

# The Relationship Between Duodenal Enterochromaffin Cell Distribution and Degree of Inflammatory Bowel Disease (IBD) In Dogs

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## ABSTRACT

Despite numerous studies carried out over the last 15 years in veterinary medicine, the pathogenesis of canine Inflammatory Bowel Disease (IBD) has still not been completely elucidated. In particular, unlike what has been demonstrated in human medicine, the influence of serotonin on clinical signs in canine IBD has not yet been clarified. The objective of this paper has been to seek a possible correlation between duodenal epithelial distribution of serotonin-producing cells (enterochromaffin cells) and disease-grading parameters (clinical, clinico-pathological, endoscopic and histopathological) in dogs with IBD. The medical records of dogs with a diagnosis of IBD were retrospectively reviewed and 21 client-owned dogs with a diagnosis of IBD were registered. Clinical score (by Canine Chronic Enteropathy Clinical Activity Index), laboratory examinations (albumin, total cholesterol, folate, cobalamin), endoscopic score and histopathological score, were compared by regression analysis with duodenal enterochromaffin cell percentage. The study results suggested a relationship between a decrease in folate absorption and an increase in duodenal enterochromaffin cell percentage (regression equation  $y=16.89-6.14x$ ; coefficient of determination  $r^2= 0.7$ ; significant level:  $P=0.007$ ). However no significant relationship was evidenced between duodenal enterochromaffin cell percentage and the other analyzed variables.

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**Keywords:** Dog; IBD; Serotonin; Enterochromaffin cells.

## INTRODUCTION

Canine Inflammatory Bowel Disease (IBD) is a disease based on evidence of clinical signs of chronic diarrhea and/or vomiting associated with weight loss, only after the exclusion of enteric infectious, parasitic, endocrine or neoplastic diseases, food responsive enteropathy (FRE) and antibiotic responsive diarrhea (ARD) (1). For more than twenty years, IBD has been the major topic of discussion and research in canine gastroenterology and the interest in understanding its pathogenesis in the veterinary field

is also related to the increasing knowledge that, currently, characterizes similar human diseases, such as Crohn's disease and ulcerative colitis. Until now, several studies about its ethio-pathogenesis and treatment have been performed in veterinary medicine, but little attention has been paid to the dysfunction of the regulation of the enteric nervous system that characterizes this syndrome. Nervous control of gastrointestinal motility and secretion is a complex process, in which serotonin (5-HT) plays an important role (2). The majority of 5-HT is stored in enterochromaffin (EC)

cells present in gut epithelium, initiates peristaltic, secretory, vasodilatory, and nociceptive reflexes, and it is removed by serotonin-selective reuptake transporter (SERT), located on enterocytes, central or peripheral serotonergic neurons and platelets (3, 4, 5). Altered serotonergic metabolism has been described in human gastrointestinal diseases (6, 7). In veterinary medicine, a recent report presented evidence for a higher concentration of 5-HT and of the EC cell marker chromogranin-A (CgA) in the intestinal mucosa of dogs with IBD when compared with healthy controls (8).

Accordingly, we have evaluated, in canine IBD, the relationship between duodenal EC cell distribution and the degree of the clinical condition, the result of laboratory tests, endoscopic appearance and duodenal histology.

## MATERIAL AND METHODS

The medical records of dogs with a diagnosis of IBD, performed between January 2011 and November 2013 at the Department of Veterinary Medical Sciences (University of Bologna – Italy) were retrospectively reviewed.

Twenty-one client-owned dogs were included (seven crossbred and 14 purebred – Rottweiler [n=2], Basset Hound [n=1], Bolognese [n=1], Boxer [n=1], Cocker Spaniel [n=1], Dachshund [n=1], Epagneul Breton [n=1], German Shepherd [n=1], Great Dane [n=1], Labrador Retriever [n=1], Maltese [n=1], Pointer [n=1], West Highlander White terrier [n=1]). Of the 4 females 2 were spayed and of the 17 males 4 were castrated. The mean age of the dogs was  $5.8 \pm 3.2$  years.

The inclusion criteria comprehended clinical signs of chronic diarrhea, vomiting and weight loss, a physical examination suggesting an enteropathy and ruling out a gastrointestinal neoplasia or infectious diseases, a complete blood count, a serum biochemistry profile, serum folate concentration, serum cobalamin concentration and a negative fecal flotation test or treatment with fenbendazole (Panacur, Intervet Italia Srl, Milano, Italy) at 50 mg/kg q 24 h for three consecutive days during the last month.

Persistence of clinical signs (vomiting and diarrhea) that followed a dietary modification (hypoallergenic diet) for three weeks was applied to exclude FRE, and treatment to exclude ARD was applied for 3 weeks with tylosin at 15 mg/kg, PO, q 12 h (Tylan, Eli Lilly Italia, Firenze, Italy) or metronidazole (Flagyl, Zambon Italia, Vicenza, Italy) at 10 mg/kg PO, q 12 h.

