cells, and lymphocytes, neutrophils, and eosinophils as the number of cells per high power field.

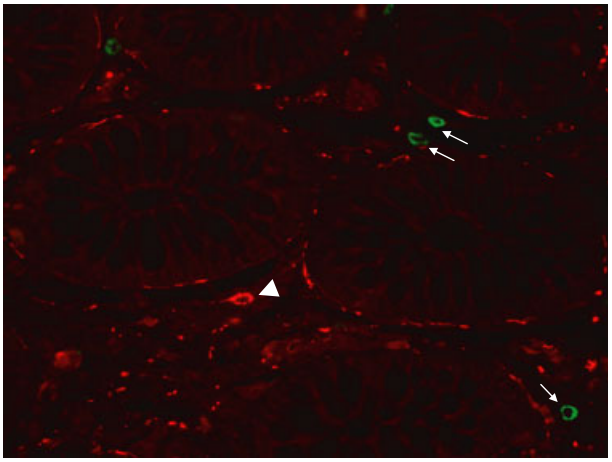

Figure 2 Immunofluorescence for PGP9.5 (red) and Tryptase (green) in the rectal mucosa of a control subject. Neurons (PGP9.5 immunoreactive cell bodies, arrowhead) and mast cells (tryptase immunoreactive cells, arrows) are detected in the rectal mucosa.

trophic factors) quantitation of NGF in an antibody sandwich format in which plates are coated with anti-NGF polyclonal antibody, which binds soluble NGF. The captured NGF is bound by a second specific monoclonal antibody and after washing, the amount of specifically bound monoclonal antibody is detected using antirat IgG conjugated to horseradish peroxidase as a tertiary reactant. The unbound conjugate is removed by washing, and following incubation with a chromogenic substrate, the color change is measured. Protein content was measured using the method of Bradford (Bio-Rad Protein Assay, Mississauga, ON).

Statistical analyses

Summary data are expressed as means (±SD). *N*-values represent the number of subjects included in the data set. Comparisons between the patients and the controls used the Student's *t*-test or Mann–Whitney *U*-test to compare means or medians, respectively, and the chi-squared test to count data. Correlation between NGF content and mast cell number was carried out using Pearson's test. Significance was expressed at the $P < 0.05$ level.

RESULTS

Quantification of inflammation and mast cells infiltration in rectal mucosa

No differences were detected between patients and controls regarding mast cells counts evaluated by tryptase or CD117 immunoreactivity. No neutrophils or eosinophils were detected in sections from patients and controls, and intraepithelial lymphocytes and CD3+ cells were similar in the rectal mucosa of both groups of subjects (Fig. 1 and Table 2).

Mucosal innervation

Neuronal bodies were detected in the rectal mucosa of patients and controls (Fig. 2). Numbers of neuronal

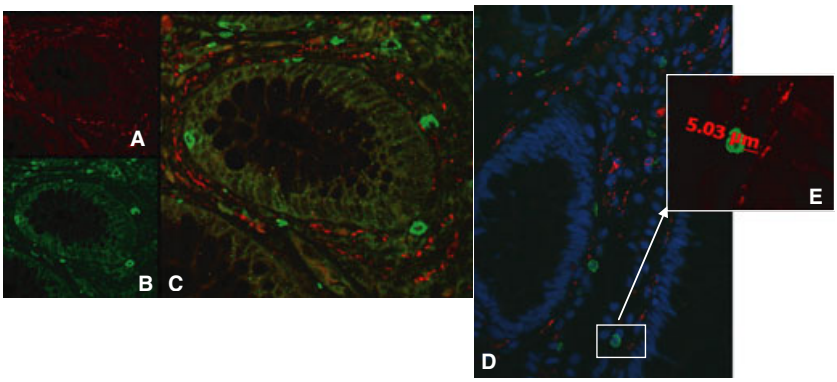

Figure 3 Immunofluorescence for PGP9.5 (red, panel A); Tryptase (green, panel B) and double immunostaining (panel C) in the rectal mucosa of a control subject. Panel D: immunofluorescence for PGP9.5 (red); Tryptase (green) and DAPI (blue) in the rectal mucosa of an IBS patient. Inset (panel E) depicts the measure of the distance between neurite (PGP9.5+) and mast cell (tryptase+) using Zeiss Axiovision software (Zeiss, Toronto, ON, Canada).

bodies were not significantly different between IBS and controls (Table 2).

