Increase in chromogranin A- and serotonin-positive cells in pouch mucosa of patients with ulcerative colitis undergoing proctocolectomy

Running head: Neuroendocrine cells in pouch mucosa

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Abbreviations: CgA, chromogranin A; CD, Crohn's disease; EC, enterochromaffin; IBD, inflammatory bowel disease; IEL, intraepithelial lymphocyte; IL, interleukin; SERT, serotonin-selective reuptake transporter; TpH, tryptophan hydroxylase; TNF, tumor necrosis factor; UC, ulcerative colitis.

Keywords: chromogranin A; Crohn's disease; pouchitis; serotonin; ulcerative colitis.

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Abstract

Background: Inflammatory bowel disease (IBD) is associated with neuroendocrine cell hyperplasia. *Aims*: We investigated neuroendocrine cells in J-pouches of patients with ulcerative colitis undergoing restorative proctocolectomy and ileal pouch-anal anastomosis. *Methods*: Sections from pouch biopsies of 17 patients and ileal biopsies of 17 active IBD patients and 16 controls were processed by immunohistochemistry for chromogranin A (CgA) and serotonin. Mucosal tryptophan hydroxylase (TpH)-1 and serotonin-selective reuptake transporter (SERT) transcripts were measured by quantitative RT-PCR. TpH-1 and SERT transcripts were detected in pouch biopsies cultured with infliximab or its isotype control, while IL-6 and IL-8 were measured in biopsy supernatants. *Results*: A significant increase in CgA-positive cells and serotonin-positive cells was observed in both pouch and IBD ileum compared to control ileum. Significantly raised transcripts of TpH-1, but not SERT, were found in IBD ileum in comparison to control ileum, with no significant difference between pouch and IBD ileum. Infliximab had no influence on ex vivo pouch expression of TpH-1 and SERT, nor on the production of IL-6 and IL-8. *Conclusion*: We here demonstrated neuroendocrine cell hyperplasia in pouch mucosa. Further studies are needed to clarify the pathophysiological implication of this finding.

Introduction

Abnormal amount and functioning of neuroendocrine cells, which comprise at least 14 cytotypes and express the pan-neuroendocrine marker chromogranin A (CgA), have been reported in several animal models of colitis[1,2] and immune-mediated intestinal disorders, including Crohn's disease (CD),[3-5] ulcerative colitis (UC)[5-11] and celiac disease.[12] However, how neuroendocrine cells modulate the inflammatory response in the aforementioned disorders is largely unknown. The dominant cytotype among the CgA-positive neuroendocrine cells populating the bowel mucosa is represented by serotonin-producing enterochromaffin (EC) cells.[13] There is evidence that serotonin induces T-cell proliferation[14] and dendritic cell activation,[15] sustains immune-cell recruitment,[16] and up-regulates pro-inflammatory cytokine production.[17] In active celiac duodenal mucosa, EC cells are increased and serotonin stimulates interferon-γ production by treated celiac biopsies grown *ex vivo*.[12]

A number of studies have been published investigating neuroendocrine cells in the pouch mucosa of UC patients undergoing restorative proctocolectomy and ileal pouch-anal anastomosis (Table 1). In particular, neurotensin-, polypeptide YY- and enteroglucagon-positive cells were found to be reduced in pouch biopsies,[18,19] while serotonin was over-expressed in the pouch mucosa of patients with irritable pouch syndrome.[20] In contrast, no difference was observed by Pietroletti et al.[19] in the mucosal expression of serotonin in pouchitis.

Based on these premises, we aimed to investigate neuroendocrine cells in the pouch mucosa of patients with ulcerative colitis who underwent restorative proctocolectomy, and to assess mucosal tryptophan hydroxylase (TpH)-1, which is an enzyme involved in serotonin synthesis, and serotonin-selective reuptake transporter (SERT) transcripts both *in vivo* and following down-regulation of inflammation obtained through tumor necrosis factor (TNF)-α blockade by

infliximab in *ex vivo* cultured pouch biopsies.

Methods

Patients and tissues. Endoscopic biopsy specimens were collected from J-pouches performed in 17 UC patients undergoing restorative proctocolectomy and ileal pouch-anal anastomosis (mean age 46.1 years, range 26-64). At biopsy collection, J-pouches had been followed-up for at least two years. None of the patients with pouch had extra-intestinal manifestations or relevant co-morbidities. In particular, coeliac disease was ruled out in all patients (negative anti-human tissue transglutaminase IgA and normal serum IgA). All patients with pouch had been treated with courses of probiotics during the year before the study, and none was being treated with antibiotics at the time of biopsy collection. Clinical, endoscopic and pathological features of patients with pouch are reported in Table 2.[21] The severity of pouch histological inflammation was assessed using Moskowitz criteria,[22] including both acute (neutrophilic infiltration and ulcerations) and chronic (lymphoplasmacellular infiltration in the lamina propria and colonic metaplasia) changes. No case of irritable pouch syndrome was included. Ileal biopsy samples were obtained from 17 active IBD patients (mean age 39.7 years, range 19-65). Amongst these patients, eight were affected by CD with ileal involvement and nine by UC with backwash ileitis. The mean grade of their histological inflammation was 4.6 (range 3-8) in CD and 1.1 (range 1-2) in UC according to the scoring system developed by Naini and Cortina.[23] Two CD patients and one UC patient suffered from concomitant spondyloarthritis. Other co-morbidities included benign essential hypertension in two CD patients, autoimmune hemolytic anaemia in one UC patient and non-alcoholic fatty liver disease in two UC patients. As regards smoking habit, one CD patient and one UC patient were current smokers. Amongst the eight CD patients, three were treated with mesalazine, one with steroids, two with antibiotics, four with thiopurines and one with methotrexate. Amongst the nine UC patients with backwash