An abdominal ultrasound examination, a complete endoscopic examination of the alimentary tract, and a histological examination of enteric bioptic samples concluded the diagnostic process, leading to the diagnosis of IBD.

## Clinical score

In order to be used for future comparisons, clinical data, obtained before treatment, were scored by the validated clinical score index (Canine Chronic Enteropathy Clinical Activity Index, CCECAI) (9). The analyzed parameters were attitude/activity, appetite, vomiting, stool consistency, stool frequency, body weight loss, serum albumin concentration, presence of peripheral edema/ascites and severity of pruritus. The parameters were analyzed with a score ranging from 0 (normal condition) to 3 (most severe condition).

The total score from 0 to 3 points indicates an insignificant disease, from 4 to 5 points indicates a mild disease, from 6 to 8 points indicates a moderate disease, from 9 to 11 point indicates a severe disease, and a score  $\geq 12$  points indicates a very severe disease.

## Laboratory analysis

From serum samples collected before treatment, albumin (reference range [RR] 2.8-3.7 g/dL), total cholesterol (RR 140-350 mg/dL), analyzed by photometric test (AU480, Beckman Coulter s.r.l., Milano, Italy), serum folate (RR 6.5-11.5  $\mu\text{g/L}$ ) and serum cobalamin (RR 250-730  $\text{ng/L}$ ), analyzed by immunoassay system (Immulite 2000, Siemens Healthineers, New Jersey, USA), were employed for further statistical analysis.

## Endoscopic score

Endoscopic video recordings of the duodenum, performed in a digital manner during the diagnostic procedure, were evaluated and scored following the endoscopic activity score index (EASI) as reported by Slovak *et al.* (2015) (10).

In particular the endoscopic parameters, evaluated with a score from 0 to 2, were: friability (bleeding on contact with endoscope or biopsy forceps), granularity (alteration in the texture of the mucosa), erosions (superficial linear mucosal defect[s] with hemorrhage) and lymphatic dilatation (multifocal to diffuse white foci within the mucosa).

## Histopathological score

Three to five duodenal biopsies, collected from each dog during the endoscopic examination, were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 $\mu$ m, and stained with hematoxylin and eosin. Sections were examined in all dogs following the histopathological standard of the World Small Animal Veterinary Association (WSAVA) Gastrointestinal Standardization Group (11).

In particular the morphological criteria (villous stunting, villous epithelial injury, crypt distension, lacteal dilation, mucosal fibrosis, goblet cells) and the parameters of inflammation (intraepithelial lymphocytes, lamina propria lymphocytes and plasma cells, lamina propria neutrophils) were scored with a 0-3 scale (0, normal state; 1, mild; 2, moderate; 3, severe).

The final score was the sum of all the considered parameters.

## Immunohistochemical analysis

Immunohistochemistry was performed with a streptavidin-biotin-peroxidase technique (Biospa, Milano, Italy) in order to demonstrate the percentage of EC cells with respect to all the cells of duodenal epithelial layer.

Replicate 4 $\mu$ m-thick sections were cut from the paraffin block of each case, incubated with hydrogen peroxide 0.3% in methanol for 20 minutes to block endogenous peroxidase activity and microwaved in citrate buffer (pH 6.0), for two cycles of 5 minutes for antigen retrieval. Sections were then incubated overnight at 4°C in a humid chamber with the primary antibody (Polyclonal rabbit anti-human chromogranin-A, Dako, Glostrup, Denmark) diluted 1:500 in PBS (pH 7.4, 0.01 M). Following washing in PBS, sections were then incubated with secondary biotinylated anti-rabbit IgG (Dako, Glostrup, Denmark) for 30 minutes at room temperature, and subsequently with streptavidin-peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 25 minutes at room temperature. After incubation in DAB chromogenic substrate solution (diaminobenzidine 0.02%, and H<sub>2</sub>O<sub>2</sub> 0.001% in PBS; Diagnostic BioSystems, Pleasanton, CA, USA) for 12 minutes, sections were immediately rinsed in PBS and in running tap water, counterstained with hematoxylin, dehydrated and mounted with DPX (Fluka, Riedel-de Haën, Germany).

