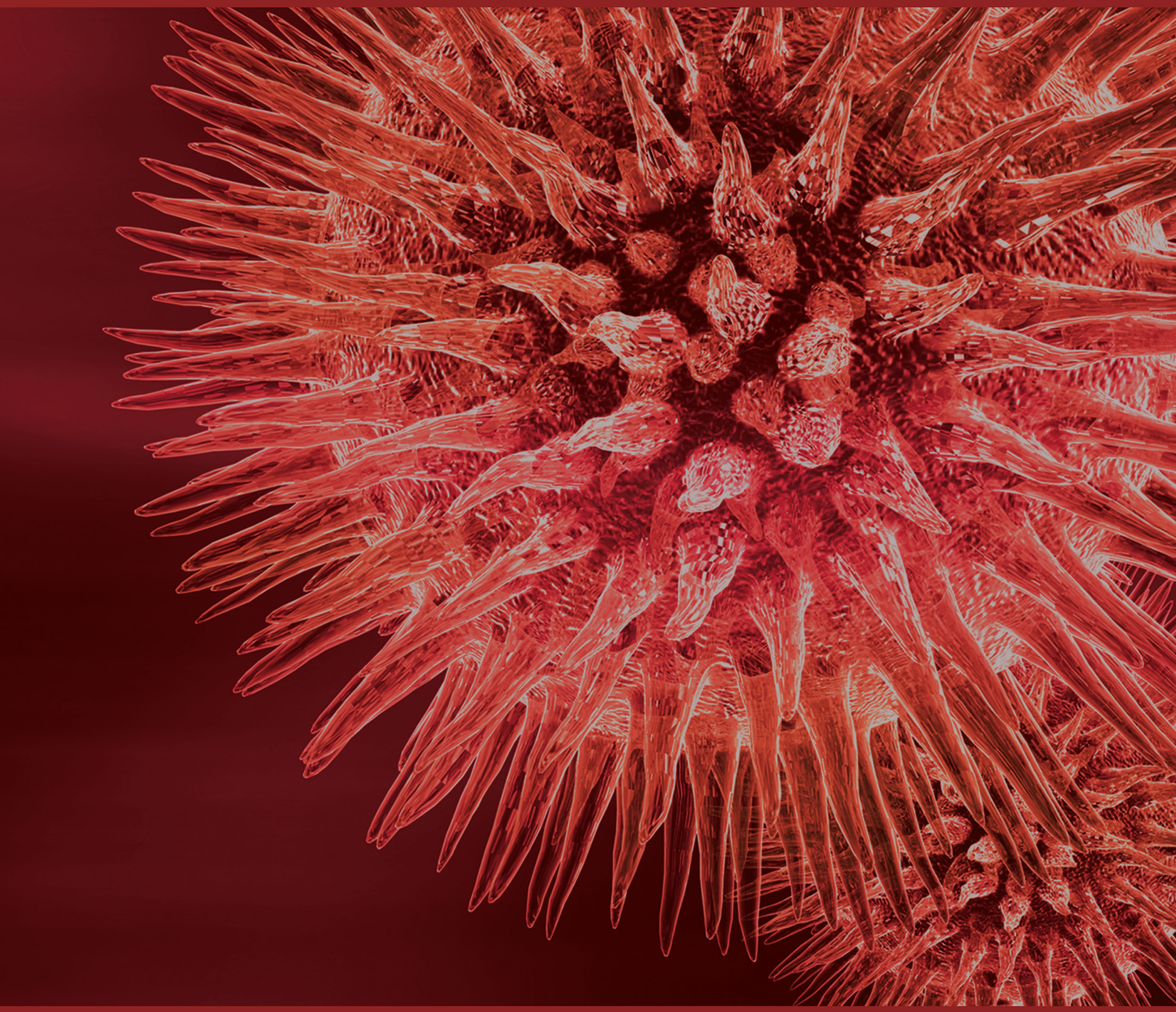
BioMed Research International

Novel Topics in Inflammatory Bowel Disease

Guest Editors: Atsushi Sakuraba, Alessandro Armuzzi, Kian Keyashian, and Makoto Naganuma



Novel Topics in Inflammatory Bowel Disease

BioMed Research International

Novel Topics in Inflammatory Bowel Disease

Guest Editors: Atsushi Sakuraba, Alessandro Armuzzi, Kian Keyashian, and Makoto Naganuma

Contents

Hindawi Publishing Corporation
BioMed Research International
Volume 2016, Article ID 8958751, 1 page
http://dx.doi.org/10.1155/2016/8958751

Editorial

Novel Topics in Inflammatory Bowel Disease

Atsushi Sakuraba,[1] Alessandro Armuzzi,[2] Kian Keyashian,[3] and Makoto Naganuma[4]

[1]*Section of Gastroenterology, Hepatology and Nutrition, University of Chicago, 5841 S. Maryland Avenue, MC 4076, Chicago, IL 60637, USA*
[2]*IBD Unit, Complesso Integrato Columbus, Gemelli Hospital-Catholic University Foundation, 00168 Rome, Italy*
[3]*Division of Gastroenterology, Oregon Health & Science University, Portland, OR 97239, USA*
[4]*Department of Gastroenterology and Hepatology, Keio University School of Medicine, Tokyo 160-8582, Japan*

Correspondence should be addressed to Atsushi Sakuraba; asakurab@medicine.bsd.uchicago.edu

Received 11 January 2016; Accepted 11 February 2016

We are pleased to announce the publication of the special issue focusing on novel topics in inflammatory bowel disease. It gained the interest of researchers from all over the world and more than 20 articles were submitted for review. Among them, our editorial team consisting of four renowned researchers in this field has selected six articles for publication. This includes basic and clinical research studies as well as translational research focusing on mechanism, epidemiology, treatment outcomes, and genetics. We are confident that this special issue advances the understanding and research of inflammatory bowel disease.

Acknowledgment

We would also like to thank all the reviewers who have participated in the review process of the articles submitted to this special issue.

Atsushi Sakuraba
Alessandro Armuzzi
Kian Keyashian
Makoto Naganuma

Hindawi Publishing Corporation
BioMed Research International
Volume 2016, Article ID 5989128, 10 pages
http://dx.doi.org/10.1155/2016/5989128

Research Article

Immunohistochemical Studies on Galectin Expression in Colectomised Patients with Ulcerative Colitis

Mattias Block,[1] Johan Mölne,[2] Hakon Leffler,[3] Lars Börjesson,[1] and Michael E. Breimer[1]

[1]*Department of Surgery, Sahlgrenska Academy, University of Gothenburg, Sweden*
[2]*Department of Pathology, Sahlgrenska Academy, University of Gothenburg, Sweden*
[3]*Department of Laboratory Medicine, Lund University Hospital, Lund, Sweden*

Correspondence should be addressed to Michael E. Breimer; michael.breimer@gu.se

Received 20 July 2015; Revised 11 November 2015; Accepted 20 December 2015

Academic Editor: Atsushi Sakuraba

Introduction. The aetiology and pathogenesis of ulcerative colitis (UC) are essentially unknown. Galectins are carbohydrate-binding lectins involved in a large number of physiological and pathophysiological processes. Little is known about the role of galectins in human UC. In this immunohistochemical exploratory study, both epithelial and inflammatory cell galectin expression were studied in patients with a thoroughly documented clinical history and were correlated with inflammatory activity. *Material and Methods*. Surgical whole intestinal wall colon specimens from UC patients ($n = 22$) and controls ($n = 10$) were studied. Clinical history, pharmacological treatment, and modified Mayo-score were recorded. Tissue inflammation was graded, and sections were stained with antibodies recognizing galectin-1, galectin-2, galectin-3, and galectin-4. *Results*. Galectin-1 was undetectable in normal and UC colonic epithelium, while galectin-2, galectin-3, and galectin-4 were strongly expressed. A tendency towards diminished epithelial expression with increased inflammatory grade for galectin-2, galectin-3, and galectin-4 was also found. In the inflammatory cells, a strong expression of galectin-2 and a weak expression of galectin-3 were seen. No clear-cut correlation between epithelial galectin expression and severity of the disease was found. *Conclusion*. Galectin expression in patients with UC seems to be more dependent on disease focality and individual variation than on degree of tissue inflammation.

1. Introduction

Ulcerative colitis (UC) is a chronic, relapsing, and remitting inflammation of the large intestine [1]. It often starts at young age and lasts throughout life. Debilitating symptoms, such as increased frequency of bloody stools, pain, fever, and lack of effective treatment, lead to the need for surgical removal of the whole large intestine in one-third of the patients [2]. For these reasons it is of great interest to understand the molecular mechanism behind the disease in order to identify new targets that can be modified as therapy. The aetiology and pathogenesis of UC are unclear [3] but are known to include the intestinal microflora, the intestinal barrier function, and the immune system and modifications of these by genetic polymorphisms [4].

Galectins, a family of soluble carbohydrate-binding proteins (lectins), have emerged as one possible therapeutic target in inflammatory bowel disease (IBD). This is based mainly on experiments in animals and cell culture [5] pointing to potential pathophysiology roles in IBD, the fact that galectins tend to be well tolerable, and the therapeutic effects of galectin inhibitors in inflammatory disease of other tissues. Galectins are defined by a conserved carbohydrate recognition domain (CRD) with affinity for β-galactosides as found in glycoproteins and glycolipids [6] and occur in different types as shown in Figure 1. Galectins are synthesized in the cytosol and may have functions there and in the nucleus [7].

An important emerging mechanism of action involves their transfer, by nonclassical secretion, into vesicles or extracellularly, where they encounter β-galactoside containing glycoproteins, which they may cross-link. This enables galectins to direct subcellular trafficking, organize membrane architecture, affect cell adhesion, and/or induce cell signals in the same or other cells [7–9]. This in turn is manifested at the organism level as rate-limiting effects on inflammation,

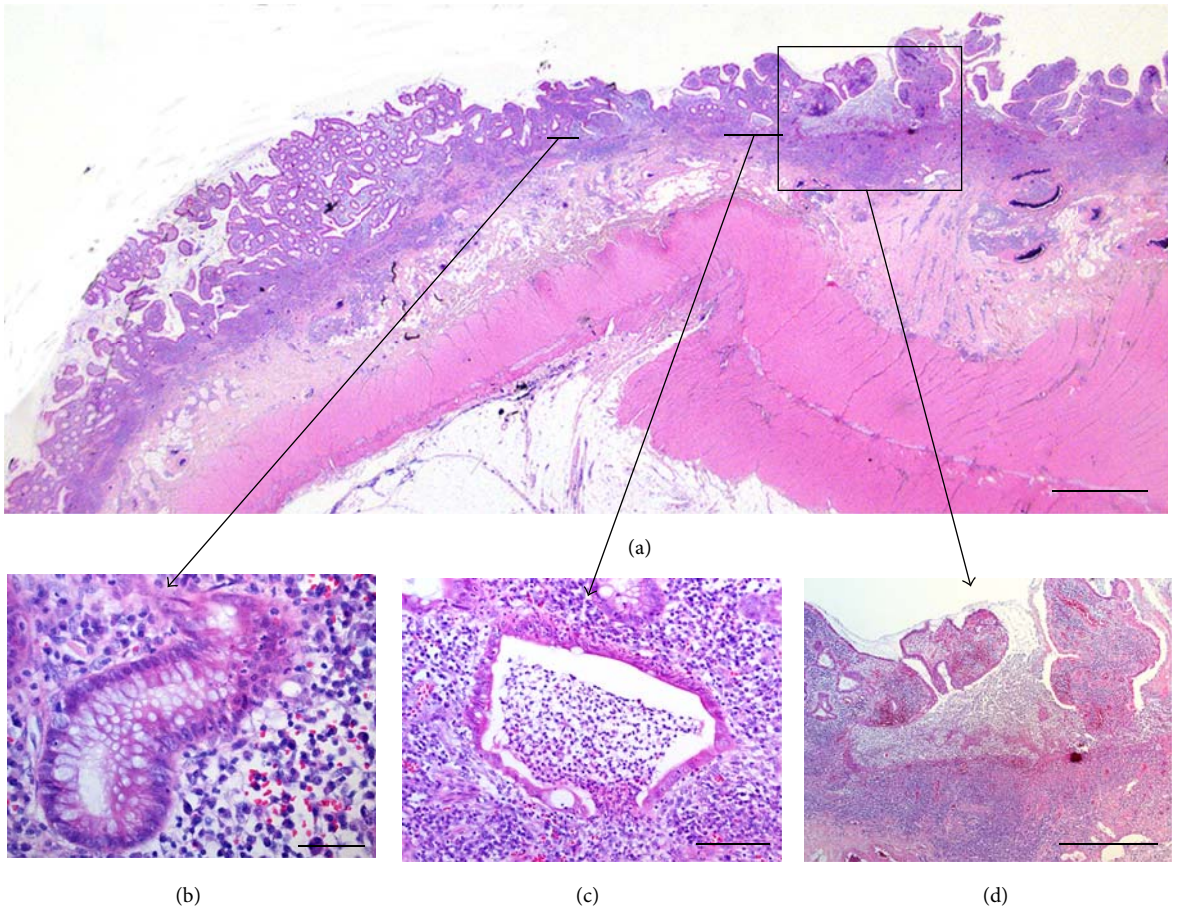


Figure 1: Illustration of the histological features for inflammatory grading of UC colon mucosa according to [17]. Top panel (a) shows a haematoxylin-eosin stained colon section from a patient with severe inflammatory activity (Table 1, patient 11, left colon). Below are shown magnifications of three selected areas illustrating cryptitis (panel (b), marked with black arrows), abscess (c), and an ulceration (d) all present in close proximity to each other. Bar in (a) = 2 mm, (b) = 50 μm, (c) = 100 μm, and (d) = 1 mm. Note that the sizes of biopsies obtained by endoscopy usually are 3-4 mm.

immunity, and cell growth [10–12]. In the large intestine, additional potential galectin mediated effects exist including modification of barrier function and interaction with microbes [13]. For this reason, galectins represent targets for therapeutic intervention of disease [14–16].