ileitis, nine were treated with mesalazine, four with steroids and two with thiopurines. Diagnosis of CD and UC was made according to clinical and histological criteria, and the site and extent of the disease were endoscopically confirmed. Mucosal samples were also collected endoscopically from normal ileum of 16 asymptomatic patients undergoing colonoscopy for colorectal cancer screening (mean age 52.2 years, range 36–68) as controls. Informed consent was obtained in all cases and the study was performed according to the Helsinki Declaration. Some tissue samples were immediately fixed in 10% neutral buffered formalin and embedded in paraffin within 24h. Consecutive 4 μm-thick sections were cut from the selected blocks, mounted on electrostatic slides (Super Frost Plus, Menzel-Glaser, Germany) and dried overnight. After dewaxing and rehydration, sections were processed for routine histology and immunohistochemistry. Some other biopsies were used for quantitative RT-PCR or organ culture experiments. The study was authorized by the Ethical Committee of the "Luigi Sacco" University Hospital, Milan, Italy (protocol number 0002846).

Intraepithelial lymphocyte (IEL) counting. Histological evaluation of hematoxylin and eosin-stained sections of pouch biopsies was undertaken by two independent observers (AV and EB) who were blinded to all clinical information. In each specimen, IEL number was counted *per* 100 epithelial cells. For each case, five different counts were carried out and the mean number of IELs per 100 epithelial cells was recorded.

Immunohistochemistry. Serial sections were taken from paraffin blocks of pouch and ileal biopsies and stained with hematoxylin-eosin or with immunoperoxidase using anti-CgA antibody (1:9000 dilution; Dako Cytomation, Glostrup, Denmark) and anti-serotonin antibody (1:1000 dilution; Monosan, Uden, The Netherlands). Neuroendocrine cell assessment was performed by counting the number of CgA-positive and serotonin-positive cells *per* 100 epithelial cells in well-oriented crypts. For each case, five different counts were carried out and the mean number of

CgA-positive and serotonin-positive cells *per* 100 crypt cells was finally recorded.

RNA extraction and analysis of mRNA expression by quantitative RT-PCR. Total RNA was extracted with the RNeasy mini-kit (Qiagen Sciences, Valencia, CA) according to the manufacturer's instructions. cDNA was synthesized using 200-400 ng total RNA and the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) in a final volume of 20 μL. Amplifications were performed on a LightCycler 2.0 instrument (Roche Diagnostics) using the LightCycler TaqMan Master kit (Roche Diagnostics) and validated fluorogenic TaqMan Gene Expression Assays-on-Demand for TpH-1 (Hs00188220_m1) and SERT (Hs00169010_m1), both from Applied Biosystems (Foster City, CA). Expression levels were calculated with the $\Delta\Delta C_T$ method following normalization to the GAPDH housekeeping gene (Universal ProbeLibrary Human GAPDH Gene Assay, Roche Diagnostics).

Organ culture. Biopsies from patients with a pouch were placed in 24-well tissue culture plates (BD Biosciences, Oxford, UK; one biopsy per well) in 300 μl serum-free HL-1 medium (Cambrex Bio Science, Walkersville, MD), supplemented with 100 U/ml penicillin and 100 g/ml streptomycin (Life Technologies Ltd, Paisley, UK), and cultured at 37°C, 5%CO_2 with the anti-TNF-α monoclonal antibody infliximab (Remicade; Merck, Milan, Italy) or its isotype-matched control (human IgG1; Sigma-Aldrich, Poole, UK). All antibodies were used at a concentration of 10 μg/ml. After 24h *ex vivo* culture, biopsies were collected in RNAlater until used for quantitative RT-PCR and supernatants were stored at -70°C prior to ELISA.

ELISA. Interleukin (IL)-6 and chemokine IL-8 levels were measured in organ culture supernatants using the specific ELISA kit (R&D Systems, Abingdon, UK), according to the manufacturer's instructions.

Statistical analysis. All analyses were performed using Stata 14.2 (StataCorp, College Station, TX, USA). A 2-sided p-value<0.05 was considered statistically significant. The Bonferroni correction was used for post-hoc comparisons of pouch, UC, CD and control and the significance was set at 0.017 (2-sided). Continuous variables were described as median and 25^{th}-75^{th} percentiles. They were compared between groups (pouch/UC/CD/controls) with the Kruskal Wallis test. The Wilcoxon signed-rank test was used for paired comparisons of biomarkers levels within patients. Correlation between biomarkers were assessed with the Spearman R, together with its 95% confidence interval (95%CI).

Results

Immunohistochemical detection of CgA and serotonin. Numerous CgA-positive cells were evident in the crypt epithelium of patients with pouch (Figure 1A), UC (Figure 1B) and CD ileum (Figure 1C). On the other hand, CgA-positivity was found in fewer cells localized in the crypts of control ileum (Figure 1D). As shown in Figure 2A, the number of CgA-positive cells *per* 100 crypt cells was significantly (p<0.001) higher in the pouch mucosa (median 13.0%, 25^{th}-75^{th} 11.0-15.7) in comparison to control ileum (median 6.6%, 25^{th}-75^{th} 5.1-8.8). No significant difference was found between pouch mucosa and both UC (median 9.7%, 25^{th}-75^{th} 9.0-10.3) and CD ileum (median 9.1%, 25^{th}-75^{th} 7.9-12.8) (after Bonferroni correction), without a significant difference between UC and CD ileum. However, pooling together UC and CD, CgA-positive cells were significantly (p=0.005) increased in IBD ileum (median 9.7%, 25^{th}-75^{th} 8.0-11.0) in comparison to control ileum, without a significant difference between IBD ileum and pouch (data not shown). No significant difference was found between pouch mucosa of the four patients undergoing restorative proctocolectomy due to dysplasia or cancer (median 11.6%, 25^{th}-75^{th} 8.5-16.1) and that of the 13 patients undergoing proctocolectomy for non-neoplastic causes (median 14.5%,

25^{th}-75^{th} 10.5-15.7). No significant correlation was found between the percentage of CgA-positive cells and the degree of acute (r_s=-0.0634; p=0.8088), chronic (r_s=-0.3211; p=0.2088) or acute plus chronic inflammation (r_s=-0.2913; p=0.2567) in pouch mucosa (data not shown).[22] We also correlated the percentage of CgA-positive cells with the proportion of IELs infiltrating the pouch mucosa whose median number was 5.4% (range 2.2-11.4); however, no significant correlation (r_s=-0.1075; p=0.6813) was observed between these two parameters (data not shown).

