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► **To cite this version:**

Gabrio Bassotti, Vincenzo Villanacci, Riccardo Nascimbeni, Moris Cadei, Stefania Manenti, et al..
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Alimentary Pharmacology and Therapeutics, 2011, 34 (1), pp.92. 10.1111/j.1365-2036.2011.04684.x .
hal-00637804

HAL Id: hal-00637804

<https://hal.science/hal-00637804>

Submitted on 3 Nov 2011

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**COLONIC MAST CELLS IN CONTROLS AND SLOW TRANSIT
CONSTIPATION PATIENTS**

Journal:	<i>Alimentary Pharmacology & Therapeutics</i>
Manuscript ID:	APT-0385-2011.R1
Wiley - Manuscript type:	Original Scientific Paper
Date Submitted by the Author:	14-Apr-2011
Complete List of Authors:	Bassotti, Gabrio; Gastroenterology & Hepatology SEction, Clinical & Experimental Medicine Villanacci, Vincenzo Nascimbeni, Riccardo Cadei, Moris Manenti, Stefania Sabatino, Giuseppe Maurer, Christoph Cathomas, Gieri Salerni, Bruno
Keywords:	Large intestine < Organ-based, Constipation < Topics, Histopathology < Topics, Motility < Topics

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COLONIC MAST CELLS IN CONTROLS AND SLOW TRANSIT CONSTIPATION PATIENTS

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Running head: colonic mast cells in constipation

Key words: colon, mast cells, slow transit constipation

CONFLICT OF INTEREST STATEMENT: NONE DECLARED

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Summary

Background. There is recent evidence that mast cells (MC) may play important roles in the gut, especially concerning visceral hypersensitivity and motor activity. However, most data are only available for clinical conditions characterized by diarrhea, where MC have chiefly investigated in the mucosal layer of the colon, and there is almost no information concerning constipation.

Aim. To investigate MC distribution in all colonic layers in controls and severely constipated patients.

Methods. Full-thickness specimens from colons of patients undergoing surgery for slow transit constipation (n= 29), compared **to controls**, were obtained and the number of MC (evaluated by **specific monoclonal antibodies**) counted as a whole and in single colonic segments (cecum, ascending, transverse, descending, and sigmoid).

Results. Compared to controls, constipated patients revealed significantly higher number of MC, both as overall number and in single colonic segments. The distribution of MC resulted fairly homogeneous in the various segment of the large bowel, in both controls and patients, **and no significant difference in the percentage of degranulated cells was found between groups.**

Conclusions. Colonic MC display a homogeneous distribution within the viscus. This cell population is shown to increase in severely constipated patients, and this might represent a compensatory mechanism trying to vicariate for the impaired propulsive activity of these patients.

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Introduction

Considered for many years to be cells specifically participating in allergic reactions, mast cells (MC) **have also relevant** roles in both innate and adaptive immunity and play a role in many different processes, including clearance of enteric pathogens, food allergies, visceral hypersensitivity, and intestinal cancer [1].

In the human gut MC, by interacting with other cell types, may influence motor and sensory functions [2]. **Thus, MC are** increased in the colonic mucosa of patients with irritable bowel syndrome (IBS) [3], **and correlated** with symptoms (especially pain and **diarrhoea**) in these subjects [4-8]. Interestingly, it has been demonstrated that mucosal MC mediators from IBS patients excite nociceptive visceral sensory nerves in experimental animal studies [9], providing some possible explanation **on the mechanisms** of visceral hypersensitivity in these patients.

However, MC could also play some role in other pathological conditions characterized by constipation as expression of an abnormal motility of the colon. For instance, it has been shown that patients with megacolon due to Chagas's disease display an increased number of MC **in the colon** [10-12], and the same is true for patients with Hirschsprung's disease [13-16].

Slow transit constipation (STC) is a subtype of chronic functional constipation

sometimes requiring surgery for symptom relief [17]. Since to date there are no data on the presence and distribution of colonic MC in **constipated patients**, purpose of the present study was to study these cells in STC patients, and compare the result with those obtained in control subjects.