Various aspects for the role of galectins in IBD pathogenesis have been studied, most of these in mouse colitis models [5]. In these models, both pro- and anti-inflammatory properties of different galectins have been identified [18–24] and related to concomitant changes in glycan structures [22, 23]. Galectin-1 is mainly anti-inflammatory, by induction of apoptosis in T-cells [5]. Galectin-2 has been studied much less but has been found mainly to be anti-inflammatory and supporting wound healing in the intestine [19]. Galectin-3 has been studied extensively and it is required for polarized targeting of some glycoproteins in intestinal epithelial cells [7, 24]. It is also highly expressed in macrophages and is mainly proinflammatory but also protects against tissue damage. Recent studies suggest rate-limiting roles of galectin-3 in the fibrosis accompanying some chronic inflammation [25]. The role of these different effects in intestinal inflammation remains unclear [5]. Galectin-4 is highly and specifically expressed in intestinal epithelial cells and may direct polarized trafficking [26] and formation of super rafts [27] as well as being bactericidal [28]. In IBD, galectin-4 has the most distinct pathogenic role as a specific activator of intestinal, but not other, CD4-positive T-cells [29].

Galectins have been studied in human IBD both as serum biomarkers [30] and in tissue samples, but there are restrictions in the clinical interpretation of data obtained since patient classification in terms of disease severity, intestinal inflammatory grade, and pharmacological treatment is limited [31–33]. Most studies were performed using tissue biopsies obtained by endoscopy that only allow evaluation of a limited amount of superficial mucosal tissue from selected areas of the intestinal wall. Considering the complexity of UC, the aim of this study was to explore the galectin expression in whole intestinal wall surgical specimens, not

Table 1: Clinical data of patients with UC included in the study. Study patients are grouped according to tissue inflammation degree as mild (cryptitis, $n = 2$), moderate (crypt abscesses, $n = 4$), and severe (ulcerations, $n = 16$) as described in Section 2. In addition, control colon tissues ($n = 10$) obtained from patients with volvulus and colon cancer were studied.

No[1]	Gender/age[2]	Performed procedure[3]	Disease duration[4]	Medical therapy SS/SL/AS/AL/IM[5]	Mayo-score[6]	CRP mg/L[7]	Infl-grade[8]
18	M/49	C	19	−/−/+/−/−	6	20	Mild
19	F/49	RR	21	−/−/−/−/−	10	5	Mild
1	M/29	C	3	+/−/+/−/+	7	5	Moderate
14	M/65	RR	10	−/−/+/−/−	10	5	Moderate
48	F/36	C	4	−/−/+/−/+	10	4	Moderate
50	F/38	C	6	−/−/+/−/+	5	9	Moderate
2	F/49	C	21	+/+/−/−/−	5	28	Severe
3[9]	F/22	C	7	+/+/−/−/−	10	5	Severe
4	F/53	C	2	+/+/+/−/−	9	5	Severe
5	F/28	RR	6	−/−/−/−/−	9	5	Severe
8	F/52	RR	25	−/−/−/−/−		5	Severe
11	F/38	C	21	+/−/+/−/+	8	44	Severe
13	F/27	RR	5	+/−/−/−/−	10	5	Severe
15	F/62	PC	8	+/−/+/+/−	7	5	Severe
17	F/33	C	7	+/+/+/+/−	8	160	Severe
22	M/42	C	14	+/−/−/+/−	11	5	Severe
26	F/39	RR	3	−/−/−/−/−	8	5	Severe
28[9]	M/24	C	9	+/−/+/−/−	11	22	Severe
29	M/20	C	7	+/+/+/−/+	6	18	Severe
40	M/35	RR	6	+/+/−/−/+	6	1	Severe
52	M/41	PC	5	−/−/−/−/+	11	2	Severe
53	F/27	C	17	−/−/−/−/+	10	10	Severe

[1]No = patient study number in IBD biobank.
[2]M = male. F = female. Age = years.
[3]C = colectomy. PC = proctocolectomy. RR = rectum resection.
[4]Disease duration = years.
[5]SS = steroids systemic. SL = steroids local. AS = azathioprine systemic. AL = azathioprine local. IM = immunomodulation.
[6]Mayo-score = clinical evaluation of the severity of the patient's disease.
[7]CRP = C-reactive protein.
[8]Infl-grade = total histopathological evaluation of the patients disease.
[9]Acute surgery.

earlier performed, as well as correlate epithelial galectin-1, galectin-2, galectin-3, and galectin-4 expression with degree of intestinal inflammation.

2. Material and Methods

2.1. Patients. All patients with IBD undergoing acute or elective colectomy, proctocolectomy, or rectal resection at the Colorectal Unit, Sahlgrenska University Hospital/Östra Campus, from 2008 to 2011 were, prior to surgery, asked to participate in a multidisciplinary IBD research project approved by the Regional Ethical Review Board, University of Gothenburg, Sweden (http://www.epn.se). Among 78 included IBD patients, 22 consecutive patients with UC disease were included in this study and patient data is listed in Table 1. Excluded patients were those with UC operated with extirpation of ileal pouch-anal anastomosis (IPAA) and those with Crohn's disease. In addition, some patients had to be excluded due to logistic reasons such as lack of laboratory staff and out-of-office-hour surgery. Prior to surgery, all patients were examined and categorized using a modified Mayo-score [34] to measure disease severity with a scale range of 0–12, 12 being most severe disease. Variables measured were frequency of bowel movements (range 0–3), blood in stool (range 0–3), quality of life (range 0–3), and endoscopic evaluation (range 0–3). Only two patients undergoing acute resection were included in this study (cases 3 and 28). Ongoing and previous medications, in particular the use of steroids, 5-ASA, and immunomodulators such as TNF inhibitors, were listed. Median age of included patients was 38 (20–65). Median laboratory tests were haemoglobin 136 (101–156) g/L, C-reactive protein (CRP) 5 (1–160) mg/L, and albumin 38 (20–46) g/L. Symptom duration was 8 (2–25) years. Total modified Mayo-score was median 8 (5–11) and there was no correlation between the Mayo-score and the histopathological inflammatory grade. Indications for

surgery were chronic colitis in 19 patients and dysplasia in one patient and two patients underwent surgery due to acute colitis.

The patients excluded due to logistic reasons did not differ concerning age, blood tests, or disease severity. Of 12 excluded patients, 7 were males. Median age was 42 (21–67). Median laboratory tests at surgery were haemoglobin 132 (107–155) g/L, C-reactive protein (CRP) 13 (1–190) mg/L, and albumin 30 (26–40) g/L. Symptom duration was 15 (3–30) years. Total Mayo-score was median 7 (4–11). Eight patients in the excluded group were on oral steroids and one was on locally administrated steroids. Six had oral 5-ASA and one had 5-ASA locally. One patient was on immunomodulating therapy.

Control tissues were obtained from patients undergoing elective resection of the sigmoid colon and right-sided hemicolectomy due to repeated sigmoid volvulus ($n = 2$) or colonic cancer ($n = 8$), respectively. Collection of control tissues was sampled as far as possible (minimum 10 cm) from the tumor.

2.2. Tissue Specimens. Resected colonic tissue was immediately embedded in a plastic bag in the operating theatre, covered with crushed ice, and transported within 45 minutes to the Department of Pathology. Tissue specimens were collected according to a standardized protocol from each colonic and rectal area; from each patient, 5-6 (rectal resection) to 15–20 separate (colectomy) samples were analysed. In addition, specimens from selected areas were collected according to the pathologist judgment. The specimens were fixed in formalin and embedded in paraffin.

Paraffin sections of full wall colon tissue samples were obtained and stained with haematoxylin-eosin. The inflammatory activity for each patient was graded, according to suggested criteria for grading disease activity in UC [17], as mild (cryptitis), moderate (crypt abscesses), or severe (ulcerations) illustrated in Figure 2. All specimens were coded and analysed in a blinded fashion by two investigators (Johan Mölne and Mattias Block). The clinical course of each patient as well as the final histopathological evaluation based on all biopsies from each specimen was unknown to the investigators when they were evaluating the inflammatory grade and galectin expression in each tissue slide.

2.3. Anti-Galectin Antibodies. Polyclonal antisera were raised in rabbits and characterized as described for anti-rat galectin-1 (diluted 1 : 800) [35–37], anti-human galectin-2 (1 : 600) [38], and anti-rat galectin-4 (1 : 50000) [39, 40]. A commercially available rat monoclonal anti-mouse galectin-3 (anti-Mac-2, clone 3/38) (1 : 500) [41] has been used extensively by us [39] and others.

2.4. Immunohistochemistry. The EnVision Flex High pH (Link) detection kit (Dako K8000, Copenhagen, Denmark) was used. The most important steps were as follows. Consecutive series of paraffin sections were produced at a 4 μm constant thickness setting. Antigen retrieval was done in tris/EDTA buffer, pH 9 (Dako K8004), by microwave oven heating and endogenous peroxidase activity was blocked by immersion in peroxidase-blocking solution (Dako K8000) for 5 minutes at RT. Immunostaining was performed in a computer-assisted Autostainer Plus processor (Dako). Incubation time for primary antibodies was 30 minutes at RT, terminated by repeated washing, followed by incubation with a dextran polymer conjugated with secondary antibodies and horseradish peroxidase (HRP) for another 30 minutes. Slides were transferred to fresh hydrogen peroxide plus 3-3-diaminobenzidine tetrahydrochloride (DAB) solutions for 4 minutes. Finally, slides were stained with Mayer's haematoxylin and permanently mounted under cover slips. Omitting or replacing the primary antibodies with irrelevant antibodies produced negative controls. Optimal primary antibody dilutions were defined by staining normal colon using serial dilutions of each antibody.

2.5. Grading of Inflammation and Immunohistochemical Labelling. Intestinal tissue specimens from each patient, 5-6 (rectal resection) to 15–20 separate (colectomy), were studied. Galectin-1, galectin-2, galectin-3, and galectin-4 expressions were examined in 3 representative tissue blocks from each patient. The epithelial cell galectin staining intensity was recorded in epithelia with cryptitis and crypt abscess and in areas adjacent to ulcerations as well as in noninflamed areas as illustrated in Figure 2. The intensity of immunoperoxidase (IP) staining was recorded on a 4-level scale: negative: 0, trace amounts: 1, weakly positive: 2, and strongly positive: 3. If galectin expression showed a focal pattern this was assigned as (f). Representative tissue sections illustrating galectin-2–galectin-4 expression in noninflamed colon tissue and IBD tissue with abscesses and ulcerations are shown in Figure 3. Leukocytes were identified by morphology (neutrophil and eosinophil granulocytes) or identified by monoclonal antibodies using CD3 (Dako, N1617) for T-cells, CD68 (Dako, N1576) for macrophages, and CD138 (Dako, IR642) for plasma cells.

3. Results

3.1. Epithelial Galectin Expression in Control Colon Tissue. Galectin-1 in normal colon epithelial cells was negative except in a few cases showing a focal, minimal staining. This is in accordance with earlier studies on normal human colon [42] and stomach [43]. In nonepithelial cells (fibroblasts, endothelium, and smooth muscle), galectin-1 was weakly expressed, as described before [44].

Galectin-2, galectin-3, and galectin-4 were all strongly positive as exemplified in Figures 2(d), 2(g), and 2(j), respectively, and did not show any individual variation (Table 2).

Galectin-2 staining was localised to the entire cytoplasm of the cells but mucus droplets were negative (Figure 2(d)). An increased staining was seen on the epithelial cell apical membrane (Figure 2(d), insert).