Numerous serotonin-positive cells were evident in the crypt epithelium of patients with pouch (Figure 1E), UC (Figure 1F) and CD ileum (Figure 1G). On the other hand, serotonin-positivity was found in fewer cells localized in the villous and crypts compartments of control ileum (Figure 1H). As shown in Figure 2B, the number of serotonin-positive cells *per* 100 crypt cells was significantly higher in the pouch mucosa (median 7.2%, 25^{th}-75^{th} 5.5-10.0, p<0.001), UC (median 8.2%, 25^{th}-75^{th} 7.2-8.6, p=0.006) and CD ileum (median 7.0%, 25^{th}-75^{th} 5.6-11.2, p=0.004) in comparison to control ileum (median 4.4%, 25^{th}-75^{th} 3.4-6.2), without a significant difference between pouch mucosa and both UC and CD ileum (after Bonferroni correction). No significant difference was found between UC and CD ileum (after Bonferroni correction). In addition, pooling together UC and CD, serotonin-positive cells were significantly (p<0.001) increased in IBD ileum (median 7.6%, 25^{th}-75^{th} 6.5-8.6) in comparison to control ileum, without a significant difference between IBD ileum and pouch (data not shown). No significant difference was found between pouch mucosa of the four patients undergoing restorative proctocolectomy due to dysplasia or cancer (median 6.5%, 25^{th}-75^{th} 5.6-8.7) and that of the 13 patients undergoing proctocolectomy for non-neoplastic causes (median 7.8%, 25^{th}-75^{th} 5.2-10.1). We found a significant, though weak, (r_s=-0.4860; p=0.0479) negative correlation between the percentage of serotonin-positive cells and the degree of acute plus chronic inflammation in pouch mucosa (data not shown). Instead, no significant correlation was found between the

percentage of serotonin-positive cells and the degree of acute (r_s=-0.47; p=0.0568) or chronic inflammation scores (r_s=-0.4219; p=0.0916) in pouch mucosa (data not shown). We also correlated the percentage of serotonin-positive cells with the proportion of IELs infiltrating the pouch mucosa, but we did not observe any significant correlation (r_s=0.2299; p=0.3748) between these two parameters (data not shown). Finally, a significant, though moderate, positive correlation (r_s=0.6233; p<0.0001) was observed in all patients and control subjects between the percentage of CgA-positive cells and serotonin-positive cells (Figure 2C).

Mucosal TpH-1 and SERT transcripts. Using quantitative RT-PCR, we measured the transcript levels of TpH-1 and SERT in the pouch mucosa of six patients undergoing restorative proctocolectomy, and in the ileal mucosa of six active IBD patients and ten control subjects. As shown in Figure 3A, TpH-1 transcript levels were significantly (p<0.001) higher in IBD ileum in comparison to control ileum. No significant difference was found between pouch and both IBD and control ileum (after Bonferroni correction). SERT transcript levels did not significantly differ among all the aforementioned groups (Figure 3B).

Ex vivo effect of infliximab on TpH-1 and SERT transcripts and on IL-6 and IL-8 production. In order to investigate the effect of infliximab on serotonin, we measured TpH-1 and SERT expression in the pouch biopsies obtained from five patients undergoing restorative proctocolectomy, and cultured *ex vivo* with infliximab or its isotype control (IgG1) (Figure 4A). Both TpH-1 and SERT transcript levels did not significantly differ between the biopsies cultured with infliximab in comparison to those cultured with IgG1.

In order to investigate the effect of infliximab on innate cytokine production, we measured IL-6 and IL-8 levels in the supernatant of mucosal pouch biopsy samples obtained from five patients undergoing restorative proctocolectomy and cultured *ex vivo* with infliximab or IgG1 (Figure 4B).

IL-6 levels did not significantly differ in the supernatants of biopsies cultured with infliximab (mean 1497 ± 1133 pg/mL) in comparison to those cultured with IgG1 (mean 634 ± 802 pg/mL). Likewise, IL-8 levels did not significantly differ in the supernatants of biopsies cultured with infliximab (mean 64458 ± 25989 pg/mL) in comparison to those with IgG1 (mean 33858 ± 14193 pg/mL).

Discussion

Herein this exploratory study, we show an increase in neuroendocrine cells in pouch mucosa. Several studies have reported changes in neuroendocrine cell number in IBD mucosa, including patients with pouch (Table 1).[3-11,18-20] Nevertheless, the current knowledge on this topic is conflicting due to the use of different counting methods (Table 1) and hormones tested. A high number of neuroendocrine cells has been observed not only in IBD mucosa,[5] but also in lymphocytic colitis,[24-26] and in the duodenum of patients with celiac disease.[12] By enumerating neuroendocrine cells through an accurate method which we already validated in the small bowel mucosa -i.e. counting *per* 100 crypt cells in well-oriented biopsy specimens-,[12] we showed an increase in the number of CgA-positive cells in pouch mucosa and IBD ileum. Our data showing a higher number of cells positive for the pan-neuroendocrine marker chromogranin A (CgA) in the ileum of CD patients are in agreement with previous studies.[3,4] Moreover, there is growing evidence of the pro-inflammatory role of serotonin in gastrointestinal disorders; in particular, serotonin triggers the release of pro-inflammatory cytokines from macrophages.[27,28] EC cell hyperplasia has been also observed in lymphocytic colitis,[26] and in a specific population with irritable pouch syndrome.[20] According to all these findings, in our series of patients with a pouch and in IBD ileum, serotonin-positive cells were significantly increased compared to controls. Notably, we here describe for the first time an increase of serotonin-positive cells in the inflamed ileum of UC patients and further studies are needed in order to assess a possible independent