Relation between mucosal mast cells and mucosal nerve fibers

The distance between tryptase-immunoreactive mast cells and PGP9.5 immunoreactive nerve fibers was counted on a mean number of 32.7 ± 10.4 measurements per subject. It was not different in IBS compared with controls (5.2 ± 0.3 *vs* 5.0 ± 0.3 μm). Fig. 3 shows a representative picture of tryptase and PGP9.5 immunolabeling. Number of mast cells in close proximity to nerve fibers (<5 or <10 μm) were not different in the two groups (Fig. 4).

Quantification of NGF in rectal mucosa

NGF content in the rectal mucosa was significantly higher in patients with IBS than in controls (0.94 ± 0.3 *vs* 0.62 ± 0.3 pg mg^{-1} protein; $P = 0.02$; Fig. 5).

DISCUSSION

This study reports that, in children suffering from diarrhea-predominant IBS, mucosal infiltration by mast cells, mucosal neuronal bodies, and relationships between mast cells and mucosal nerve fibers are not different from controls. However, we found that, in absence of mucosal inflammation, NGF content, a neurotrophin involved in the sensitization of visceral afferents in animal models of visceral hypersensitivity,[15,17,18] is increased in the rectal mucosa of children suffering from diarrhea-predominant IBS.

Increased mast cell numbers and close proximity to mucosal enteric nerve fibers in the colonic mucosa have been reported in rat models of visceral hypersensitivity, such as acute stress[20,21] and maternal separation.[18] In adult patients with IBS, increased[11,12,22] or decreased[13] mast cell numbers in descending colon and close proximity to mucosal nerve fibers[11,13] have also been reported. In this study, the first conducted in children, we did not find any difference between IBS patients and controls regarding mast cell infiltration and distance between mast cells and nerve fibers present in the rectal mucosa. However, we cannot exclude that mast cell activation and degranulation may be increased in the IBS patients. As mast cells quantification can be technically difficult and questionable, we paid a special attention to label these cells with antibodies to tryptase and CD117 which stain, respectively, secretory granules and a membrane marker. There was no difference between IBS and controls using CD117 or tryptase staining. The discrepancies with other studies conducted in adults may be related to the time course of evolution in the pediatric patients. One cannot exclude that as children with IBS have a relatively shorter evolution than adults (mean duration of symptoms 1–2 years[3] *vs* 15–20 years in adults), the time lag between the onset of symptoms implies that modifications seen in adults may not reflect initial pathophysiological mechanisms, but

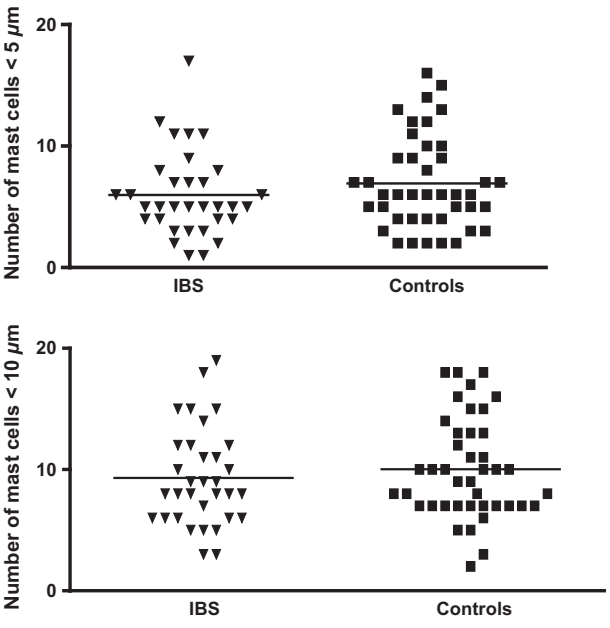

Figure 4 Individual values of mast cell numbers (tryptase immunoreactive cells) located <5 and <10 μm from nerve fibers (PGP9.5 immunoreactive nerve fibers) in rectal mucosa of children with non-constipated irritable bowel syndrome (IBS) and controls. Values were counted on three non-overlapping fields for each subject.