Galectin-3 staining was localised to the entire cytoplasm of the cell but mucus droplets were negative (Figure 2(g)). There was a gradient of galectin-3 staining intensity with

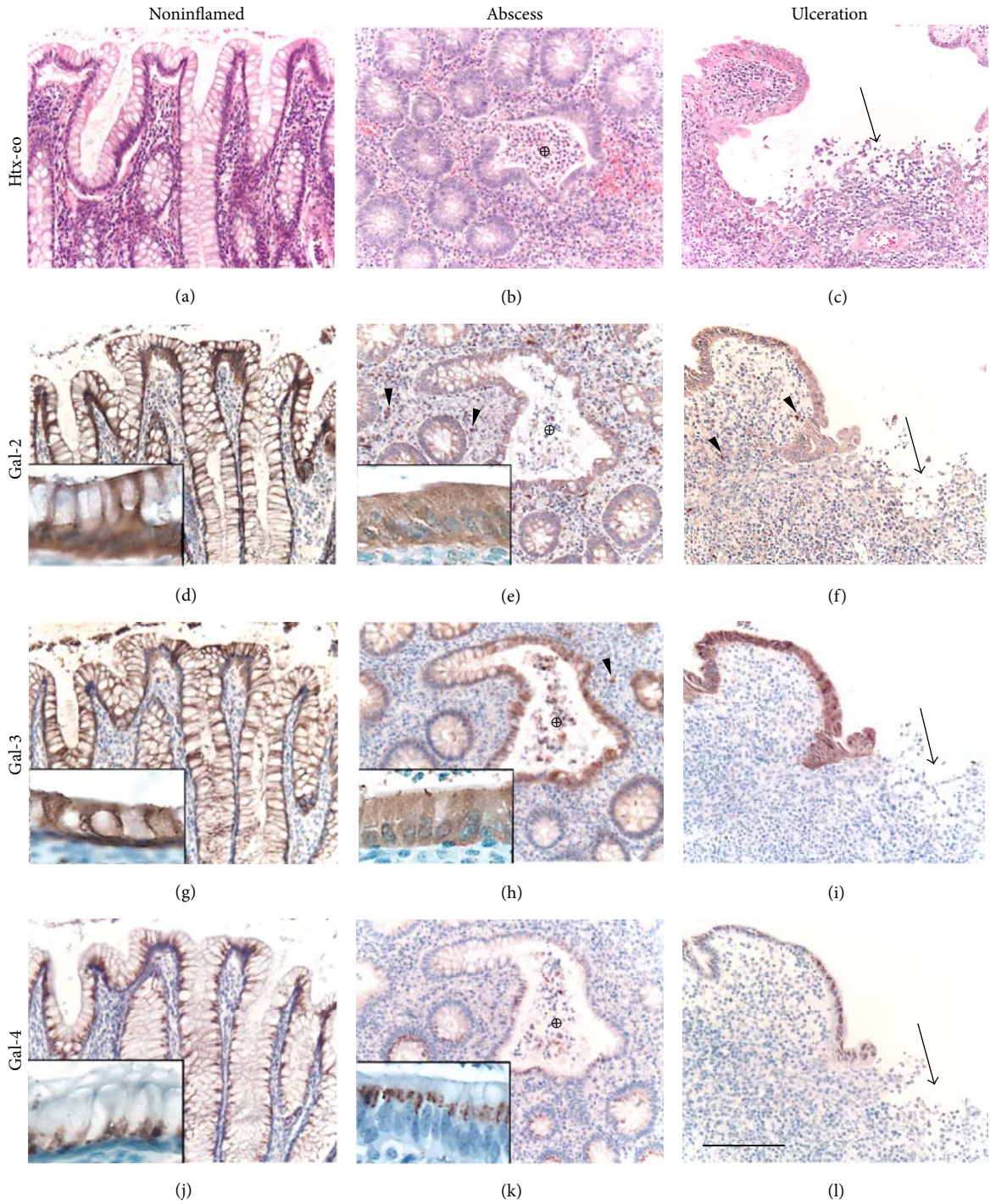


FIGURE 2: Immunostaining of galectin-2–galectin-4 in UC colon. Haematoxylin-eosin staining of noninflamed colon (a), ulcerative colitis with an abscess (marked ⊕ in (b)), and an ulceration (arrow in (c)) obtained from patient No 53 in Table 1. The noninflamed epithelium (a) contains cells filled with mucous and the crypts have a normal architecture. Immunohistochemical staining of serial sections from the same specimen for galectin-2, galectin-3, and galectin-4 is shown in rows 2 to 4, respectively. Galectins are predominantly expressed in the colonic epithelium. The inserts illustrate that galectin-2 and galectin-3 are homogeneously distributed in the cytoplasm while galectin-4 has a distinct perinuclear distribution most easily seen in (k) where mucus droplets are less abundant. In addition, a low number of inflammatory cells express a strong staining for galectin-2 and galectin-3, but not galectin-4, and this is illustrated for galectin-2 and galectin-3 by arrowheads in panels (e), (f), and (h). Bar = 200 μm.

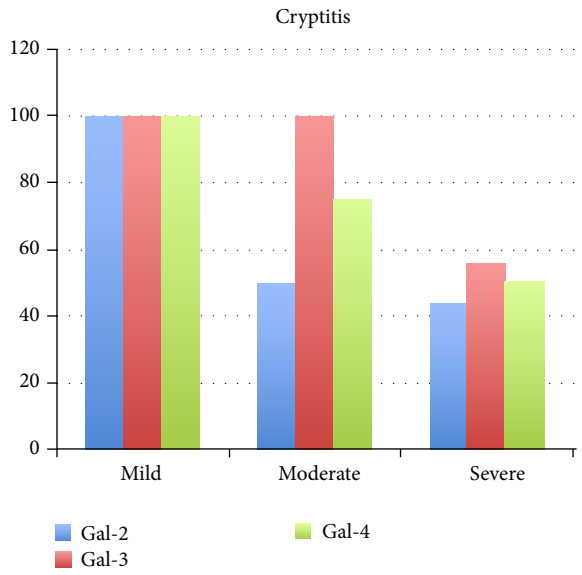


Figure 3: Summarized epithelial galectin-2, galectin-3, and galectin-4 expression in areas of cryptitis for the different individuals grouped according to the inflammatory grade (mild, moderate, and severe). Figures on the y-axis represent mean percentage of epithelial cell staining intensity; see Table 2.

strong surface epithelial expression and diminishing expression in crypts.

Galectin-4 in the control group showed, like galectin-2 and galectin-3, a strong expression pattern in all cases. However, compared to galectin-2 and galectin-3, labelling was different with a localised supranuclear distribution (Figures 2(j), insert, and 2(k)) and no staining was present in the remaining cytosol or plasma membrane.

3.2. Epithelial Galectin Expression in UC Colon (Study Group)

Galectin-1. Most of the UC patients, as well as the control individuals, lacked galectin-1 expression in the epithelial cells (not shown). Only a few cases showed a weak focal epithelial cell staining.

Galectin-2. In patients with mild inflammatory activity, the epithelial cell galectin-2 expression was identical to that seen in control individuals (Table 2). In those with moderate activity, most patients had a strong galectin-2 expression but some patients with severe activity showed a reduced-to-minimal epithelial expression in all 3 slides (Figures 2(e) and 2(f) and Table 2). Due to a reduced amount of mucus the entire cytoplasm was galectin-2 positive in areas of inflammation (Figures 2(e) and 2(f)).

Galectin-3. The colon epithelium showed the same gradient of galectin-3 staining intensity with strong surface expression and diminishing expression in crypts, as in the control group (Figure 2(g)). In inflamed areas, staining was seen in the whole cytoplasm, as for galectin-2 (Figures 2(h) and 2(i)). A reduced expression of galectin-3 in some individuals with increased inflammatory activity was observed but the majority of patients expressed normal levels in severely inflamed specimens (Table 2).

Galectin-4. A decreased galectin-4 expression in several patients with severe inflammatory activity (Figures 2(k) and 2(l) and Table 2) was observed but there was a great interindividual variation. The supranuclear distribution was seen also in inflamed epithelium (Figure 2(k) insert).

3.3. Individual Changes in Epithelial Cell Galectin Expression. In addition to Table 2, the epithelial galectin expression in areas with cryptitis, for the different individuals grouped according to the inflammatory grade (mild, moderate, and severe), is summarized in Figure 3. This shows that the galectin expression in the epithelial cells is decreased related to the severity of inflammation. However, when comparing individual patients, changes in epithelial cell galectin expression varied considerably between individual patients reflecting an individual pattern rather than a general reduction in galectin expression (Table 2). In the two cases with mild inflammation there was no change in galectin expression related to the inflammatory grade. Of the 4 cases with moderate inflammatory grade, 2 cases (No 48 and No 50) showed a galectin expression in the normal epithelial cells that was identical to that of the healthy controls (all positive) and no change in their expression was found irrespectively of inflammatory grade. Patients 1 and 14 had very small changes in galectin expression. The 16 patients with severe inflammation showed different pattern of changes in their epithelial cell galectin-2, galectin-3, and galectin-4 expression. These heterogenic patterns can be explained by the focality of UC disease and also individual factors among the patients as shown in Figure 1, where a patient with severe inflammation showed cryptitis, abscess, and ulceration in the same specimen.

3.4. Galectin Expression in Inflammatory Cells. In control colon tissue, inflammatory cells were seen in the lamina propria. Individual cells showed a labelling intensity comparable to inflammatory cells in the study. As expected, the number of inflammatory cells in the control tissue was considerably lower than in the study group.

The majority of inflammatory cells found in the intestinal wall in the study group were macrophages, lymphocytes, and plasma cells identified by morphology and specific CD markers. As expected, neutrophilic granulocytes were seen in areas of cryptitis, crypt abscesses, and ulcerations but were otherwise sparse. In the mucosa, approximately 50% of the leukocytes were plasma cells, 25% macrophages, and 25% T-cells. In the submucosa, the dominating cell type was macrophages, while only occasional and focal infiltrates of lymphocytes and plasma cells were seen.

Galectin-1 and galectin-4 were not present in any inflammatory cells.

Galectin-2 was strongly expressed in inflammatory cells in the majority of the patients irrespectively of the intestinal inflammatory grade. The labelling intensity was comparable to the galectin-2 expression in epithelial cells in very few

Table 2: Galectin expression in colon epithelial cells in patients with UC. Study patients are grouped according to degree of tissue inflammation (see Table 1) and normal controls ($n = 10$). Epithelial cell staining intensity for individual anti-galectin antibodies was graded as 0 to 3. Galectin expression is shown for the epithelial cells present in the uninflamed, cryptitis, abscess, and ulceration areas. Areas not applicable (i.e., ulceration in mild inflammation cases) are marked with —. Staining for galectin-1 was negative in the epithelial cells of both controls and UC cases and is not listed.

Patient number	Inflammatory grade*	Epithelial cell galectin expression#											
		Noninflamed area			Cryptitis			Abscess			Ulceration		
		Gal-2	Gal-3	Gal-4	Gal-2	Gal-3	Gal-4	Gal-2	Gal-3	Gal-4	Gal-2	Gal-3	Gal-4
18	Mild	3†	3	3	3	3	3	—¶	—	—	—	—	—
19	Mild	3	3	3	3	3	3	—	—	—	—	—	—
1	Moderate	2	3	1	2	3	1	2	3	1	—	—	—
14	Moderate	3	3	3	2	3	3	2	3	3	—	—	—
48	Moderate	3	3	3	3	3	3	3	3	3	—	—	—
50	Moderate	3	3	3	3	3	3	3	3	3	—	—	—
2	Severe	1	3	2	3	1	1	3	1	0	3	3	0
3	Severe	3	3	2	2	1	1	1	1	1	1	3	2
4	Severe	3	3	3	1	1	3	1	3	2	2	2	3
5	Severe	2 (f)‡	3	3	1	1	3	1	3	3	1	2	3
8	Severe	3	3	3	2	3	2	—	—	—	1	1	1
11	Severe	3	3	3	2	3	2	2	3	2	2	3	2
13	Severe	2	3	2	—	—	—	—	—	—	1	1	2
15	Severe	3	3	3	3	3	2	3	3	2	3	3	3 (f)
17	Severe	3	3	3	2	3	3	2	3	3	3	3	3
22	Severe	3	3	3	3	3	3	3	3	3	2	3	2
26	Severe	3	3	3	1	3	3	1	3	3	3 (f)	3	2
28	Severe	1	3	1	3	3 (f)	3	3 (f)	3 (f)	3	3 (f)	3 (f)	3
29	Severe	3	3	3	3	2	3	3	2	3	2	2	2
40	Severe	3	3	2	—	—	—	2	2	3	3	3	3
52	Severe	3	3	1	3	3	1	—	—	—	3	3	1
53	Severe	3	3	3	3	3	3	3	3	3	3	3	3
Controls $n = 10$	Normal	3	3	3	—	—	—	—	—	—	—	—	—

*Histopathological classification of inflammatory activity.
#Galectin-2, galectin-3, and galectin-4 expression in epithelial cells present in the area.
†Galectin expression is graded according to a 4-level scale as follows: 0 = negative, 1 = trace, 2 = weak expression, and 3 = strong expression.
¶Empty fields (not marked with 0–3) mean no presence of cryptitis, abscess, or ulceration.
‡Focal distribution in some cells in the area.

inflammatory cells (<2%), while about 50% of the cells showed a weak expression (Figures 2(e) and 2(f)). Almost all of the strongly galectin-2 positive cells were identified as macrophages. However, in total, only a minority (10–20%) of the macrophages were positive. The majority of plasma cells (75–100%) were weakly positive as well as few (<5%) of the T-cells.