Patients

Surgical full-thickness specimens from twenty-nine patients (24 women, 5 men, age range 27-75 yrs) undergoing subtotal colectomy with ileorectostomy for severe intractable constipation were **evaluated**. Twenty of these patients have previously been investigated in a study on enteric nervous system (ENS) abnormalities [18]. Inclusion criteria were: 1) long-standing history of constipation (more than three years); 2) one or fewer evacuations per week; 3) no frequent (more than two episodes per month) or chronic abdominal pain; 4) sensation of incomplete evacuation in >1/4 defecations; 5) negative history for (sub)occlusive episodes; 6) unresponsiveness to appropriate and intensive medical treatment, including high fiber diet, stimulant and osmotic laxatives, and enemas. Intestinal transit time, measured by means of radiopaque markers, was delayed in all patients (up to more than 240 hours). Causes of secondary constipation were excluded by drug history, physical examination and laboratory screening (blood chemistry, thyroid hormones and, where appropriate, oral glucose tolerance test, sex hormone profiles and antinuclear antibodies). To exclude organic diseases or mechanical causes of constipation and megacolon or megarectum, each patient underwent double-contrast barium enema and/or colonoscopy. Absence of Hirschsprung's disease was demonstrated by normal relaxation of the internal anal sphincter at anorectal manometry [19]. No patient had evidence of obstructed defecation, as documented by anorectal manometry and/or defecography.

At the time of surgery all patients were treated with osmotic laxatives (polyethylene glycol); two patients used antihypertensive drugs (lisinopril).

Controls

Since right and left sections of the colon were analyzed in patients, for cecum, ascending and transverse we obtained control samples from 10 patients (8 women, 2 men, age range

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3 38-70 years) undergoing right hemicolectomy, and for descending and sigmoid from 10
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5 patients (6 women, 4 men, age range 41-78 years) undergoing left hemicolectomy, both
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7 for non-obstructing cancer. These patients were not constipated and had no colonic
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9 dilatation. **At the time of surgery the patients were prepared with polyethylene glycol;**
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11 **three subjects used antihypertensive drugs (two lisinopril and one propranolol).** The
12
13 control specimens were taken at least 3 cm from the resection margin in tumour free
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15 areas.
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22 **Methods**

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24 Tissues were fixed in 10% neutral-buffered formalin for 24 hours, then full-thickness
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26 samples **from multiple colonic segments** (cecum, ascending, transverse, descending,
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28 and sigmoid areas) were taken and transversal sections obtained. **Sections were**
29
30 **processed for both conventional histology (H&E and Trichrome stain) and**
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32 **immunohistochemistry (IHC).**
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36 **MC detection.** A specific antibody targeting MC tryptase [20] (monoclonal mouse clone
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38 10D11, dilution 1:200, Novocastra, UK) was used. Paraffin sections were dewaxed and
39
40 rehydrated through decreasing alcohol series up to distilled water. Sections were then
41
42 subjected to heat-induced epitope retrieval by immersion in a heat-resistant container filled
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44 with citrate buffer solution (pH 6.0) placed in a pressure cooker and microwaved for 20
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46 minutes. Endogenous peroxidase activity was suppressed by incubation with 3% solution
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48 of H₂O₂ for 5 minutes.
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53 **In addition, MC were also assessed by an anti-Kit antibody (CD117, rabbit polyclonal**
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55 **antibody, IgG, dilution 1:50, Dako, Carpinteria, CA); sections were processed as**
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57 **above and Kit immunostaining was carried out using a peroxidase-based**
58
59 **visualization kit (Dako EnVision™), following the manufacturer's recommendations.**
60

Data analysis

Slides from patients and controls were coded and read in blind **by one pathologist (VV)**.

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 at x 40 magnification (Olympus BX 40). For each anatomic area (cecum, ascending, transverse, descending, and sigmoid) MC were counted in the mucosa, submucosa, inner muscle layer, outer muscle layer, and serosa. **The percentage of degranulated MC in the mucosa was also calculated for both groups. We categorized MC as intact or degranulated according to previously described criteria [21]. Cells were counted as intact if they had discrete, focal, and well circumscribed immunostaining for tryptase, whereas the degranulated ones had discrete and relatively focal staining for tryptase but did not have well circumscribed immunostaining.**

Moreover, since in previous investigations MC were found closely to nerve fibres [22,23], we evaluated whether these cells were also present at the level of the submucous or myenteric plexus.