role of these cells in UC-associated ileitis.[29] All the studies on serotonin-positive cells in UC patients have been conducted in colonic or rectal mucosa and reported controversial data.[5,8-11] Finally, the presence of dysplasia or cancer leading to proctocolectomy did not influence neither CgA-positive cells nor serotonin-producing cells in pouch mucosa.

As EC cells might be underestimated by immunohistochemistry and may not reflect the increase of serotonin production, we measured serotonin rate-limiting enzyme TpH-1 in the pouch biopsies by quantitative RT-PCR. Besides TpH-2 that is only expressed in human brain, TpH-1 is the only enzyme that synthetizes serotonin, therefore could be considered a reliable indirect measure of serotonin production.[29] Our results showing higher TpH-1 transcript levels in IBD ileum compared to controls are in keeping with the known pro-inflammatory effects of this molecule and the EC cell hyperplasia identified in this group. TpH-1 transcript levels in patients with pouch are increased compared to controls, but to a level that is just below statistical significance. Moreover, there is a weak negative correlation between the percentage of CgA-positive cells or that of serotonin-positive cells and the degree of acute plus chronic inflammation. Therefore, it is reasonable to assume that the small number of patients with a high degree of pouch inflammation may justify these findings, as well as other possible mechanisms of inflammation we have not looked at in the present study (i.e., dysbiosis, mucosal ischemia, genetic susceptibility, immune dysregulation).[31] Peripheral TpH-1 inhibitors have been successfully tested in murine models of IBD.[32] As serotonin is rapidly removed from the interstitial space by enterocytes through SERT, we also detected the mucosal transcripts of this transporter, reported to be reduced in the rectum of UC patients.[11] However, we did not find any change in the mucosal transcript level of SERT in patients with pouch in comparison to control subjects. We then measured TpH-1 and SERT expression in the pouch biopsies cultured *ex vivo* with infliximab, an anti-TNF-α monoclonal antibody that has been demonstrated to exert a therapeutic effect on patients with pouchitis.[33] Nonetheless, infliximab did not modify TpH-1 and

SERT levels. In order to validate our *ex vivo* experiments, in the biopsy culture supernatants we detected IL-6, a cytokine of the innate immunity, and IL-8, a chemokine, known to be increased in pouch mucosa.[34,35] Infliximab was able to reduce both IL-6 and IL-8, although not significantly. A likely explanation for this finding is the lack of statistical power, with the subsequent high risk of β-error, given that most of the enrolled patients with a pouch only had mild mucosal inflammation. As already mentioned, serotonin causes the release of these cytokines from macrophages[28] and this may explain the modest efficacy of infliximab that blocks a different pathway of inflammation. Several 5-HT_3 receptor antagonists have been reported to ameliorate intestinal inflammation, but none of them has been approved so far.[36] We therefore envisage that future studies will provide new insights to this regard. Finally, in contrast with Schaeffer et al.,[37] none of our pouch biopsies was associated with increased IELs infiltration, and no correlation between IELs count and CgA- or serotonin-positive cells was observed in our pouch biopsies.

In conclusion, neuroendocrine cells are increased in the mucosa of patients with pouch, although no clear correlation was seen with the degree of inflammation, nor with the presence of previous dysplasia. We cannot exclude that the different immunological and microbial luminal milieu in which the “orthotopically-transplanted ileum” is transposed when the pouch is crated might have an influence on the neuroendocrine cell compartment. The significance of this finding is yet to be fully understood, but could represent a future target of intervention. Larger studies are needed to clarify whether the lack of change of TpH-1 and SERT transcript levels may be attributed to a small sample size or to other unexplored causes.

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Figure legend

Figure 1. A-H. Immunohistochemical detection of chromogranin A (CgA) and serotonin. The immunohistochemical detection of CgA showed numerous CgA-positive cells in the pouch mucosa of a patient undergoing restorative proctocolectomy and ileal pouch-anal anastomosis for ulcerative colitis (A) and in the ileal crypt epithelium of a patient with active ulcerative colitis (B) and Crohn's disease (C). On the contrary, CgA positivity was limited to a few cells in the ileal crypt epithelium of a control subject (D). Serotonin immunostaining revealed numerous positive cells in the pouch mucosa of the patient shown in A (E), in the ileal mucosa of the patients with ulcerative colitis shown in B (F) and Crohn's disease shown in C (G), compared with serotonin-positive cells in the ileal crypt epithelium of the control subject shown in D (H). Data are representative of staining performed in the pouch of 17 patients undergoing restorative proctocolectomy, and in the ileum of 17 patients with active inflammatory bowel disease and 16 control subjects.

Figure 2. A-B. Number of chromogranin A (CgA)-positive cells and serotonin-positive cells counted *per* 100 crypt cells, and their correlation. Number of CgA-positive cells (A) and serotonin-positive cells (B), both counted *per* 100 crypt cells, in the pouch mucosa of 17 patients undergoing restorative proctocolectomy and ileal pouch-anal anastomosis for ulcerative colitis, and in the ileal crypt epithelium of 17 patients with active inflammatory bowel disease (IBD) and 16 control subjects. Horizontal bars represent mean values. (C) Significant positive correlation between the number of chromogranin A (CgA)-positive cells *per* 100 crypt cells and the number of serotonin-positive cells *per* 100 crypt cells.