Galectin-3 expression in the inflammatory cell infiltrates varied between individual patients. Both negative and positive as well as trace amount of staining were noted for patients irrespectively of the intestinal inflammatory grade. For patients expressing galectin-3 in inflammatory cells, the expression was weaker compared to galectin-2, with an overall strong expression in <1% of the inflammatory cells and a weak expression in 10–15%. The majority of the positive inflammatory cells were macrophages and 20–30% of macrophages were positive for galectin-3. About 10% of the plasma cells and <1% of T-cells were positive for galectin-3 (Figure 2(h)).

4. Discussion

The UC tissues analysed in this study were obtained after bowel resection. This permits evaluation of the entire intestinal wall as well as several different intestinal regions compared to a limited number of small biopsies collected during endoscopy [32, 33]. Another difference compared to previous studies is that the surgical specimens were obtained from patients being at the end of the road regarding medical treatment of their disease and therefore representing, on average, a later state of the disease. However, there was still

considerable heterogeneity regarding the degree of inflammation within a single intestine (exemplified in Figure 1) and also between individual patients. The control tissue in this study was from both the sigmoid area ($n = 2$) and ascending area of colon ($n = 8$) and no difference in galectin pattern was seen irrespective of which anatomical part the samples were collected from, nor was there any difference in galectin pattern depending on the anatomical localisation found in the UC study patients (Table 1). Therefore, it is not likely that the differences in galectin expression depend on the anatomical localisation of the tissue sections.

The galectin expression did not differ considerably between the colon epithelium in the control group and that of epithelial cells having a normal histological appearance in the UC study group. When the galectin expression in areas with cryptitis was summarized for each inflammatory grade (Figure 3) it seems that a decrease in epithelial galectin expression is correlated to inflammation. However, since the expression pattern for each individual patient was very complex (Table 2) we argue that there was no clear-cut correlation between the expressions of galectin-2, galectin-3, and galectin-4 in the colonic epithelium and the inflammatory activity in our study group of consecutive patients. This is in contrast to some reports that suggest decreased galectin expression in conjunction with intestinal inflammation [18, 20, 32]. There may be several explanations for this discrepancy. The evaluation of a certain antigen(s) in tissues/cells involves many technical aspects of the methodologies used that affect the quality of analysis. Immunohistochemistry has a well-known variation depending on the techniques for tissue handling and antibody reagents used. Molecular analysis strategies using homogenized biopsies also have limitations due to sampling errors. For example, galectin-3 has been quantified using mRNA reverse transcription technique [20]. However, there is usually a patchy appearance of the intestinal inflammatory process both macroscopically and, especially, at the microscopic level, with differences in intensity between different cryptitis, abscess, and ulceration areas (Figures 1 and 2). Therefore, quantification of whole biopsies collected by endoscopy has to be carefully evaluated regarding representativeness of tissue specimens analysed. Furthermore, changes in galectin expression in biopsies will be due to either loss of epithelial cells, reduced expression in individual cells, the amount of infiltrating inflammatory cells, or a combination of these factors. Many of the individual patients express a stable amount of galectins in their epithelial cells independently of the degree of inflammation (Table 2). Therefore, results from previous studies indicating that a reduced galectin expression correlates with inflammation [20, 32] may be due to lack of epithelial cells due to tissue damage as well as sampling error of small biopsies and not to a specific downregulation of epithelial galectin biosynthesis. Furthermore, to increase the number of individual cases analysed in this investigation will most likely not result in a statistical significant correlation between galectin expression and inflammation due to the great heterogeneity of epithelial cell galectin expression both within and between individual cases as shown in Table 2. Even if a statistical significant correlation should be obtained, the biological significance of this must be highly questioned.

The tissue localisation of galectin-1–galectin-4 found here agreed in general with previous immunohistochemical studies of human and mouse intestines. In addition, we also found some features not described before. Galectin-1, one of the most studied galectins, has typically not been found in human epithelial cells [42, 43], and this was also the case here. This is in contrast to mouse where galectin-1 is expressed in the intestinal epithelial cells [45, 46] but also shows a strain variation [46]. Variable expression of galectin-1 has been found in human nonepithelial tissue like muscle, lymphocytes, and fibroblasts [42] but here no or low expression was found. However, this does not rule out a role in colitis, as galectin-1 can bind both epithelial cells and lymphocyte glycans, resulting in apoptosis and other cell regulations [47].

Galectin-2 is the closest compared to galectin-1 but much less studied in mammals. Similar to galectin-1, it is a noncovalent dimer, but unlike galectin-1 it has characteristic localisation to the gastrointestinal tract and it has a different carbohydrate-binding specificity, seemingly adapted to intestinal glycans [18, 19]. Thus, it can be regarded as an intestinal paralogue of galectin-1. Here we found high expression of galectin-2 in colon epithelial cells, as reported before, but also the novel observation of high expression in some submucosal macrophages, suggesting an immunoregulatory role. Added galectin-2 can support epithelial wound healing and suppress lamina propria T-cells to ameliorate experimental colitis in mice [18].

Galectin-3, the other most studied galectin, was abundant in epithelial cells and also in some macrophages, in agreement with previous studies [20, 24]. Galectin-3 behaves mainly as a proinflammatory protein, and studies using null mutant mice support a rate-limiting role in chronic inflammation with fibrosis in many tissues. In a mice colitis experimental model, intraperitoneally administrated galectin-3 reduced inflammation [48].

In contrast to galectin-2 and galectin-3, which were distributed in the entire cytoplasm, galectin-4 had specific supranuclear localisation presumed to be in the Golgi network. To our knowledge, this has not been reported before. Galectin-4 is expressed only in the digestive tract and restricted to epithelial cells. Previous studies have showed that there is no significant difference in the expression of galectin-4 in epithelial cells from inflamed colon versus controls [18] which is confirmed by this study.

Information regarding galectin expression in the infiltrating inflammatory cells in IBD intestine is very limited. In the macrophages, we found a strong expression of galectin-2 and a weak expression of galectin-3 with a significant interindividual variability, while staining for galectin-1 and galectin-4 was completely negative. There was no increase in the expression of galectin-2 and galectin-3 in the inflammatory infiltrates in the UC study group compared to the expression in inflammatory cells in controls. Reports on galectin expression in intestinal inflammatory cells have, to our knowledge, only been reported for galectin-3. A study of patients with ileal pouch-anal anastomosis [49]

revealed a significant decrease in the galectin-3 staining index in subepithelial macrophages in patients with chronic or recurrent acute pouchitis compared to the noninflamed controls. A reduced expression of galectin-3 has been found in intestinal macrophages of patients with Crohn's disease but not in UC patients [50]. The observation of a high specific expression of galectin-2 in submucosal macrophages has not been reported earlier. Galectin-2 is evolutionary and structurally most related to galectin-1, but it has a tissue expression and carbohydrate-binding specificity more adapted to glycan structures found in intestinal epithelial cells (galactosides with blood group determinants) [47] and does not bind sialylated glycans as found in serum glycoproteins bound by galectin-1 [51].

5. Conclusions

The findings that several UC patients did not show any changes in colon epithelial cell galectin expression while others showed an individual specific change not correlated to the inflammatory grade indicate that the variation in epithelial galectin expression may not be related primarily to the inflammatory grade but rather to the focal presentation of the disease as well as individual factors not defined at present.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contribution

Mattias Block, Hakon Leffler, Lars Börjesson, and Michael E. Breimer conceived and designed the experiments. Mattias Block, Johan Mölne, Lars Börjesson, and Michael E. Breimer analysed the data. Mattias Block, Johan Mölne, Hakon Leffler, and Michael E. Breimer wrote the paper. All the authors read and approved the final paper.

Acknowledgments

The Ihre Foundation, Sahlgrenska University Hospital, and Gothenburg Medical Society supported this study.

References

[1] D. K. Podolsky, "Inflammatory bowel disease," *The New England Journal of Medicine*, vol. 347, no. 6, pp. 417–429, 2002.

[2] T. Öresland, "Review article: colon-saving medical therapy vs. colectomy in ulcerative colitis—the case for colectomy," *Alimentary Pharmacology and Therapeutics*, vol. 24, supplement 3, pp. 74–79, 2006.

[3] C. Abraham and J. H. Cho, "Inflammatory bowel disease," *The New England Journal of Medicine*, vol. 361, no. 21, pp. 2066–2078, 2009.

[4] B. Khor, A. Gardet, and R. J. Xavier, "Genetics and pathogenesis of inflammatory bowel disease," *Nature*, vol. 474, no. 7351, pp. 307–317, 2011.

[5] A. Hokama, E. Mizoguchi, and A. Mizoguchi, "Roles of galectins in inflammatory bowel disease," *World Journal of Gastroenterology*, vol. 14, no. 33, pp. 5133–5137, 2008.

[6] H. Leffler, S. Carlsson, M. Hedlund, Y. Qian, and F. Poirier, "Introduction to galectins," *Glycoconjugate Journal*, vol. 19, no. 7–9, pp. 433–440, 2002.

[7] D. Delacour, A. Koch, and R. Jacob, "The role of galectins in protein trafficking," *Traffic*, vol. 10, no. 10, pp. 1405–1413, 2009.

[8] D. Schneider, C. Greb, A. Koch et al., "Trafficking of galectin-3 through endosomal organelles of polarized and non-polarized cells," *European Journal of Cell Biology*, vol. 89, no. 11, pp. 788–798, 2010.

[9] J. P. Cerliani, S. R. Stowell, I. D. Mascanfroni, C. M. Arthur, R. D. Cummings, and G. A. Rabinovich, "Expanding the universe of cytokines and pattern recognition receptors: galectins and glycans in innate immunity," *Journal of Clinical Immunology*, vol. 31, no. 1, pp. 10–21, 2011.

[10] J. W. Dennis, K. S. Lau, M. Demetriou, and I. R. Nabi, "Adaptive regulation at the cell surface by N-glycosylation," *Traffic*, vol. 10, no. 11, pp. 1569–1578, 2009.

[11] F.-T. Liu and G. A. Rabinovich, "Galectins: regulators of acute and chronic inflammation," *Annals of the New York Academy of Sciences*, vol. 1183, pp. 158–182, 2010.

[12] A. C. MacKinnon, M. A. Gibbons, S. L. Farnworth et al., "Regulation of transforming growth factor-β1-driven lung fibrosis by galectin-3," *American Journal of Respiratory and Critical Care Medicine*, vol. 185, no. 5, pp. 537–546, 2012.

[13] L. G. Baum, O. B. Garner, K. Schaefer, and B. Lee, "Microbe-host interactions are positively and negatively regulated by galectin-glycan interactions," *Frontiers in Immunology*, vol. 5, article 284, 2014.

[14] C.-I. Lin, E. E. Whang, D. B. Donner et al., "Galectin-3 targeted therapy with a small molecule inhibitor activates apoptosis and enhances both chemosensitivity and radiosensitivity in papillary thyroid cancer," *Molecular Cancer Research*, vol. 7, no. 10, pp. 1655–1662, 2009.

[15] C.-I. Lin, E. E. Whang, D. B. Donner et al., "Galectin-3 targeted therapy with a small molecule inhibitor activates apoptosis and enhances both chemosensitivity and radiosensitivity in papillary thyroid cancer," *Molecular Cancer Research*, vol. 7, no. 10, pp. 1655–1662, 2009.

[16] P. Nangia-Makker, S. Nakahara, V. Hogan, and A. Raz, "Galectin-3 in apoptosis, a novel therapeutic target," *Journal of Bioenergetics and Biomembranes*, vol. 39, no. 1, pp. 79–84, 2007.

[17] K. Geboes, R. Riddell, A. Öst, B. Jensfelt, T. Persson, and R. Löfberg, "A reproducible grading scale for histological assessment of inflammation in ulcerative colitis," *Gut*, vol. 47, no. 3, pp. 404–409, 2000.

[18] D. Paclik, U. Berndt, C. Guzy et al., "Galectin-2 induces apoptosis of lamina propria T lymphocytes and ameliorates acute and chronic experimental colitis in mice," *Journal of Molecular Medicine*, vol. 86, no. 12, pp. 1395–1406, 2008.

[19] D. Paclik, K. Lohse, B. Wiedenmann, A. U. Dignass, and A. Sturm, "Galectin-2 and -4, but not Galectin-1, promote intestinal epithelial wound healing in vitro through a TGF-beta-independent mechanism," *Inflammatory Bowel Diseases*, vol. 14, no. 10, pp. 1366–1372, 2008.

[20] S. Müller, T. Schaffer, B. Flogerzi et al., "Galectin-3 modulates T cell activity and is reduced in the inflamed intestinal epithelium in IBD," *Inflammatory Bowel Diseases*, vol. 12, no. 7, pp. 588–597, 2006.

[21] G. A. Rabinovich, L. G. Baum, N. Tinari et al., "Galectins and their ligands: amplifiers, silencers or tuners of the inflammatory response?" *Trends in Immunology*, vol. 23, no. 6, pp. 313–320, 2002.