Statistical analysis

Data were firstly analyzed with the D'Agostino-Pearson test, and resulted normally distributed; therefore, comparisons were made by the Student's t-test for independent values, the analysis of variance (ANOVA) for repeated measures, **and the test for difference of proportions**. Values of $p < 0.05$ were chosen for rejection of the null hypothesis. Data are presented as median (95% CI).

Ethical considerations

Since this was a retrospective study, no individual patient identification was involved and no study-driven clinical intervention was performed; therefore a simplified IRB approval was obtained.

Results

Conventional histology. No increased amount of fibrosis was found in patients, compared to controls, for both H&E and Trichrome stain.

Immunohistochemistry. As a first observation, it was found that the overall number of MC, in both controls and patients, decreased from the mucosal to the serosal surface.

Table 1 shows MC distribution in the mucosa of controls and patients and representative pictures are shown in Figure 1 (A-D).

Overall, MC were significantly increased in patients compared to controls ($p < 0.001$), even in single colonic segments. Interestingly, MC distribution was fairly homogeneous within different colonic segments in controls (ANOVA, $p = 0.79$) and STC patients (ANOVA, $p = 0.38$).

Table 2 shows MC distribution in the submucosa of controls and patients. Again, MC were significantly increased in patients compared to controls in the entire colon ($p = 0.001$), and in the various colonic segments; each separate segment of the viscus displayed homogeneous distribution of this cell population in both controls (ANOVA, $p = 0.32$) and STC patients (ANOVA, $p = 0.61$).

Table 3 shows MC distribution in the inner muscle layer of controls and patients. No significant differences were found between the two groups ($p = 0.09$), the single colonic segments, and the cells' distribution within segments in controls (ANOVA, $p = 0.7$) and patients (ANOVA, $p = 0.52$).

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3 Table 4 shows MC distribution in the outer muscle layer of controls and patients. The
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5 overall number of cells was significantly increased in patients compared to controls ($p=$
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7 0.002), whereas comparison of single segments showed no differences between groups in
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9 the ascending and descending colon. Distribution of MC within different colonic segments
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11 was slightly but significantly different in controls (ANOVA, $p= 0.0048$), but not in STC
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13 (ANOVA, $p= 0.5$).
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17 Table 5 shows MC distribution in the serosa of controls and patients. Again, no significant
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19 differences were found between the two groups ($p= 0.36$), the single colonic segments,
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21 and the cells' distribution within segments in controls (ANOVA, $p= 0.07$) and STC patients
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23 (ANOVA, $p= 0.50$).
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27 **MC numbers obtained by CD117 stain almost overlapped those obtained by trypsinase**
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29 **stain (data not shown), and the MC were morphologically differentiated by**
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31 **interstitial cells of Cajal by their different aspect. The percentage of degranulated**
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33 **MC (Figure 2 A) in the mucosa was not different between controls (1.07%) and**
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35 **patients (2.5%, $p= 0.13$), and in H&E sections these cells were always associated to**
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37 **the presence of eosinophils (Figure 2 B). No MC were found contacting neurons**
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39 **within the submucous or myenteric plexus (Figure 2, C and D).**
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45 46 Discussion