Figure 3. A-B. Tryptophan hydroxylase (TpH)-1 and serotonin-selective reuptake

transporter (SERT) transcripts. (A) TpH-1 and (B) SERT transcripts, measured by quantitative RT-PCR, in the pouch mucosa of six patients undergoing restorative proctocolectomy and ileal pouch-anal anastomosis for ulcerative colitis, and in the ileal mucosa of six patients with active inflammatory bowel disease (IBD), and seven control subjects. The values, normalized for GAPDH and representing the fold change in transcript expression compared to control subjects, are reported as means.

Figure 4. A-B. Effect of infliximab on the pouch mucosa grown *ex vivo*. (A) Tryptophan hydroxylase (TpH)-1 and serotonin-selective reuptake transporter (SERT) transcripts, measured by quantitative RT-PCR, in organ culture pouch biopsies collected from five patients undergoing restorative proctocolectomy and ileal pouch-anal anastomosis for ulcerative colitis and cultured for 24h with IgG1 or infliximab. The values, normalized for GAPDH and representing the fold change in transcript expression compared to IgG1, are reported as means. (B) Interleukin (IL)-6 and IL-8 concentrations were detected by ELISA in the supernatants of the same organ culture biopsies used for quantitative RT-PCR for TpH-1 and SERT. Results are reported as means.