[22] A. Nishida, K. Nagahama, H. Imaeda et al., "Inducible colitis-associated glycome capable of stimulating the proliferation of memory $CD4^+$ T cells," *The Journal of Experimental Medicine*, vol. 209, no. 13, pp. 2383–2394, 2012.

[23] J. M. Rhodes, B. J. Campbell, and L. G. Yu, "Lectin-epithelial interactions in the human colon," *Biochemical Society Transactions*, vol. 36, part 6, pp. 1482–1486, 2008.

[24] A. Koch, F. Poirier, R. Jacob, and D. Delacour, "Galectin-3, a novel centrosome-associated protein, required for epithelial morphogenesis," *Molecular Biology of the Cell*, vol. 21, no. 2, pp. 219–231, 2010.

[25] N. C. Henderson and T. Sethi, "The regulation of inflammation by galectin-3," *Immunological Reviews*, vol. 230, no. 1, pp. 160–171, 2009.

[26] D. Delacour, V. Gouyer, J.-P. Zanetta et al., "Galectin-4 and sulfatides in apical membrane trafficking in enterocyte-like cells," *Journal of Cell Biology*, vol. 169, no. 3, pp. 491–501, 2005.

[27] E. M. Danielsen and G. H. Hansen, "Lipid raft organization and function in the small intestinal brush border," *Journal of Physiology and Biochemistry*, vol. 64, no. 4, pp. 377–382, 2008.

[28] S. R. Stowell, C. M. Arthur, M. Dias-Baruffi et al., "Innate immune lectins kill bacteria expressing blood group antigen," *Nature Medicine*, vol. 16, no. 3, pp. 295–301, 2010.

[29] A. Hokama, E. Mizoguchi, K. Sugimoto et al., "Induced reactivity of intestinal $CD4^+$ T cells with an epithelial cell lectin, galectin-4, contributes to exacerbation of intestinal inflammation," *Immunity*, vol. 20, no. 6, pp. 681–693, 2004.

[30] L. Froľová, K. Smetana Jr., D. Borovská et al., "Detection of galectin-3 in patients with inflammatory bowel diseases: new serum marker of active forms of IBD?" *Inflammation Research*, vol. 58, no. 8, pp. 503–512, 2009.

[31] E. Jensen-Jarolim, C. Neumann, G. Oberhuber et al., "Anti-Galectin-3 IgG autoantibodies in patients with Crohn's disease characterized by means of phage display peptide libraries," *Journal of Clinical Immunology*, vol. 21, no. 5, pp. 348–356, 2001.

[32] E. Jensen-Jarolim, R. Gscheidlinger, G. Oberhuber et al., "The constitutive expression of galectin-3 is downregulated in the intestinal epithelia of Crohn's disease patients, and tumour necrosis factor alpha decreases the level of galectin-3-specific mRNA in HCT-8 cells," *European Journal of Gastroenterology and Hepatology*, vol. 14, no. 2, pp. 145–152, 2002.

[33] P. Demetter, N. Nagy, B. Martin et al., "The galectin family and digestive disease," *The Journal of Pathology*, vol. 215, no. 1, pp. 1–12, 2008.

[34] J. D. Lewis, S. Chuai, L. Nessel, G. R. Lichtenstein, F. N. Aberra, and J. H. Ellenberg, "Use of the noninvasive components of the Mayo score to assess clinical response in ulcerative colitis," *Inflammatory Bowel Diseases*, vol. 14, no. 12, pp. 1660–1666, 2008.

[35] D. N. W. Cooper and S. H. Barondes, "Evidence for export of a muscle lectin from cytosol to extracellular matrix and for a novel secretory mechanism," *Journal of Cell Biology*, vol. 110, no. 5, pp. 1681–1691, 1990.

[36] D. N. W. Cooper, S. M. Massa, and S. H. Barondes, "Endogenous muscle lectin inhibits myoblast adhesion to laminin," *Journal of Cell Biology*, vol. 115, no. 5, pp. 1437–1448, 1991.

[37] F. A. van den Brûle, C. Buicu, A. Berchuck et al., "Expression of the 67-kD laminin receptor, galectin-1, and galectin-3 in advanced human uterine adenocarcinoma," *Human Pathology*, vol. 27, no. 11, pp. 1185–1191, 1996.

[38] M. A. Gitt, S. M. Massa, H. Leffler, and S. H. Barondes, "Isolation and expression of a gene encoding L-14-II, a new human soluble lactose-binding lectin," *The Journal of Biological Chemistry*, vol. 267, no. 15, pp. 10601–10606, 1992.

[39] M. E. Huflejt, E. T. Jordan, M. A. Gitt, S. H. Barondes, and H. Leffler, "Strikingly different localization of galectin-3 and galectin-4 in human colon adenocarcinoma T84 cells. Galectin-4 is localized at sites of cell adhesion," *The Journal of Biological Chemistry*, vol. 272, no. 22, pp. 14294–14303, 1997.

[40] M. E. Huflejt and H. Leffler, "Galectin-4 in normal tissues and cancer," *Glycoconjugate Journal*, vol. 20, no. 4, pp. 247–255, 2004.

[41] M. K. Ho and T. A. Springer, "Mac-2, a novel 32,000 Mr mouse macrophage subpopulation-specific antigen defined by monoclonal antibodies," *The Journal of Immunology*, vol. 128, no. 3, pp. 1221–1228, 1982.

[42] X. Sanjuan, P. L. Fernandez, A. Castells et al., "Differential expression of galectin 3 and galectin 1 in colorectal cancer progression," *Gastroenterology*, vol. 113, no. 6, pp. 1906–1915, 1997.

[43] A. F. T. Rossi, M. C. Duarte, A. B. Poltronieri et al., "Deregulation of annexin-A1 and galectin-1 expression in precancerous gastric lesions: Intestinal metaplasia and gastric ulcer," *Mediators of Inflammation*, vol. 2014, Article ID 478138, 11 pages, 2014.

[44] X. Yu, R. Siegel, and R. G. Roeder, "Interaction of the B cell-specific transcriptional coactivator OCA-B and galectin-1 and a possible role in regulating BCR-mediated B cell proliferation," *The Journal of Biological Chemistry*, vol. 281, no. 22, pp. 15505–15516, 2006.

[45] L. Santucci, S. Fiorucci, N. Rubinstein et al., "Galectin-1 suppresses experimental colitis in mice," *Gastroenterology*, vol. 124, no. 5, pp. 1381–1394, 2003.

[46] A. Mathieu, N. Nagy, C. Decaestecker et al., "Expression of galectins-1, -3 and -4 varies with strain and type of experimental colitis in mice," *International Journal of Experimental Pathology*, vol. 89, no. 6, pp. 438–446, 2008.

[47] S. R. Stowell, C. M. Arthur, P. Mehta et al., "Galectin-1, -2, and -3 exhibit differential recognition of sialylated glycans and blood group antigens," *The Journal of Biological Chemistry*, vol. 283, no. 15, pp. 10109–10123, 2008.

[48] E. Lippert, M. Stieber-Gunckel, N. Dunger, W. Falk, F. Obermeier, and C. Kunst, "Galectin-3 modulates experimental colitis," *Digestion*, vol. 92, no. 1, pp. 45–53, 2015.

[49] E. Brazowski, I. Dotan, H. Tulchinsky, I. Filip, and A. Eisenthal, "Galectin-3 expression in pouchitis in patients with ulcerative colitis who underwent Ileal Pouch-Anal Anastomosis (IPAA)," *Pathology Research and Practice*, vol. 205, no. 8, pp. 551–558, 2009.

[50] E. Lippert, M. Gunckel, J. Brenmoehl et al., "Regulation of galectin-3 function in mucosal fibroblasts: potential role in mucosal inflammation," *Clinical and Experimental Immunology*, vol. 152, no. 2, pp. 285–297, 2008.

[51] C. Cederfur, E. Salomonsson, J. Nilsson et al., "Different affinity of galectins for human serum glycoproteins: galectin-3 binds many protease inhibitors and acute phase proteins," *Glycobiology*, vol. 18, no. 5, pp. 384–394, 2008.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 921730, 8 pages
http://dx.doi.org/10.1155/2015/921730

Research Article

Incidence Trends and Geographical Variability of Pediatric Inflammatory Bowel Disease in Slovenia: A Nationwide Study

Darja Urlep,[1] Rok Blagus,[2] and Rok Orel[1]

[1]*Department of Gastroenterology, Hepatology and Nutrition, University Children's Hospital, Ljubljana, Slovenia*
[2]*Institute for Biostatistics and Medical Informatics, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia*

Correspondence should be addressed to Darja Urlep; darja.urlep@gmail.com

Received 24 July 2015; Revised 4 November 2015; Accepted 4 November 2015

Academic Editor: Alessandro Armuzzi

Background. The aims of the study were to determine the incidence rate of pediatric inflammatory bowel disease (PIBD) and its trends for the period of 2002–2010 and to assess the geographical distribution of PIBD in Slovenia. *Materials and Methods*. Medical records of patients (0–18 years) with newly diagnosed IBD during the study period were retrospectively reviewed. *Results*. The mean incidence rate for IBD in 2002–2010 was 7.6 per 100,000 children and adolescents per year, 4.5 for Crohn's disease (CD), 2.9 for ulcerative colitis (UC), and 0.2 for IBD-unclassified, respectively. The incidence rate increased from 5.8 per 100,000 per year in 2002–2004 to 8.6 in 2005–2007 and remained stable afterwards. Statistically significant difference in the incidence rate between the Northeastern and Southwestern parts of the country was observed ($p = 0.025$). *Conclusion*. This nationwide study demonstrates that Slovenia is among the European countries with the highest PIBD incidence. During the study period a substantial rise of PIBD incidence was observed during the first half of the study and it seems to have stabilized in the second half. The significant difference in PIBD incidence between Northeastern and Southwestern parts of the country merits further exploration of the possible environmental factors.

1. Introduction

Over the last decades a number of epidemiologic studies on inflammatory bowel disease (IBD) in children reported an increasing trend of pediatric incidence in developed [1–24] as well as in developing countries [25–31]. The highest incidence reported is still from the Northern countries of Europe and America. In Europe, the highest incidence of childhood IBD has been observed in Scandinavian countries and it seems that the incidence is still on the rise, mainly that of CD [18–21]. The increasing incidence of pediatric IBD (PIBD) has also been reported in the studies from other Western European countries [11–13, 22–24]. Recently, some new epidemiological data suggest that the incidence of PIBD has been increasing in Eastern Europe as well [25, 26, 28, 29].

Two epidemiologic studies on PIBD incidence were performed in Slovenia and both demonstrated an increasing trend of incidence [30, 31]. The first study was done for the period from 1994 to 2005 and covered the country's Central/Southwestern (CSW) area which is inhabited by approximately two thirds of the Slovenian pediatric population (0–18 years). It showed the mean annual PIBD incidence of 4.03 per 100,000 per year. The trend of the incidence was observed to have risen from 3.04 in the period from 1994 to 1999 to 5.14 in the period from 2000 to 2005 [30]. The second study for the period from 2002 to 2010 covered the area of the wider Northeastern Slovenia (WNE) and demonstrated a higher PIBD incidence rate of 7.6 per 100,000 children (0–18 years) per year [31].

The aims of this nationwide study were to determine the incidence rates of PIBD, Crohn's disease (CD), and ulcerative colitis (UC) and their trends for the whole country of Slovenia during the period between 2002 and 2010. Another aim was to assess the geographical distribution of PIBD throughout Slovenia.

2. Materials and Methods

All pediatric cases (0–18 years) of IBD newly diagnosed in the period from 2002 to 2010 and residing in well-defined

statistical regions of the whole Slovenia were included in our study by a detailed retrospective review of the patients' medical records.

Although Slovenia is a small Central/Southeastern European country with only 2 million inhabitants, it is very diverse. In the North, there is the mountain range of the Alps with the appropriate mountain climate, in the East lies the Pannonian plain with the continental climate, and the Southwest side is under the influence of the Mediterranean Sea and thusly has a Mediterranean climate.

Data on the background pediatric population were obtained from the Statistical Office of the Republic of Slovenia [32]. The childhood population (0–18 years) of Slovenia was 439,448 in 2002 and 384,375 in 2010, showing a decline of 12.5% in the 9-year study period.

Due to their relatively small size, the statistical regions were grouped into three bigger units: Northeastern unit (Mura, Carinthia, and Drava regions), Central unit (Savinja, Upper Carniola, Central Slovenia, Central Sava, Lower Sava, and Southeast Slovenia regions), and Southwestern unit (Gorizia, Coastal-Karst, and Littoral-Inner Carniola regions) (Figure 1). The mean annual pediatric populations in the period of 2002–2010 (0–18 years) for Northeastern, Central, and Southwestern geographical units were 100,308, 255,623, and 52,349, respectively.