Histological sections of normal canine pancreas were used as positive control to assess the specificity of the reactions. As

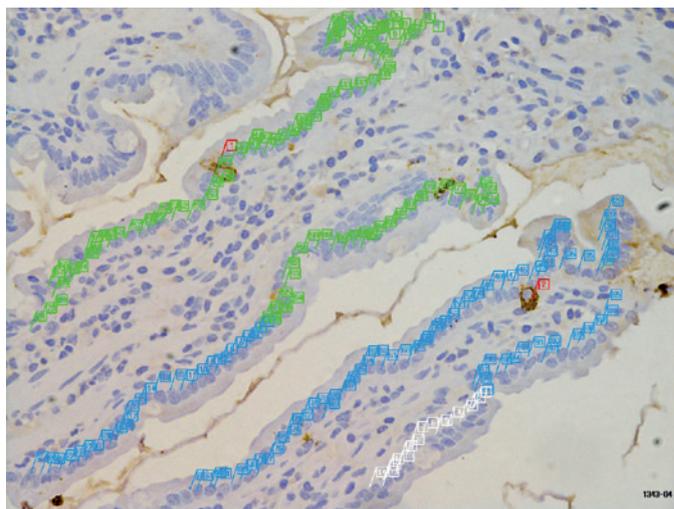
a negative control, an isotype-matched antibody of irrelevant specificity (NeoMarkers, Fremont, CA, USA) was used in place of the primary antibody.

The slides were first evaluated at low magnification, assessing the sites with a higher concentration of positive cells. These sites were then digitally captured at higher magnification (200X) with a digital capture system (Nikon DS-L3, Nikon Instruments S.p.A, Firenze, Italy) connected to a light microscope (Nikon Eclipse 55i, Nikon Instruments S.p.A, Firenze, Italy) and five fields per slide were stored.

Fields were considered adequate for evaluation if villi were sectioned longitudinally, and the images were captured in order to include as many epithelial cells as possible.

Through the Nikon DS-L3 digital software (Nikon Instruments S.p.A, Firenze, Italy) the cells positively labeled for CgA (i.e. dark brown color) were marked in red and counted; thereafter, the other enterocytes were marked in green, blue and white (color was changed each 100 cells in order to facilitate the counting procedure) and totaled (Figure 1).

The positively-labeled cells were then expressed as a percentage with respect to the total number of cells in the



**Figure 1:** Exemplification of the system used to quantify the density of enterochromaffin cells in biopsy sections. In chromogranin-A immunostained sections, the positive labeled cells (enterochromaffin cells) are marked in red, and the enterocytes are marked in green, blue or white (color was changed each 100 cells in order to facilitate the counting procedure). The density of enterochromaffin cells is then expressed with respect to the enterocytes number, regardless of the area occupied by the inflammatory infiltrate. Streptavidin-biotin-peroxidase technique, hematoxylin counterstain, 200x.

**Table 1:** Regression between the percentage of enterochromaffin cells (EC cells %) and clinical score (CCECAI), serum albumin, serum cholesterol, serum folate, serum cobalamin, endoscopic activity score index (EASI) and histopathological score, respectively.

Dependent Y	CCECAI	Albumin (g/dL)	Serum cholesterol (mg/dL)	Serum folate ( $\mu\text{g/L}$ )	Serum cobalamin (ng/L)	EASI	Histopathological score
Independent X	EC cells (%)	EC cells (%)	EC cells (%)	EC cells (%)	EC cells (%)	EC cells (%)	EC cells (%)
R <sup>2</sup>	0.06	0.16	0.10	0.67	0.01	< 0.01	<0.01
Regression equation	Y=8.9999-0.8328x	Y=1.1174+0.7604x	Y=118.3226+22.5887x	Y=16.8928-6.1360x	Y=326.5332-36.5200x	Y=2.0598-0.09406x	Y=11.6543-0.3543x
F-ratio	0.6503	2.1287	1.0523	13.9130	0.098187	0.02837	0.05889
Significance Level	P=0.439	P=0.173	P=0.332	P=0.007	P=0.763	P=0.869	P=0.812

epithelial layer, which allowed a reliable comparison among fields with different cellularity.

### Statistical analysis

Statistical analysis was performed with a commercially available program (MedCalc software, Ostend, Belgium). Assessment of data for normality was calculated by applying the D'Agostino-Pearson test. Data were expressed as median (95% confidence interval). A linear regression was applied between the percentage of duodenal EC cells and CCECAI, serum albumin, total cholesterol, serum folate, serum cobalamin, EASI, and histopathological index, respectively. Values of  $P < 0.05$  were considered statistically significant.

## RESULTS

CCECAI was performed in 19/21 dogs; albumin was analyzed in 20/21; total cholesterol in 18/21; serum folate in 16/21; serum cobalamin in 15/21 dogs; endoscopic score was calculated in 20/21; histopathological score in 18/21, and EC cell percentage with respect to duodenal epithelial layer cells in 14/21 dogs.