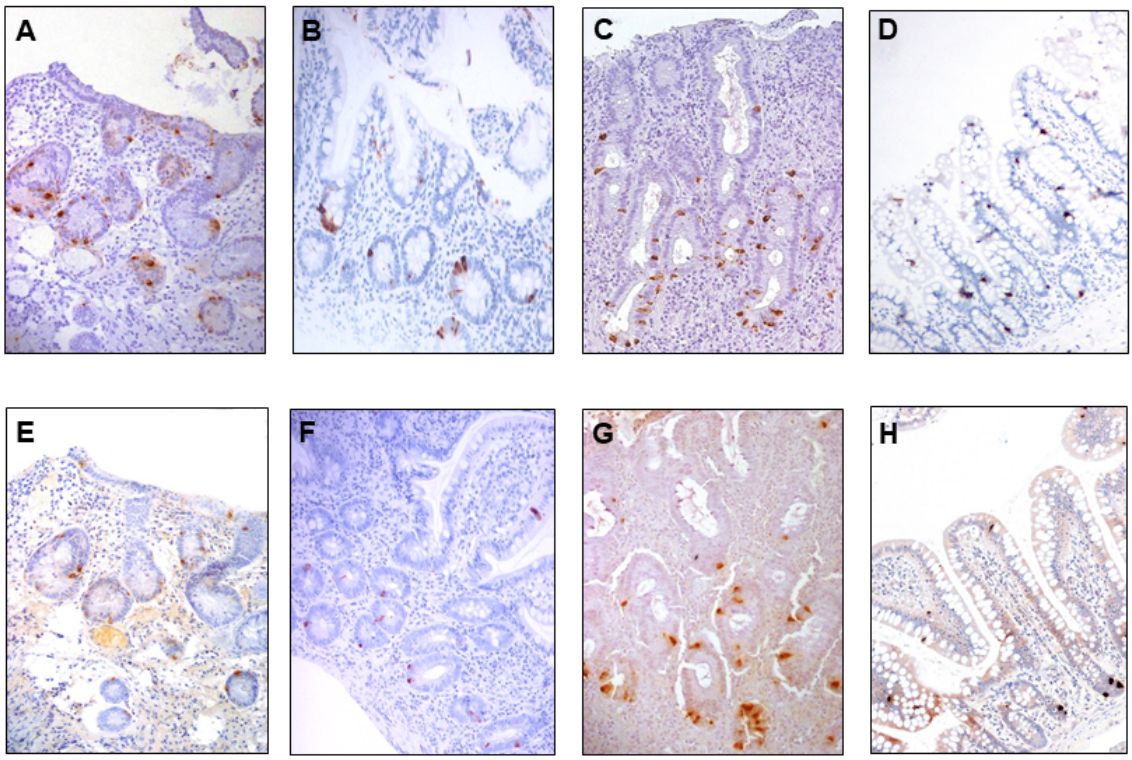

Figure 1

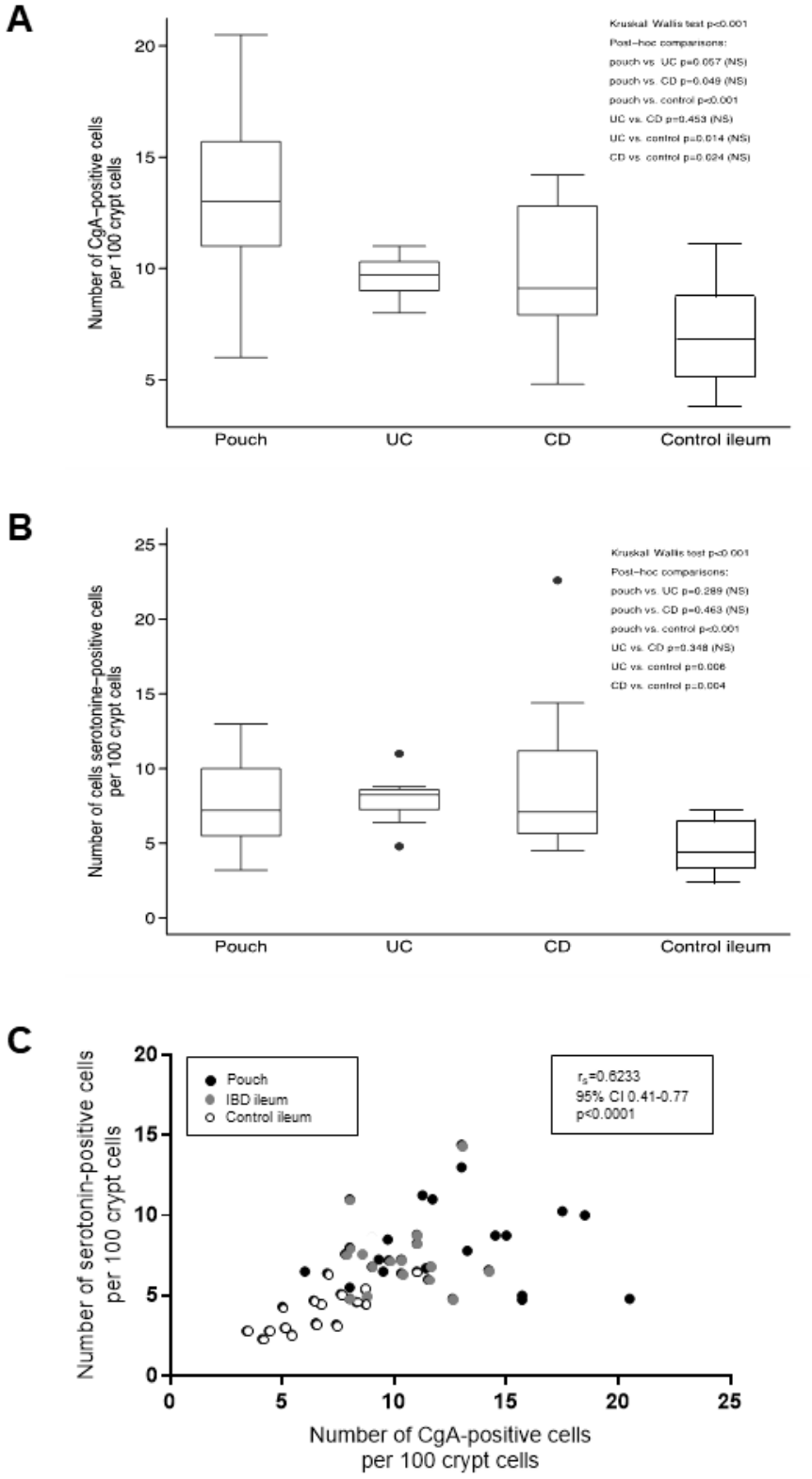
A
Kruskal Wallis test p<0.001
Post-hoc comparisons:
pouch vs. UC p=0.057 (NS)
pouch vs. CD p=0.049 (NS)
pouch vs. control p<0.001
UC vs. CD p=0.453 (NS)
UC vs. control p=0.014 (NS)
CD vs. control p=0.024 (NS)
Number of CgA-positive cells per 100 crypt cells
20
15
10
5
Pouch
UC
CD
Control ileum
B
Kruskal Wallis test p<0.001
Post-hoc comparisons:
pouch vs. UC p=0.289 (NS)
pouch vs. CD p=0.463 (NS)
pouch vs. control p<0.001
UC vs. CD p=0.348 (NS)
UC vs. control p=0.006
CD vs. control p=0.004
Number of cells serotonine-positive cells per 100 crypt cells
25
20
15
10
5
0
Pouch
UC
CD
Control ileum
C
Pouch
IBD ileum
Control ileum
r_s=0.6233
95% CI 0.41-0.77
p<0.0001
Number of serotonin-positive cells per 100 crypt cells
Number of CgA-positive cells per 100 crypt cells
0
5
10
15
20
25

Figure 2

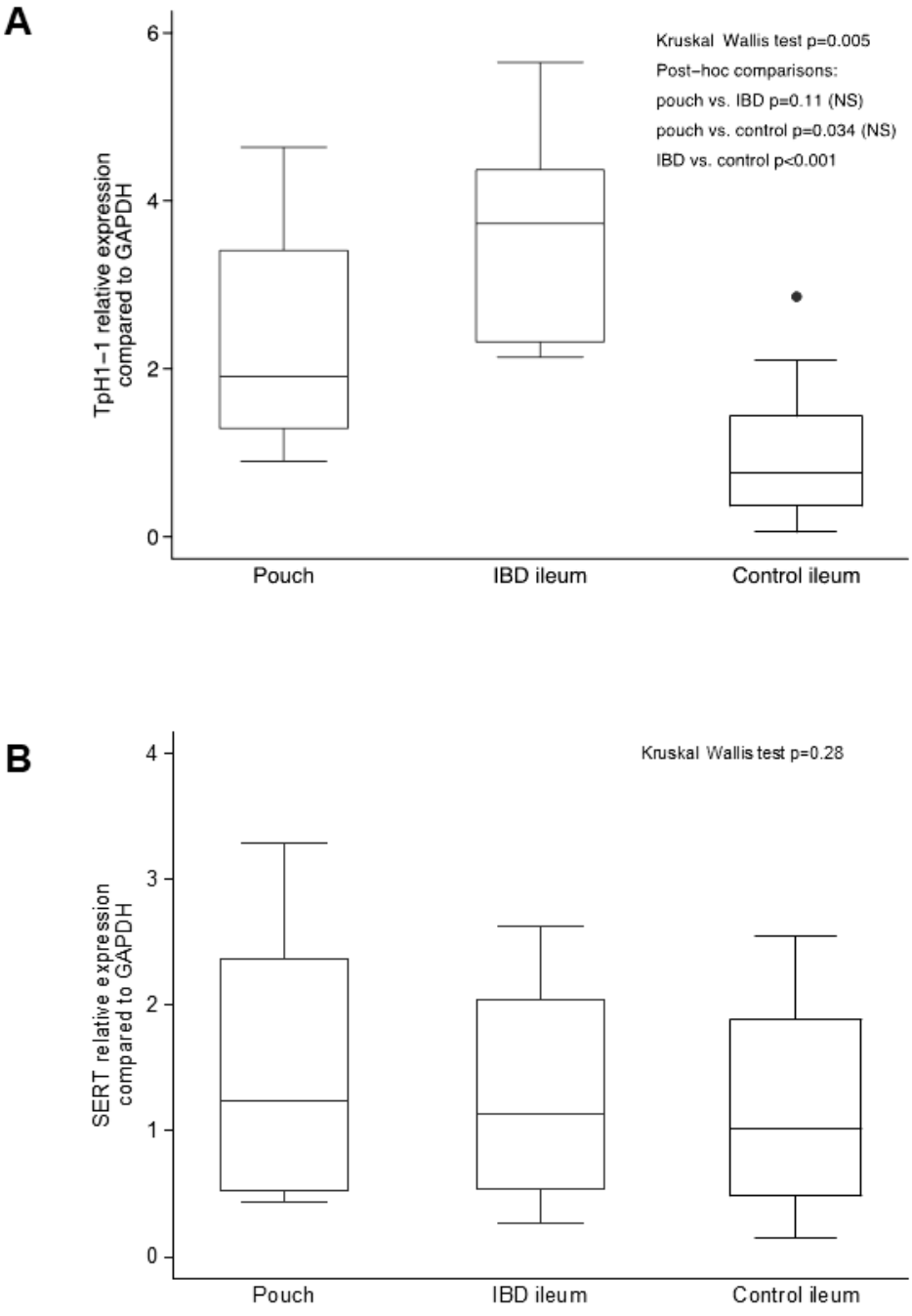
A
TpH1−1 relative expression compared to GAPDH
0
2
4
6
Kruskal Wallis test p=0.005
Post−hoc comparisons:
pouch vs. IBD p=0.11 (NS)
pouch vs. control p=0.034 (NS)
IBD vs. control p<0.001
Pouch
IBD ileum
Control ileum
B
SERT relative expression compared to GAPDH
0
1
2
3
4
Kruskal Wallis test p=0.28
Pouch
IBD ileum
Control ileum