The health care system in Slovenia recommends that all children (0–18 years) with suspected IBD are to be referred to one of the two pediatric gastroenterology tertiary centres (in Ljubljana and Maribor). General pediatricians working at regional hospitals and primary health centers do not diagnose or treat patients with IBD by themselves. In Slovenia, a consensus between pediatric and adult gastroenterologists was made (both being members of the Slovenian Association for Gastroenterology and Hepatology), which states that children younger than 18 are always to be managed by pediatric and not by adult gastroenterologists. Despite the agreement, adult gastroenterologists in Slovenia were personally contacted to find out whether any young patients with IBD were under their care during the study period. There were indeed 9 adolescents under 16 years, diagnosed by adult gastroenterologists; however, they were then referred to a pediatric centre for appropriate treatment and were included in the study.

Newly diagnosed cases of IBD (0–18 years) were retrospectively identified based on the admission register and the endoscopic register. In the admission register run at the gastroenterology unit (both for inpatients and outpatients) only the data on diagnoses (IBD without subtypes), names, surnames, dates of birth, dates of diagnosis, and the individual inpatients' and outpatients' numbers were recorded. Based on these data we were able to find the medical inpatient and/or outpatient records which provided us with other required information. Because we were not sure that all newly diagnosed patients with IBD were indeed recorded at admission we also reviewed the endoscopic register, where all upper and lower endoscopies performed, along with their endoscopic results, are recorded. Such an endoscopic register must necessarily be maintained due to the Slovenian health insurance policy. We obtained all

Figure 1: Statistical regions of Slovenia.

required data (pathohistological, clinical data and data on the small bowel investigations and additional endoscopic investigations) from the patients' medical records. Only those patients with a confirmed diagnosis of IBD were included in our study.

The diagnosis of IBD was based on clinical signs and symptoms, a physical examination, endoscopic appearance, histological assessment of mucosal biopsy specimens, and small bowel imaging studies. Patients who did not fulfill the diagnostic criteria for IBD regarding the macroscopic changes at endoscopy and typical histological findings were excluded from the study. The diagnosis was never based only on clinical presentation and radiological or/and ultrasound findings. Data from the patients' medical records about diagnostic workup were compared with the Porto diagnostic criteria (Table 1) [33].

Disease location according to the Paris classification was evaluated only in patients with CD and UC with a complete diagnostic workup [34]. The complete diagnostic workup consisted of upper GI endoscopy and the small bowel investigations (small bowel follow-through, capsule endoscopy, MR enterography, or CT imaging of small bowel). Capsule endoscopy was performed in all of the patients with suspected or diagnosed CD after the year 2006 as part of the routine diagnostic workup. Only reliable changes (aphthous lesions, ulcers, and pseudopolyps) were assessed in order to define the localization of inflammation and therefore the extent of the disease. CD patients with pathological findings of the small bowel observed only by abdominal ultrasound imaging were not included in the evaluation of disease location.

The disease extent in the colon was defined only by macroscopic lesions visible during endoscopy, whereas the involvement of the upper GI tract was defined by the presence of either typical macroscopic changes or highly specific microscopic lesions (granulomas).

Incidence rates of total IBD, CD, UC, and IBD-U were calculated for whole Slovenia, for each of the twelve statistical regions, and for the three aforementioned geographical units. Mean annual incidences for the three time periods (2002–2004, 2005–2007, and 2008–2010, resp.) were determined as well.

Table 1: Diagnostic procedures performed at diagnosis.

	CD ($n = 167$)	UC ($n = 105$)	IBD-U ($n = 7$)
Colonoscopy	167 (100%)	105 (100%)	7 (100%)
Terminal ileocolonoscopy	153 (90.3%)	91 (86.4%)	6 (85.8%)
Upper GI endoscopy	158 (93.2%)	81 (76.9%)	7 (100%)
Abdominal ultrasound	167 (100%)	105 (100%)	7 (100%)
Small bowel follow-through	42 (24.7%)	5 (4.7%)	0 (0%)
Scintigraphy with labeled leucocytes	21 (12.4%)	8 (7.6%)	0 (0%)
Capsule endoscopy*	87 (51.3%)	11 (10.4%)	4 (57.2%)
Magnetic resonance imaging (MRI)+	18 (10.6%)	2 (1.9%)	2 (28.6%)
Computed tomography (CT)	5 (2.9%)	0 (0%)	0 (0%)

*Capsule endoscopy has been available since 2006.
+MR enterography has been available since 2009.

Figure 2: Age at diagnosis for girls and boys with IBD.

2.1. Statistical Analysis. The population data for the period from 2002 to 2010 were obtained by the Statistical Office of the Republic of Slovenia (SURS) [32]. Incidence rates were calculated as events per 100,000 person-years and their 95% confidence intervals (CI) were based on the Poisson distribution.

The differences between periods or regions were estimated with the Poisson model, where the number of events was considered as a dependent variable and the population size was included in the model as an offset term.

Starting from 31 December 2008 the Statistical Office began to use a different definition of the population which reduced the number of inhabitants by around 1%. It is estimated that this decrease occurred mainly due to not including foreigners who live in Slovenia for less than one year, which are in majority estimated to be over 18 years of age, suggesting that this change of the definition only had a marginal effect on our study population. It should be noted however that it is still possible that due to the change in the methodology the incidence rates in the period from 2009 to 2010 can be slightly overinflated in comparison with 2002–2008.

The study was approved by the National Medical Ethics Committee.

3. Results

In total, 279 cases of IBD in Slovenian childhood and adolescent population (0–18 years) were newly diagnosed during the study period from 2002 to 2010 (167 CD, 105 UC, and 7 IBD-U). The median age at IBD diagnosis was 13.7 years (range: 1.5–18) (Figure 2). The female/male ratio was 1.1, 1.2, and 0.8 for CD, UC, and IBD-U, respectively.

Of 279 newly diagnosed IBD patients, 89/279 (31.9%) were treated at Maribor's centre, 188/279 (67.7%) of them were treated at Ljubljana's centre, and 2/279 (0.7%) patients were treated at both centers.

The main characteristics of IBD patients are shown in Table 2.

3.1. Nationwide Incidence Rates. The mean annual incidence per 100,000 children and adolescents between 0 and 18 years of age for entire Slovenia in the period from 2002 to 2010 was 7.59 (95% CI: 6.72–8.53) for all IBD, 4.54 (95% CI: 3.88–5.28) for CD, 2.85 (95% CI: 2.33–3.45) for UC, and 0.19 (95% CI: 0.07–0.39) for IBD-U, respectively.

The nationwide incidence rates of total IBD, CD, UC, and IBD-U and their 95% CI for each year of the study period are presented in Table 3.

PIBD incidence rates for 3-year periods are shown in Figure 3. There was a statistical significant increase in the incidence rate of PIBD between the periods from 2005 to 2007 and from 2002 to 2004 ($p = 0.009$) and the periods from 2008 to 2010 and from 2002 to 2004 ($p = 0.017$); however, no statistical significant difference was seen between the periods from 2005 to 2007 and from 2008 to 2010 ($p = 0.841$).

3.2. Geographical Distribution of the PIBD Incidence Rates in Slovenia. The mean annual incidence rates of PIBD for the twelve Slovenian statistical regions for the whole period of 2002–2010 are presented in Figure 4. The highest PIBD incidence of 13.8 per 100,000 per year was observed in Carinthia, which is the most Northern Slovenian statistical region, positioned along the border with Austria. On the contrary, the lowest incidence rate of 2.4 was seen in the most Western Gorizia region bordering Italy.

When the three bigger regional units were taken into account, the highest incidence of PIBD was found to be in the Northeastern Slovenia (9.4 per 100,000 per year) and the lowest in the Southwestern Slovenia (5.7 per 100,000 per year), while the incidence in Central Slovenia was between the aforementioned two (7.3 per 100,000 per year) (Figure 5). There was a statistically significant difference in the mean annual incidence rate of PIBD between the Northeastern and Southwestern regional units (incidence ratio (IR) 1.64, 95% CI from 1.07 to 2.53, and $p = 0.025$) but not between the Central unit and any of these two (IR = 0.77, 95% CI from 0.59 to 1.01, and $p = 0.051$ and IR = 1.27, 95% CI from 0.84 to 1.91, and $p = 0.255$ for the comparison

Table 2: IBD patient characteristics ($n = 279$).

	n	%
Female	145	52
Male	134	48
Age at diagnosis (y)		
0–4	9	3.2
5–9	24	8.6
10–14	96	34.4
15–18	150	53.7
Positive family history	44	15.7
Location of CD (Paris)		
CD patients with available data	112	66
L1 ileal/ileocecal	21	18.7
L2 colon	11	9.8
L3 ileocolonic	24	21.4
L4a gastric	2	1.8
L4b proximal ileum and jejunum	1	0.9
L1 + L4a	5	4.5
L1 + L4b	6	5.3
L3 + L4a	26	23.1
L3 + L4b	11	9.8
L3 + L4a + L4b	5	4.5
Location of UC (Paris)		
E4 pancolitis	68	64.6
E3 distal to hepatic flexure	19	18.1
E2 left-sided colitis	13	12.35
E1 proctitis	5	4.75
Behaviour of CD		
B1 inflammatory	143	85.7
B2 stricturing	10	6.0
B3 fistulizing	14	8.3

Table 3: Annual incidence (per 100,000, 95% CI) of IBD, CD, and UC among children and adolescents with 0–18 years of age in Slovenia, 2002–2010.

Annual incidence	IBD ($n = 279$)	CD ($n = 167$)	UC ($n = 105$)
2002	5.7 (3.7–8.4)	3.6 (2.1–5.9)	2.0 (0.9–3.9)
2003	6.1 (3.9–8.9)	3.7 (2.1–6.1)	2.3 (1.1–4.3)
2004	5.7 (3.7–8.5)	3.6 (1.9–5.9)	2.1 (0.9–4.0)
2005	6.8 (4.5–9.8)	3.9 (2.2–6.3)	2.9 (1.5–5.1)
2006	9.4 (6.6–12.9)	5.7 (3.6–8.5)	3.5 (1.9–5.8)
2007	9.7 (6.9–13.3)	5.7 (3.6–8.6)	3.2 (1.7–5.5)
2008	7.3 (4.9–10.5)	5.1 (3.1–7.8)	2.0 (0.9–3.9)
2009	8.4 (5.8–11.8)	5.1 (3.1–7.9)	3.3 (1.8–5.7)
2010	9.4 (6.6–12.9)	4.6 (2.7–7.2)	4.3 (2.5–6.9)

with the Northeastern Slovenia and Southwestern Slovenia, resp.). Similar differences between Northeastern Slovenia, Central Slovenia, and Southwestern Slovenia were found for the incidences of CD and UC, but they were not statistically significant ($p > 0.05$) (Table 4).

Table 4: Mean annual incidence (per 100,000, 95% CI) of IBD, CD, and UC among children and adolescents in the three Slovenian geographical units, 2002–2012.

	IBD	CD	UC
Northeastern Slovenia	9.4 (7.5–11.6)	5.6 (4.2–7.4)	3.4 (2.3–4.8)
Central Slovenia	7.3 (6.2–8.4)	4.3 (3.5–5.3)	2.7 (2.1–3.5)
Southwestern Slovenia	5.7 (3.8–8.3)	3.4 (1.9–5.5)	2.3 (1.2–4.2)

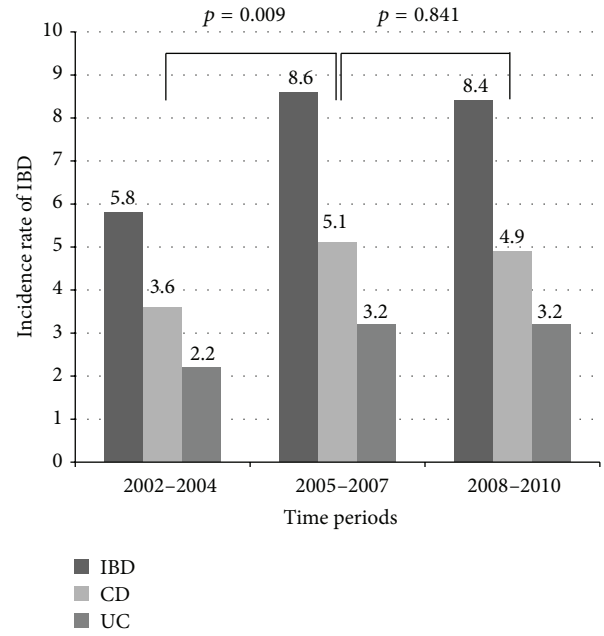


Figure 3: Incidence rates for pediatric IBD, CD, and UC per 100,000 children and adolescents for 3-year periods per year in 2002–2010.

4. Discussion

This study is the third on the incidence rate of PIBD in Slovenia, but it is the first nationwide study covering the whole country.

Two previous studies were conducted in two different time periods and for different geographical regions of Slovenia. The first study was made for the period of 1994–2005 and only for the CSW Slovenia. The observed mean annual PIBD incidence in the 1994–2005 study was 4.03 per 100,000 per year [30]. The second study did cover the area of WNE but it also included the Savinja statistical region which is closer to Central Slovenia than Northeastern statistical region from the geographical view. The second study showed a higher incidence rate of 7.6 per 100,000 per year in comparison with the first one [31]. Partially different time periods and different geographical areas did not allow us to draw any conclusions on whether there has been a real rise in the PIBD incidence in Slovenia in the last years.