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48 Gastrointestinal MC are considered as a heterogeneous population of immunocytes that
49
50 may be subdivided in mucosal MC (mainly located in the mucosa) and connective tissue
51
52 MC (distributed in the serosal layer) [24]. There are **only a few** data on the distribution of
53
54 this cell population **within** the gut [25]; studies in experimental animal models showed that
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56 MC are evenly distributed along the **gastrointestinal tract** [26], **whereas data available**
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3 **for** the human colon (but only from the mucosa) reported a decreasing density from the
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5 cecum to the rectum [3], and an age-related reduction of MC in the rectal mucosa [27].
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8 The present study, conducted on full thickness surgical specimens, **investigated** the
9
10 distribution of colonic MC in normal subjects and patients with STC. We showed that this
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12 cell population displays a decreasing inward-outward (from mucosa to serosa) gradient,
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15 **that its density** is relatively constant in the different bowel layers of various colonic
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17 segments in both controls and constipated patients, and that the latter display a significant
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19 increase of MC, compared to controls, in the colonic layers of all colonic segments, except
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21 for the inner muscle layer and the serosa.
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24 **We chose to detect MC by IHC for tryptase and CD117, two sensitive and specific**
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26 **methods [28]. Although tryptase may be considered more specific for MC [29], the**
27
28 **results of tryptase and CD117 stains show a highly significant correlation (as in the**
29
30 **present study) [30,31], so that staining for mast cell tryptase alone apparently is**
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32 **sufficient.**
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36 What is the significance of these findings? MC have been chiefly investigated in the
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38 **duodenal and** colonic mucosa of IBS patients [32-34], where they are thought to
39
40 represent an important pathogenetic factor and a potential therapeutic target [36-38].
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43 However, **MC** have been less extensively studied in patients complaining of constipation.
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46 Apart from sporadic observations of an increased number of **colonic** mucosal MC in
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48 constipation-predominant IBS patients [39], the only available studies in constipated
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50 patients were obtained in chagasic patients [10-12] and in those with Hirschsprung's
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52 disease [13-16]. Concerning Chagas' disease, **the increase of MC** found in these subjects
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54 **has** been put in relation to the increased fibrosis found in the colon of these patients [11],
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57 whereas in Hirschsprung's disease **this** increase has been hypothesized as a possible
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59 mechanism of ENS reparation [15].
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3 Although we did not find an increased amount of fibrosis in the colon of our patients, we
4 feel that, since MC are a source of nerve growth factor [40,41], an increased number of
5 these cells could indeed, as hypothesized for congenital megacolon, represent an attempt
6 of the viscus at repairing the damaged neuroenteric circuitries present in these subjects
7 [18,20]. Moreover, the highest number of MC in constipated patients was found in the
8 mucosa and submucosa; in both human beings [4-8] and experimental animal models
9 [42,43] an increased number of mucosal MC has been reported described associated to
10 **diarrhoea**, probably due to the release of a wide variety of bioactive mediators by these
11 cells [44,45]. **The percentage of degranulated MC was higher in patients, although**
12 **not significantly compared to controls; interestingly, the degranulated MC were**
13 **always accompanied by the presence of eosinophils, enforcing the concept of the**
14 **eosinophil-MC axis [46] that is considered to play an important role in functional**
15 **gastrointestinal disorders.**

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34 Physiological data available on propulsive activity in severely constipated patients (i.e.,
35 those sometimes requiring surgery for refractoriness to other therapeutic options)
36 consistently show that the main events, represented by the high-amplitude propagated
37 contractions [47,48], are consistently reduced or absent [49-54]. However, **there is**
38 **evidence** that compensative mechanisms exist in these **patients, represented** by the
39 preservation of the low-amplitude propagated contractions [55], that try to vicariate the
40 propulsive deficit [56]. Interestingly, these contractions are identical to those recorded in
41 clinical conditions characterized by **diarrhoea** [57-59]. Thus, it could be hypothesized that
42 this increase could be an attempt by a deranged colon to counterbalance an impaired
43 propulsive function due to ENS abnormalities [18,20].

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In conclusion, **we** investigated the distribution of MC **in all colonic layers** of controls and
severely constipated patients, **and demonstrated** a significant increase of this cell

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3 population in **patients**, increase that could have a pathophysiologic meaning as an
4 attempt to boost, at least in part, a defective propulsive activity of these patients. Further
5 studies, possibly supported by experimental animal models, are obviously needed to
6 confirm **and establish whether** these findings **may have clinical values and therapeutic**
7 **implications.**
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20 **Financial support for this study: none**
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Table 1. MC distribution (median (95% CI)) in the mucosa of controls and patients. Data are expressed as median (95% CI)

	C	STC	p
Total	35 (29-41)	88 (86-92)*	*0.001
Cecum	7.5 (5.5-8.5)	19 (18-20)*	*0.0001
Ascending	7 (5-9)	17 (16-19)*	*0.0001
Transverse	8 (6-10)	19 (15-19)*	*0.0001
Descending	8.5 (4.5-11.5)	18 (16-20)*	*0.0001
Sigmoid	7 (6-8)	18 (16-19)*	*0.0001