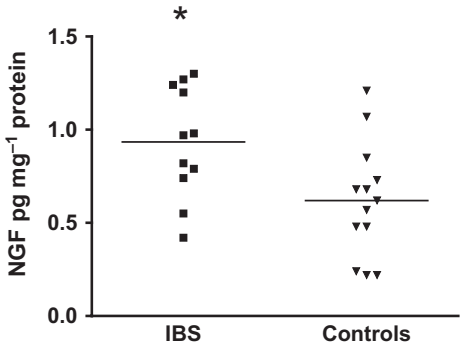

Figure 5 Individual values and mean (SD) of Nerve growth factor (NGF) content in the rectal mucosa of children with irritable bowel syndrome (IBS) compared with controls (*$P = 0.02$ *vs* controls).

rather their long-term consequences. For instance, studies of animal models of visceral hypersensitivity have clearly reported differences in colonic nerve density between 4- and 12-week old rats after maternal deprivation.[17] In that context, we believe that the pediatric IBS is the best 'model' to study the mechanisms leading to IBS and visceral hypersensitivity in humans.

Recent data support the existence of neuronal cells within the intestinal mucosa in swine[23] and humans.[24–26] The role of these mucosal neurons remains unknown, but could play significant role in the visceral sensitivity. We therefore hypothesized that they could be involved in IBS. We confirmed the presence of neuronal cell bodies in the rectal mucosa of children, (Fig. 3) but no difference was found between IBS and controls.

Animal models of visceral hypersensitivity have permitted to demonstrate the critical role of peripheral NGF in the sensitization of visceral afferents. In a model of maternal separation, Barreau *et al.* have demonstrated that NGF expression and messenger RNA were increased in the colon in maternal deprived rats having colonic hypersensitivity.[18] Anti-NGF antibodies abolished the effects of maternal separation, whereas neonatal administration of NGF mimicked them.[27] In a subsequent study, the same group showed that NGF was responsible for increased mast cell infiltration and close proximity of mucosal nerve fibers to mast cells in the colon.[17] Using a different model of visceral hypersensitivity induced by chronic stress, Winston *et al.* have recently confirmed the key role of peripheral NGF in peripheral visceral sensitization, but independently of mast cell infiltration and inflammation. They showed that NGF is upregulated in colonic wall in chronic stress in response to norepinephrine/epinephrine and, after retrograde transport of the complex NGF-TrkA (the specific receptor to NGF) to the thoracolumbar dorsal root ganglia sensitizes afferent sensory neurons.[15] This model of chronic stress-induced visceral sensitization demonstrates a mast cell-independent mechanism of visceral hypersensitivity. In the present study, the high NGF content of rectal mucosa in IBS patients is in keeping with the previously described experimental models. The origin of NGF in the intestinal wall is currently unknown. As inflammation is known to influence NGF signaling (and vice versa),[14,28,29] the mucosal inflammatory condition was carefully determined for each individual included in the study. In keeping with the chronic stress model,[15] we did not notice any associated mucosal inflammation, modifications of mast cells number, or distance to nerve fibers.

Potential limitations of this study are related to the relatively low number of patients included. However, despite the difficulties to obtain tissue specimens in children, we were able to recruit children who can be considered as appropriate controls, as they were asymptomatic regarding painful symptoms and bowel habits. We are also aware that the participants were recruited from a tertiary pediatric center and therefore those with IBS may be at the more severe end of the spectrum of the functional disorders. Thus, we cannot conclude that the present findings are valid for all pediatric patients with IBS in the community. If this is related to increased NGF content, it may result in an overestimation of the difference in IBS patients. Finally, these results do not necessarily apply to constipated predominant IBS patients as this study was not designed to specifically include constipated patients.

In conclusion, we report for the first time in humans that NGF content in rectal mucosa is increased in subjects with diarrhea-predominant IBS. Further studies are needed to understand which structures and mechanisms are responsible for this increased local NGF production, what is its role in visceral hypersensitivity, and which proportion of patients suffering from IBS are affected by this mechanism.

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DISCLOSURES

None.

AUTHOR CONTRIBUTIONS

SW was involved in the study design, immunofluorescence experiments, and analysis of the data, and wrote the manuscript; CG was involved in the acquisition of data and brought technical support for quantification of neurotrophins; NP performed and analyzed all the pathological data; CF designed the study. He obtained funds from the Canadian Association of Gastroenterology. He analyzed the data and critically revised the manuscript.

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