Surprisingly, the current study covering whole Slovenia has demonstrated the mean annual PIBD incidence rate of 7.6 per 100,000 per year, which is the same incidence that was reported in the previous study that covered only the WNE (wide Northeastern Slovenia) for the same time period. This observation has confirmed that PIBD throughout whole

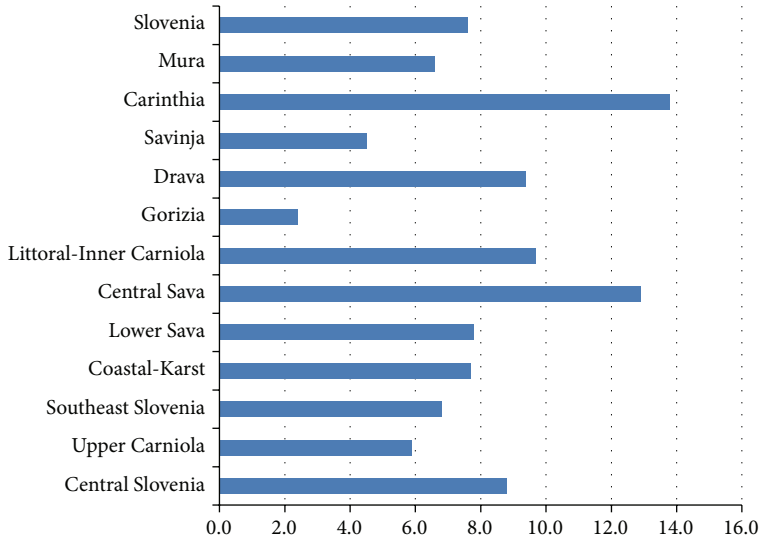


Figure 4: Twelve Slovenian statistical regions and mean annual PIBD incidence rates (per 100,000 children and adolescents) in 2002–2010.

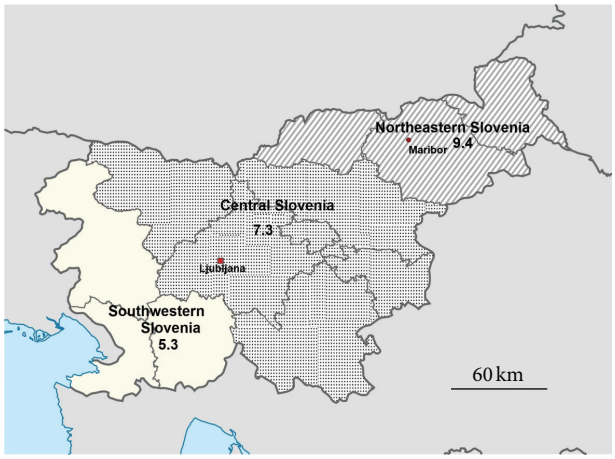


Figure 5: Three geographical units of Slovenia and mean annual PIBD incidence rates (per 100,000 children and adolescents) in 2002–2010.

Slovenia is high and comparable to the reported incidences from Western European countries [12, 19, 22–24]. During the study period a substantial rise of PIBD incidence was observed during the first half of the study and it seems to have stabilized in the second half. Whether the incidence of PIBD in Slovenia has already reached a plateau is yet to be discovered. Therefore, more prospective epidemiologic data are needed along with a nationwide PIBD register.

This study has shown that the PIBD incidence varies greatly among different statistical regions. The results are biased by the relatively small number of newly diagnosed IBD patients per year and a small population number in each region as well. However, when the three bigger geographical units were taken into account, a statistically significant difference in PIBD between Northeastern (NE) Slovenia and Southwestern (SW) Slovenia was found. The incidence of PIBD observed in NE Slovenia was 9.4 per 100,000 per year and the one in SW Slovenia was 5.7 per 100,000 per year. We can only speculate that this difference is at least partially due to the regions being affected by the different climate conditions. In the NE Slovenia, which borders Austria and Hungary, a subalpine and continental climate is present in contrast to the typical Mediterranean climate with abundant sunlight exposure over the whole year in SW Slovenia.

The North-South gradient has long been known for IBD [35, 36]. In several countries including UK, France, USA, and Finland, North-South gradients have been reported as well [11, 37–39]. Recently, several studies have pointed out an important immunological role of vitamin D and its association with autoimmune diseases such as IBD [40–44].

Although there are some indicators that may explain the role of sunlight exposure in the observed difference between the most Northeastern and Southwestern parts of the country, we believe that there may be some other more important environmental factors that contribute to the observed geographical difference of the PIBD incidence in Slovenia.

Northeastern regions of Slovenia are known for their traditional continental diet which is characterized by a large amount of saturated fats and refined carbohydrates with a high content of red meat (mainly pork) and a consequently high content of omega-6 fatty acids. On the contrary, the population of the Southwestern part of the country is accustomed to the Mediterranean diet with large amount of fish, olive oil, vegetables, and fruits and therefore higher amounts of fiber and omega-3 fatty acids and lower amounts of saturated fats. Several studies have pointed out the role of a diet as a possible risk factor for IBD development [45–52]. There is no doubt that genetic factors may also account for some of the observed differences in the PIBD incidence between NE Slovenia and SW Slovenia; therefore studies on the genetical background of these two Slovenian populations could be of importance. On the other hand, NE Slovenia borders Austria and Hungary and thusly the NE Slovenian population has a

similar cuisine to that of those two neighbouring countries, with a typical heavy, high fat continental diet largely resulting from the historically common Austro-Hungarian diet. On the contrary, the cuisine in the SW parts of the country is similar to the Italian Mediterranean cuisine. It has been postulated that there is an important interplay between genetic and environmental factors which are involved in the IBD aetiopathogenesis. Dietary factors may influence the expression of genes involved in the IBD pathogenesis, and the genetic background determines which environmental factors may have a role in the development of IBD in genetically predisposed individuals [53, 54].

This study has shown that PIBD incidence in Central Slovenia is lower when compared to NE Slovenia. This observation is in contrast with our expectations, because the Central parts of Slovenia are economically more developed, and the standard of living is higher when compared to the Northeastern part. Therefore, our study did not confirm the hypothesis that economic development is a potential risk factor for IBD.

Although we are aware that this study shares some of the disadvantages of a retrospective approach, we believe it is representative, mainly because of the consistency of our healthcare system with only two tertiary pediatric gastroenterology centers, where all pediatric IBD patients up to 18 years of age are to be diagnosed and treated. Nevertheless there may be some patients below 18 years of age who may have been diagnosed by adult gastroenterologists. Therefore, our data on the PIBD incidence may be slightly underestimated and must therefore be interpreted with caution considering the retrospective design of the study.

On the other hand, this study is one of the rare studies that were truly nationwide and it covered the whole population of the country.

5. Conclusion

This study has demonstrated a high incidence rate of PIBD in Slovenia. During the study period a substantial rise of the PIBD incidence was observed only during the first half of the study and it seems to have stabilized in the second half. Whether the incidence of PIBD in Slovenia has already reached a plateau is yet to be discovered.

The significant difference in PIBD incidence rates between the most Northeastern and Southwestern regions of Slovenia merits further exploration. Prospective epidemiological studies are needed to clarify the role of environmental factors in the pediatric inflammatory bowel disease in Slovenia and worldwide.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] J. R. Barton, S. Gillon, and A. Ferguson, "Incidence of inflammatory bowel disease in Scottish children between 1968 and 1983: marginal fall in ulcerative colitis, three-fold rise in Crohn's disease," *Gut*, vol. 30, no. 5, pp. 618–622, 1989.

[2] H. Hildebrand, B. Fredrikzon, L. Holmquist, B. Kristiansson, and B. Lindquist, "Chronic inflammatory bowel disease in children and adolescents in Sweden," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 13, no. 3, pp. 293–297, 1991.

[3] H. Hildebrand, M. Brydolf, L. Holmquist, I. Krantz, and B. Kristiansson, "Incidence and prevalence of inflammatory bowel disease in children in South-Western Sweden," *Acta Paediatrica*, vol. 83, no. 6, pp. 640–645, 1994.

[4] E. Langholz, P. Munkholm, P. A. Krasilnikoff, and V. Binder, "Inflammatory bowel diseases with onset in childhood. Clinical features, morbidity, and mortality in a regional cohort," *Scandinavian Journal of Gastroenterology*, vol. 32, no. 2, pp. 139–147, 1997.

[5] E. Armitage, H. Drummond, S. Ghosh, and A. Ferguson, "Incidence of juvenile-onset Crohn's disease in Scotland," *The Lancet*, vol. 353, no. 9163, pp. 1496–1497, 1999.

[6] J. Askling, L. Grahnquist, A. Ekbom, and Y. Finkel, "Incidence of paediatric Crohn's disease in Stockholm, Sweden," *The Lancet*, vol. 354, no. 9185, p. 1179, 1999.

[7] E. Lindberg, B. Lindquist, L. Holmquist, and H. Hildebrand, "Inflammatory bowel disease in children and adolescents in Sweden, 1984–1995," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 30, no. 3, pp. 259–264, 2000.

[8] K. Hassan, F. J. Cowan, and H. R. Jenkins, "The incidence of childhood inflammatory bowel disease in Wales," *European Journal of Pediatrics*, vol. 159, no. 4, pp. 261–263, 2000.

[9] A. J. M. Watson, A. T. Johnston, P. M. Barker, G. G. Youngson, W. M. Bisset, and A. A. Mahomed, "The presentation and management of juvenile-onset chronic inflammatory bowel disease in Northeastern Scotland," *Journal of Pediatric Surgery*, vol. 37, no. 1, pp. 83–86, 2002.

[10] B. S. Bentsen, B. Moum, and A. Ekbom, "Incidence of inflammatory bowel disease in children in southeastern Norway: a prospective population-based study 1990–94," *Scandinavian Journal of Gastroenterology*, vol. 37, no. 5, pp. 540–545, 2002.

[11] E. L. Armitage, M. C. Aldhous, N. Anderson et al., "Incidence of juvenile-onset Crohn's disease in Scotland: association with northern latitude and affluence," *Gastroenterology*, vol. 127, no. 4, pp. 1051–1057, 2004.

[12] S. Auvin, F. Molinié, C. Gower-Rousseau et al., "Incidence, clinical presentation and location at diagnosis of pediatric inflammatory bowel disease: a prospective population-based study in northern France (1988–1999)," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 41, no. 1, pp. 49–55, 2005.

[13] M. Ahmed, I. H. Davies, K. Hood, and H. R. Jenkins, "Incidence of paediatric inflammatory bowel disease in South Wales," *Archives of Disease in Childhood*, vol. 91, no. 4, pp. 344–345, 2006.

[14] P. Turunen, K.-L. Kolho, A. Auvinen, S. Iltanen, H. Huhtala, and M. Ashorn, "Incidence of inflammatory bowel disease in Finnish children, 1987–2003," *Inflammatory Bowel Diseases*, vol. 12, no. 8, pp. 677–683, 2006.

[15] T. Grieci and A. Bütter, "The incidence of inflammatory bowel disease in the pediatric population of Southwestern Ontario," *Journal of Pediatric Surgery*, vol. 44, no. 5, pp. 977–980, 2009.

[16] E. I. Benchimol, A. Guttmann, A. M. Griffiths et al., "Increasing incidence of paediatric inflammatory bowel disease in Ontario, Canada: evidence from health administrative data," *Gut*, vol. 58, no. 11, pp. 1490–1497, 2009.

[17] H. M. Malaty, X. Fan, A. R. Opekun, C. Thibodeaux, and G. D. Ferry, "Rising incidence of inflammatory bowel disease among children: a 12-year study," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 50, no. 1, pp. 27–31, 2010.

[18] G. Perminow, S. Brackmann, L. G. Lyckander et al., "A characterization in childhood inflammatory bowel disease, a new population-based inception cohort from south-eastern Norway, 2005-07, showing increased incidence in crohn's disease," *Scandinavian Journal of Gastroenterology*, vol. 44, no. 4, pp. 446–456, 2009.

[19] C. Jakobsen, A. Paerregaard, P. Munkholm et al., "Pediatric inflammatory bowel disease: increasing incidence, decreasing surgery rate, and compromised nutritional status: a prospective population-based cohort study 2007–2009," *Inflammatory Bowel Diseases*, vol. 17, no. 12, pp. 2541–2550, 2011.

[20] P. Lehtinen, M. Ashorn, S. Iltanen et al., "Incidence trends of pediatric inflammatory bowel disease in Finland, 1987–2003, a nationwide study," *Inflammatory Bowel Diseases*, vol. 17, no. 8, pp. 1778–1783, 2011.

[21] Ú. Agnarsson, S. Björnsson, J. H. Jóhansson, and L. Sigurdsson, "Inflammatory bowel disease in Icelandic children 1951–2010. Population-based study involving one nation over six decades," *Scandinavian Journal of Gastroenterology*, vol. 48, no. 12, pp. 1399–1404, 2013.