Abbreviations: C= controls; MC= mast cells; STC= slow transit constipation

Table 2. MC distribution in the submucosa of controls and patients. Data are expressed as median (95% CI)

	C	STC	p
Total	26 (22-30)	48 (43-51)*	*0.0001
Cecum	5 (4-7)	9 (8-9)*	*0.0004
Ascending	6 (4-7)	10 (9-10)*	*0.0004
Transverse	3 (2-7)	9 (8-12)*	*0.0002
Descending	5 (4-7)	9 (8-10)*	*0.0002
Sigmoid	6 (3-7)	9 (8-11)*	*0.0003

Abbreviations: C= controls; MC= mast cells; STC= slow transit constipation

Table 3. MC distribution in the inner muscle layer of controls and patients. Data are expressed as median (95% CI)

	C	STC	p
Total	23 (21-234)	24(23-26)*	*0.07
Cecum	5 (2-6)	5 (4-7)*	*0.56
Ascending	5 (3-6)	4 (4-5)*	*0.85
Transverse	3 (3-5)	5 (4-6)*	*0.08
Descending	5 (4-5)	5 (4-6)*	*0.87
Sigmoid	5 (4-6)	5 (5-6)*	*0.52 vs C

Abbreviations: C= controls; MC= mast cells; STC= slow transit constipation

Table 4. MC distribution in the outer muscle layer of controls and patients. Data are expressed as median (95% CI)

	C	STC	p
Total	19 (18-21)	28(25-29)*	*<0.0001
Cecum	3 (2-4)	6 (5-6)*	*0.05
Ascending	4 (4-6)	5 (4-7)*	*0.13
Transverse	2 (2-5)	5 (4-6)*	*0.001
Descending	5 (4-5)	6 (5-7)*	*0.09
Sigmoid	3 (3-5)	5 (5-6)*	*0.0006

Abbreviations: C= controls; MC= mast cells; STC= slow transit constipation

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Table 5. MC distribution in the serosa of controls and patients. Data are expressed as median (95% CI)

	C	STC	p
Total	12 (12-15)	12 (12-14)*	*0.64
Cecum	2 (1-3)	2 (2-3)*	*0.4
Ascending	3 (2-4)	3 (2-3)*	*0.08
Transverse	2 (1-3)	2 (1-4)*	*0.6
Descending	3 (1-4)	3 (3-3)*	*0.7
Sigmoid	2 (2-3)	3 (2-3)*	*0.9

Abbreviations: C= controls; MC= mast cells; STC= slow transit Constipation

Figure legend

Figure 1. Representative images of MC in STC. A. Mucosal MC (tryptase, original magnification x 20). B. Submucosal MC (tryptase, original magnification x 40). C. Presence of MC in the inner muscle layer (tryptase, original magnification x 20). D. Serosal MC (tryptase, original magnification x 40).

Figure 2. A. Representative image showing a degranulated mast cell (arrow) compared to intact ones (tryptase stain, original magnification x40). B. H&E stain showing the presence of a degranulated mast cell (thin arrow) associated to the presence of eosinophils (thick arrow). Original magnification x100. C. Tryptase stain showing two mast cells (arrows) well outside a myenteric ganglion (original magnification, x40). D. CD117 stain showing interstitial cells of Cajal (arrows) near a myenteric ganglion, but no mast cells near or within the ganglion (original magnification, x40).