Median (95% confidence index) for CCECAI was 8 (6-10), for albumin was 1.59 g/dL (1.22-3.20), for serum cholesterol 160 mg/dL (107-192), for serum folate 8.09  $\mu\text{g/L}$  (4.55-12.68), for serum cobalamin 232 ng/L (159-271), for duodenal endoscopic score (EASI) 1.5 (1-3.8), for duodenal histopathological score 10.5 (8.4-13.2) and for duodenal EC cell percentage, with respect to duodenal epithelial layer cells, was 1.16% (0.94-1.98).

A significant negative relationship was found only between duodenal EC cell percentage and serum folate (regression equation  $y=16.89-6.14x$ ; coefficient of determination  $r^2=0.7$ ; significant level:  $P=0.007$ ); no significant relationship was

found between duodenal EC cell percentage and the other analyzed variables (Table 1).

## DISCUSSION

One of the central results of the research is represented by the lack of relationship between duodenal EC cell concentration and the clinical condition, serum albumin, total cholesterol, serum cobalamin, endoscopic index and histopathological score, respectively.

Essentially, this was not a unexpected result. Even if each of the employed grading systems (CCECAI, clinicopathological, EASI, histological) appeared able to define the severity of canine IBD (9, 11, 12, 13), it is questionable if a significant association between, clinical signs, serum biomarkers and intestinal histopathological findings exists in the canine IBD (14, 15).

With the exception of a recent paper by Bailey *et al.* (8), no previous studies have been performed on 5-HT metabolism in dogs with IBD. Moreover these researchers (8) showed a significant increase in 5-HT and CgA expression in duodenal biopsies of dogs with IBD compared to healthy dogs, but no analysis was performed within the IBD group in relation to IBD severity.

On the other hand, it is not possible to compare our results for CgA duodenal expression with Bailey *et al.* study due to different cellular count criteria.

In fact, they (8) manually counted the number of cells stained positively for CgA in 20 random high power fields per slide and calculated average data, while we counted the CgA positive cells in five fields per slide which was digitally stored and then expressed as a percentage with respect to the total number of epithelial layers.

The only relationship that we have been able to identify,

was the negative relationship between serum folate concentration and duodenal EC percentage, indicating the probable involvement of EC cells in the damage of the proximal small bowel. In fact, even if its sensitivity/specificity is not high, the decrease of serum folate could be interpreted as an indication of proximal small intestinal malabsorption (16).

One of the limits of our study is that we counted the CgA positive cells, assuming incorrectly, that they all produce 5-HT, with the consequence of a possible 5-HT producing cells over-count. Although CgA could mark all EC cells and not only the 5-HT producing EC cells, the duodenal CgA cell density has been elected in human gastroenterology as a reliable biomarker for the diagnosis of irritable bowel syndrome (7). Furthermore, Bailey *et al.* (8) substantiated, in duodenal histologic samples examined from healthy dogs and dogs with IBD, a significant relationship between duodenal CgA positive cells and duodenal 5-HT producing EC cells.

In colonic biopsies of human beings, 50–60% of CgA positive cells were also positive for 5-HT (6). We must also point out that we consider that the count of CgA cells instead 5-HT producing EC cells, in our study, involved the entire population, and, although there may have been some degree of over-count, it poorly affects the results.

A limitation of our retrospective study was represented by the evaluation of the EC cell count alone, omitting the 5-HT metabolic pathway, starting from tryptophan hydroxylase-1 (enzyme for 5-HT synthesis) up to 5-HT reuptake transport protein (SERT), expressed by platelets, nerve terminals, mucosal enterocytes, and vascular endothelial cells. These mediate the intracellular reuptake of 5-HT, reducing its availability (3, 4, 5). In fact, an increase in mucosal 5-HT content could be due to a decrease of SERT level, rather than an hyperplasia of EC cells producing 5-HT (17). The membrane expression of SERT can be altered by phosphorylation by protein kinase C (PKC) leading to internalization of SERT and reduction in 5-HT uptake rates in SERT-expressing cell lines (18).

Interferon- $\gamma$  and tumour necrosis factor  $\alpha$  significantly and synergistically impair SERT functions, thus inducing a decrease in both SERT mRNA and protein levels (19).

Instead, IL-10 has showed to have a dual effect on SERT, in relation to its concentration. At a high concentration, IL-10 has been shown to induce an increase of SERT activity and expression in the cell membrane, while a low concentration inhibits SERT activity (20).

In conclusion, since the previous study (8) has only compared healthy dogs and dogs with IBD, without addressing what were the changes in the metabolic pathway of serotonin within IBD group, further research is required to improve our understanding of the involvement of 5-HT in the pathogenesis of canine IBD, evaluating if SERT activity is related with IBD severity, and therefore if the decrease in 5-HT reuptake is linked to nociception and clinical signs in these patients.

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#### CONFLICT OF INTEREST STATEMENT

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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