Figure 3

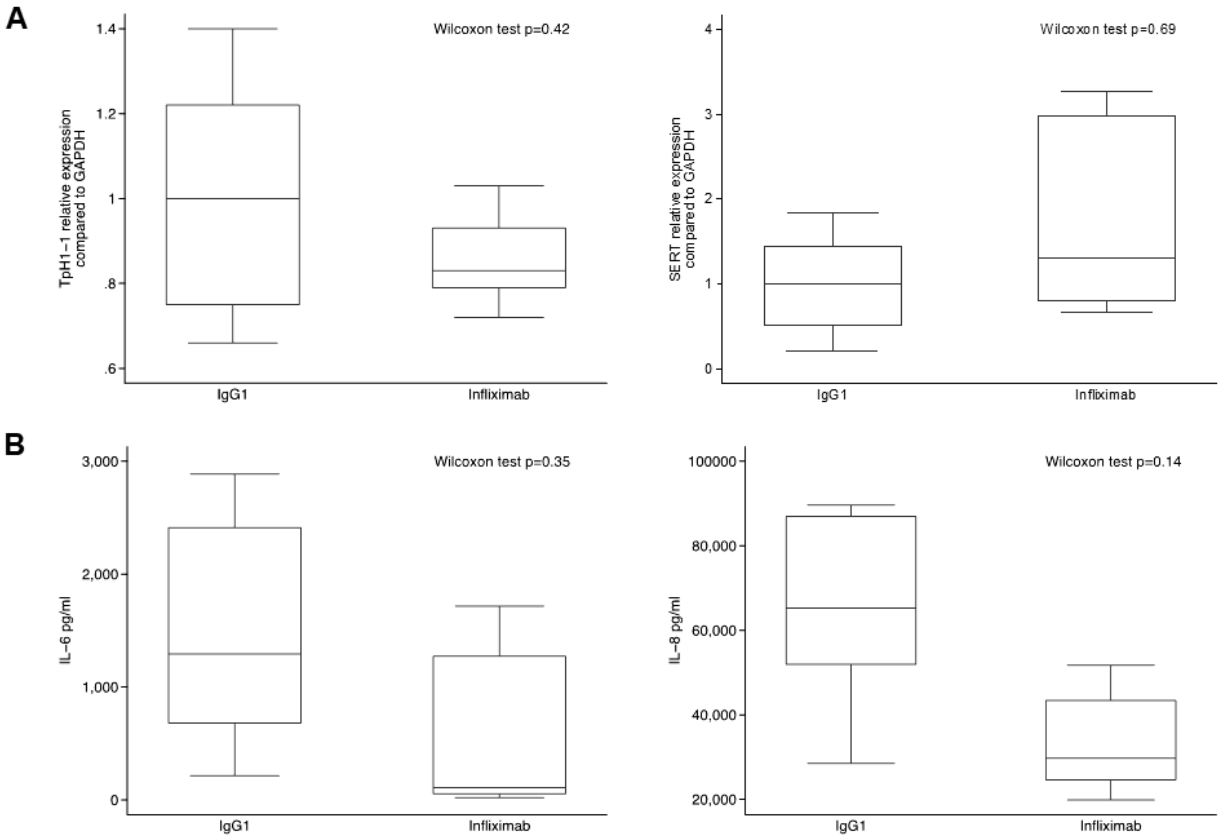
A
TpH1−1 relative expression compared to GAPDH
1.4
1.2
1
.8
.6
Wilcoxon test p=0.42
IgG1
Infliximab
SERT relative expression compared to GAPDH
4
3
2
1
0
Wilcoxon test p=0.69
IgG1
Infliximab
B
IL−6 pg/ml
3,000
2,000
1,000
0
Wilcoxon test p=0.35
IgG1
Infliximab
IL−8 pg/ml
100000
80,000
60,000
40,000
20,000
Wilcoxon test p=0.14
IgG1
Infliximab