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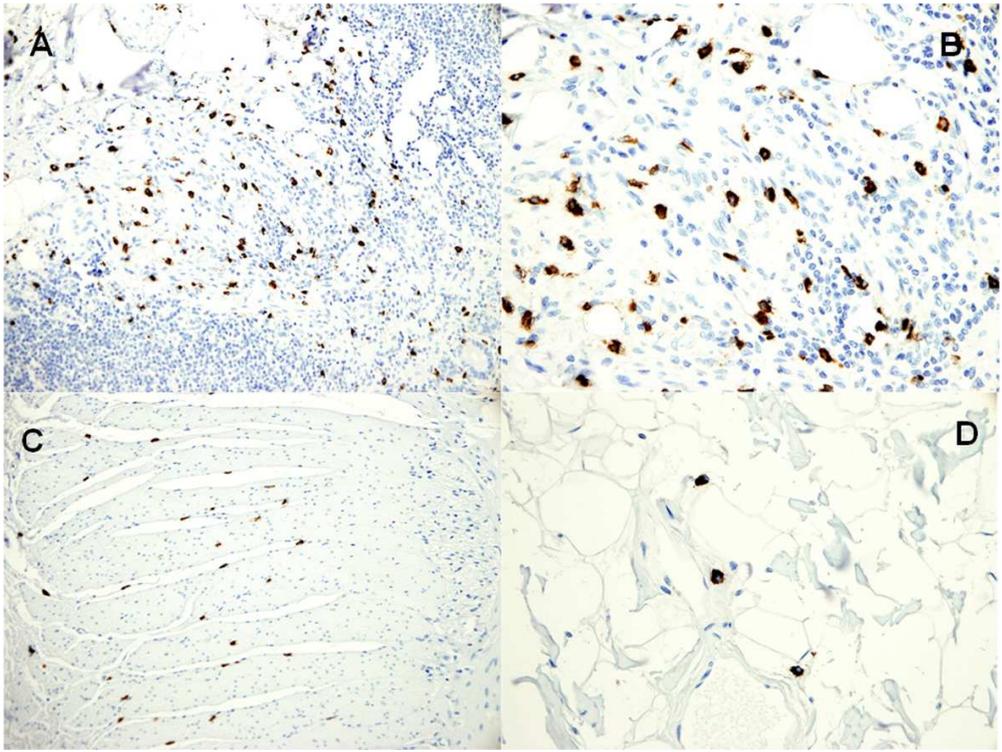


figure 1
99x75mm (300 x 300 DPI)

Review

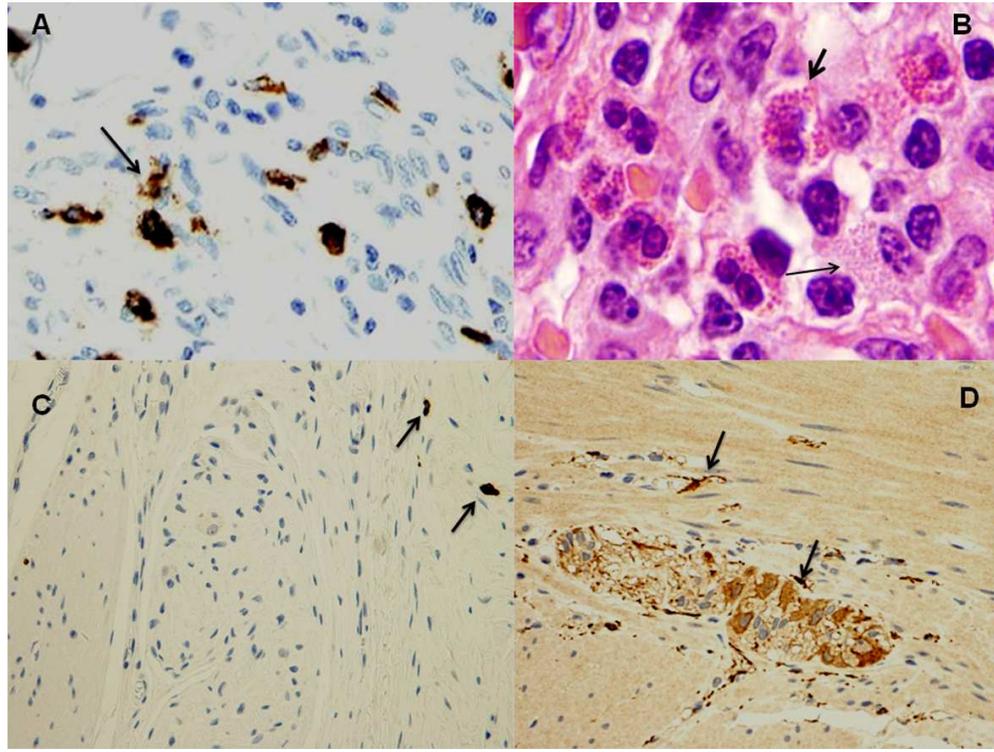


figure 2
99x75mm (300 x 300 DPI)

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