Figure 4

Table 1. Studies analyzing neuroendocrine cells in inflammatory bowel disease

Authors	Disease type	N	Site	Detection method	Counting method	Findings
Greenberg et al.[18]	Pouchitis	12	Pouch	IHC for neurotensin, enteroglucagon and PYY	*Per* mm of epithelium	Decrease in neurotensin-, enteroglucagon- and PYY-positive cells in pouch mucosa (compared to normal ileal mucosa)
Pietroletti et al.[19]	Pouchitis	9	Pouch	IHC for CgA, enteroglucagon, serotonin, PYY, neurotensin and somatostatin	*Per* visual field (x10)	Decrease in neurotensin-, enteroglucagon-, PYY-positive cells in pouch mucosa (compared to normal ileal mucosa). No difference of CgA- and somatostatin-positive
Shen B et al.[20]	IPS	36	Pouch	IHC for serotonin	*Per* 4200 epithelial cells	Increase in serotonin-positive cells in IPS (compared to normal pouch mucosa)
Verity et al.[10]	UC	8	Colon	Gomori silver technique and diazo method	*Per* 100 crypts	Decrease in serotonin-positive cells in UC (compared to normal colonic mucosa)
Watanabe et al.[7]	UC	25	Colon	IHC for somatostatin	*Per* 1000 epithelial cells	Decrease in somatostatin-positive cells in CD (compared to normal colonic mucosa)
El-Salhy et al.[5]	UC	17	Colon	IHC for CgA, enteroglucagon, serotonin, PYY and PP	Area of positive cells/area of epithelial cells	Increase in CgA- and serotonin-positive cells in UC (compared to normal colonic mucosa)
Ahonen et al.[8]	UC	6	Rectum	Formaldehyde-induced fluorescence after freeze-drying and Epon embedding	*Per crypt*	Decrease in serotonin-positive cells in UC (compared to normal rectal mucosa)
Kyösola et al.[9]	UC	13	Rectum	Glyoxylic-acid-induced fluorescence histochemical method	On a scale from 0 to 10	Decrease in serotonin-positive cells in UC (compared to normal rectal mucosa)
Gledhill et al.[6]	UC	10	Rectum	Grimelius's silver impregnation	*Per* crypt or mm of epithelium or mm of *muscularis mucosae*	Increase in neuroendocrine cells in UC (compared to normal rectal mucosa)
Coates et al.[11]	UC	22	Rectum	IHC for CgA and serotonin	*Per* mm of *muscularis mucosae*	Decrease in serotonin-positive cells in UC (compared to normal rectal mucosa)
Bishop et al.[3]	CD	10	Ileum	IHC for CgA, glucagon (N-terminal), somatostatin, neurotensin and PYY	*Per* mm of *muscularis mucosae*	Increase in CgA-positive and serotonin-positive cells (compared to normal ileal mucosa)
Moran et al.[4]	CD	38	Ileum	IHC for CgA and PYY; immunofluorescence for GLP-1	*Per* villous-crypt unit/HPF	Increase in CgA-positive and GLP-1-positive cells, but not in PYY-positive cells (compared to normal ileal mucosa)

CD, Crohn's disease; CgA, chromogranin; GLP, glucagon-like peptide; HPF, high power field; IHC, immunohistochemistry; IPS, irritable pouch syndrome; PP, pancreatic polypeptide; PYY, peptide YY; UC, ulcerative colitis.

Table 2. Clinical, endoscopic and pathological features of 17 patients undergoing restorative proctocolectomy and ileal pouch-anal anastomosis for ulcerative colitis

Pt	Sex	Age (yr)	Age at UC diagnosis (yr)	Age at proctocolectomy (yr)	Smoking habit	Reasons leading to proctocolectomy	Endoscopic Inflammation according to PDAI	Moskowitz criteria			IELs (%)
								Acute (0-6)	Chronic (0-6)	Total (0-12)	
1	F	44	34	39	Smoker	Unresponsive to medical treatment	0	1	2	3	6.5
2	F	46	25	41	Never smoker	Unresponsive to medical treatment	0	1	5	6	5.4
3	M	56	33	52	Past smoker	Dysplasia	2	3	4	7	6.0
4	M	28	15	23	Past smoker	Toxic megacolon	1	1	5	6	3.4
5	M	57	45	53	Never smoker	Unresponsive to medical treatment	2	3	6	9	5.2
6	F	61	28	58	Smoker	Dysplasia	2	3	5	8	10.2
7	F	26	17	22	Never smoker	Unresponsive to medical treatment	3	4	6	10	4.0
8	F	40	32	37	Never smoker	Unresponsive to medical treatment	2	1	5	6	4.0
9	F	40	22	35	Never smoker	Unresponsive to medical treatment	2	1	2	3	5.8
10	M	43	10	37	Past smoker	Dysplasia	1	1	5	6	3.0
11	M	42	38	40	Past smoker	Unresponsive to medical treatment	0	1	4	5	5.2
12	M	64	61	62	Never smoker	Unresponsive to medical treatment	2	1	5	6	4.6
13	M	28	26	32	Past smoker	Toxic megacolon	1	1	4	5	9.8
14	F	63	40	58	Past smoker	Cancer (T_1N_0)	3	3	5	8	2.2
15	F	45	28	39	Smoker	Unresponsive to medical treatment	2	1	5	6	5.6
16	M	37	26	32	Past smoker	Unresponsive to medical treatment	3	3	2	5	6.6
17	M	64	59	60	Never smoker	Unresponsive to medical treatment	3	6	6	12	11.4

F, female; IEL, intraepithelial lymphocyte; M, male; PDAI, Pouchitis Disease Activity Index; Pt, patient; UC, ulcerative colitis; yr, years.