[22] P. Henderson, R. Hansen, F. L. Cameron et al., "Rising incidence of pediatric inflammatory bowel disease in Scotland," *Inflammatory Bowel Diseases*, vol. 18, no. 6, pp. 999–1005, 2012.

[23] B. Hope, R. Shahdadpuri, C. Dunne et al., "The rising incidence of paediatric-onset inflammatory bowel disease," *Archives of Disease in Childhood*, vol. 97, no. 7, pp. 585–586, 2012.

[24] J. Martín-de-Carpi, A. Rodríguez, E. Ramos et al., "The complete picture of changing pediatric inflammatory bowel disease incidence in Spain in 25 years (1985–2009): the EXPERIENCE registry," *Journal of Crohn's and Colitis*, vol. 8, no. 8, pp. 763–769, 2014.

[25] O. Pozler, J. Maly, O. Bonova et al., "Incidence of Crohn disease in the Czech Republic in the years 1990 to 2001 and assessment of pediatric population with inflammatory bowel disease," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 42, no. 2, pp. 186–189, 2006.

[26] K. Karolewska-Bochenek, I. Lazowska-Przeorek, P. Albrecht et al., "Epidemiology of inflammatory bowel disease among children in Poland. A prospective, population-based, 2-year study, 2002–2004," *Digestion*, vol. 79, no. 2, pp. 121–129, 2009.

[27] M. I. El Mouzan, O. Saadah, K. Al-Saleem et al., "Incidence of pediatric inflammatory bowel disease in Saudi Arabia: a multicenter national study," *Inflammatory Bowel Diseases*, vol. 20, no. 6, pp. 1085–1090, 2014.

[28] K. E. Müller, P. L. Lakatos, A. Arató et al., "Incidence, paris classification, and follow-up in a nationwide incident cohort of pediatric patients with inflammatory bowel disease," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 57, no. 5, pp. 576–582, 2013.

[29] K. E. Müller, P. L. Lakatos, M. Papp, and G. Veres, "Incidence and paris classification of pediatric inflammatory bowel disease," *Gastroenterology Research and Practice*, vol. 2014, Article ID 904307, 10 pages, 2014.

[30] R. Orel, T. Kamhi, G. Vidmar, and P. Mamula, "Epidemiology of pediatric chronic inflammatory bowel disease in central and Western Slovenia, 1994–2005," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 48, no. 5, pp. 579–586, 2009.

[31] D. Urlep, T. K. Trop, R. Blagus, and R. Orel, "Incidence and phenotypic characteristics of pediatric IBD in Northeastern Slovenia, 2002–2010," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 58, no. 3, pp. 325–332, 2014.

[32] Statistical Office of the Republic of Slovenia, 2015, http://www.stat.si/eng/tema_demografsko_prebivalstvo.asp.

[33] IBD Working Group of the European Society for Paediatric Gastroenterology. Hepatology and Nutrition, "Inflammatory bowel disease in children and adolescents: recommendations for diagnosis—the Porto criteria," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 41, no. 1, pp. 1–7, 2005.

[34] A. Levine, A. Griffiths, J. Markowitz et al., "Pediatric modification of the Montreal classification for inflammatory bowel disease: the Paris classification," *Inflammatory Bowel Diseases*, vol. 17, no. 6, pp. 1314–1321, 2011.

[35] A. Sonnenberg, D. J. McCarty, and S. J. Jacobsen, "Geographic variation of inflammatory bowel disease within the United States," *Gastroenterology*, vol. 100, no. 1, pp. 143–149, 1991.

[36] S. Shivananda, J. Lennard-Jones, R. Logan et al., "Incidence of inflammatory bowel disease across Europe: is there a difference between north and south? Results of the European collaborative study on inflammatory bowel disease (EC-IBD)," *Gut*, vol. 39, no. 5, pp. 690–697, 1996.

[37] V. Nerich, E. Monnet, A. Etienne et al., "Geographical variations of inflammatory bowel disease in France: a study based on national health insurance data," *Inflammatory Bowel Diseases*, vol. 12, no. 3, pp. 218–226, 2006.

[38] M. D. Kappelman, S. L. Rifas-Shiman, K. Kleinman et al., "The prevalence and geographic distribution of Crohn's disease and ulcerative colitis in the United States," *Clinical Gastroenterology and Hepatology*, vol. 5, no. 12, pp. 1424–1429, 2007.

[39] A. Jussila, L. J. Virta, V. Salomaa et al., "High and increasing prevalence of inflammatory bowel disease in Finland with a clear North-South difference," *Journal of Crohn's and Colitis*, vol. 7, pp. e256–e262, 2013.

[40] M. F. Holick, "Vitamin D deficiency," *The New England Journal of Medicine*, vol. 357, no. 3, pp. 266–281, 2007.

[41] S. Ardizzone, A. Cassinotti, D. Trabattoni et al., "Immunomodulatory effects of 1,25-dihydroxyvitamin D3 on Th1/Th2 cytokines in inflammatory bowel disease: an in vitro study," *International Journal of Immunopathology and Pharmacology*, vol. 22, no. 1, pp. 63–71, 2009.

[42] N. Narula and J. K. Marshall, "Management of inflammatory bowel disease with vitamin D: beyond bone health," *Journal of Crohn's and Colitis*, vol. 6, no. 4, pp. 397–404, 2012.

[43] T. L. Van Belle, C. Gysemans, and C. Mathieu, "Vitamin D in autoimmune, infectious and allergic diseases: a vital player?" *Best Practice and Research: Clinical Endocrinology and Metabolism*, vol. 25, no. 4, pp. 617–632, 2011.

[44] V. Nerich, P. Jantchou, M.-C. Boutron-Ruault et al., "Low exposure to sunlight is a risk factor for Crohn's disease," *Alimentary Pharmacology and Therapeutics*, vol. 33, no. 8, pp. 940–945, 2011.

[45] R. Orel and D. Urlep, "Food and intestinal microorganisms: factors in pathogenesis, prevention and therapy of ulcerative colitis," in *Ulcerative Colitis—Treatments, Special Populations and the Future*, M. O'Connor, Ed., chapter 7, pp. 99–114, InTech, Rijeka, Croatia, 1st edition, 2011.

[46] C. A. Chapman-Kiddell, P. S. W. Davies, L. Gillen, and G. L. Radford-Smith, "Role of diet in the development of inflammatory bowel disease," *Inflammatory Bowel Diseases*, vol. 16, no. 1, pp. 137–151, 2010.

[47] A. N. Ananthakrishnan, H. Khalili, G. G. Konijeti et al., "A prospective study of long-term intake of dietary fiber and risk of Crohn's disease and ulcerative colitis," *Gastroenterology*, vol. 145, no. 5, pp. 970–977, 2013.

[48] A. N. Ananthakrishnan, H. Khalili, G. G. Konijeti et al., "Long-term intake of dietary fat and risk of ulcerative colitis and Crohn's disease," *Gut*, vol. 63, no. 5, pp. 776–784, 2014.

[49] A. Cárdeno, M. K. Magnusson, H. Strid, C. Alarcón De La Lastra, M. Sánchez-Hidalgo, and L. Öhman, "The unsaponifiable fraction of extra virgin olive oil promotes apoptosis and attenuates activation and homing properties of T cells from patients with inflammatory bowel disease," *Food Chemistry*, vol. 161, pp. 353–360, 2014.

[50] C. M. Yates, P. C. Calder, and G. E. Rainger, "Pharmacology and therapeutics of omega-3 polyunsaturated fatty acids in chronic inflammatory disease," *Pharmacology & Therapeutics*, vol. 141, no. 3, pp. 272–282, 2014.

[51] T. Pfeffer-Gik and A. Levine, "Dietary clues to the pathogenesis of Crohn's disease," *Digestive Diseases*, vol. 32, no. 4, pp. 389–394, 2014.

[52] C. De Filippo, D. Cavalieri, M. Di Paola et al., "Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 33, pp. 14691–14696, 2010.

[53] I. Costea, D. R. MacK, R. N. Lemaitre et al., "Interactions between the dietary polyunsaturated fatty acid ratio and genetic factors determine susceptibility to pediatric Crohn's disease," *Gastroenterology*, vol. 146, no. 4, pp. 929–931, 2014.

[54] L. R. Ferguson, "Nutrigenetics, nutrigenomics and inflammatory bowel diseases," *Expert Review of Clinical Immunology*, vol. 9, no. 8, pp. 717–726, 2013.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 416838, 12 pages
http://dx.doi.org/10.1155/2015/416838

Research Article

Genetic Polymorphisms of *IL-17F* and *TRAF3IP2* Could Be Predictive Factors of the Long-Term Effect of Infliximab against Crohn's Disease

Shigetoshi Urabe,[1] Hajime Isomoto,[1] Tetsuya Ishida,[2] Kazumi Maeda,[3] Tatsuo Inamine,[3] Shinji Kondo,[3] Norihide Higuchi,[4] Kayoko Sato,[4] Ryohei Uehara,[1] Hiroyuki Yajima,[1] Haruhisa Machida,[1] Chun Chuan Chen,[1] Yasuhiro Fukuda,[1] Fuminao Takeshima,[1] Kazuhiko Nakao,[1] and Kazuhiro Tsukamoto[3]

[1]*Department of Gastroenterology and Hepatology, Nagasaki University Hospital, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan*
[2]*Department of Gastroenterology, Oita Red Cross Hospital, 3-2-27 Chiyo-machi, Oita 870-0033, Japan*
[3]*Department of Pharmacotherapeutics, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan*
[4]*Department of Hospital Pharmacy, Nagasaki University Hospital, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan*

Correspondence should be addressed to Kazuhiro Tsukamoto; ktsuka@nagasaki-u.ac.jp

Received 8 July 2015; Accepted 27 September 2015

Academic Editor: Atsushi Sakuraba

Background. We aimed to identify certain genes related to response to infliximab (IFX) and biomarkers to predict the IFX effect for Japanese Crohn's disease (CD) patients by performing an association study of single nucleotide polymorphisms (SNPs) in candidate genes in the interleukin- (IL-) 17 signaling pathway with response to IFX after 1 year of treatment. *Methods*. A total of 103 patients were divided into two groups, responders and nonresponders. Twenty-eight tag SNPs in 5 genes were genotyped. The frequencies of alleles and genotypes of each SNP were compared between responders and nonresponders in three different inheritance models. A genetic test was performed using a combination of the associated SNPs as biomarkers. *Results*. Multivariate logistic regression analysis indicated that the four variable factors, concomitant use of immunomodulators, penetrating disease, a G/G genotype of rs766748 in *IL-17F*, and a C/C or C/A genotype of rs1883136 in *TRAF3IP2*, independently contributed to response to IFX after 1 year of treatment. Genetic test using the polymorphisms of these genes perfectly predicted the responder and nonresponder CD patients with both concomitant use of immunomodulators and penetrating disease. *Conclusion*. *IL17F* and *TRAF3IP2* are one of IFX-related genes, useful as biomarkers of IFX response, and may be target molecules for new therapeutic drugs.

1. Introduction

Crohn's disease (CD) is involved in idiopathic inflammatory bowel disease (IBD) and is mainly characterized by chronic granulomatous inflammatory changes in the gastrointestinal tract. Although the etiology of CD is unknown, it can be attributed to numerous environmental factors, genetic predisposition, and excessive immune and inflammatory responses [1, 2]. In most cases, CD develops at a young age and its symptoms, such as abdominal pain, diarrhea, and bloody stool, undergo cycles of remission and relapse, eventually resulting in the impairment of the quality of the life of CD patients [3].

Treatments of CD are selected on the basis of the present site of the lesions, the degree of inflammation, the presence or absence of complications, and the previous response to treatment. Among medical therapies, 5-amino salicylic acid is often used for patients with mild disease severity, whereas

steroids and/or anti-TNF-α antibodies, such as infliximab (IFX) and adalimumab, are used for patients with moderate or severe disease severity [4].

IFX is a chimeric anti-TNF-α monoclonal antibody that consists of the variable region of the murine anti-TNF-α antibody and the constant region of human IgG1. IFX inhibits the action of TNF-α by neutralizing the biological activity of soluble TNF-α, by damaging cells on membrane-bound TNF-α, and by dissociating TNF-α from its receptor [5]. IFX is widely available for the treatment of CD since 1991, when its usefulness has been confirmed in clinical settings worldwide. In Japan, clinical trials of IFX were started in 1996. In the ACCENT I randomized clinical trial carried out in North America, Europe, and Israel, about 58% of patients responded within 2 weeks to a single infusion of 5 mg/kg IFX. However, thereafter only 39% of these responders, who received repeated infusions of IFX every 8 weeks, were still in remission after 54 weeks of treatment [6]. Therefore, identification of biomarkers to predict the long-term therapeutic effect of IFX is warranted.

Interleukin- (IL-) 17 is an inflammatory cytokine that is secreted from Th17 cells. Within the IL-17 families, there are six ligands (IL-17A to F) and five receptors (IL-17RA to RE). In particular, intestinal Paneth cells express IL-17A and colonic epithelial cells produce IL-17F [7, 8]. After IL-17A forms a homodimerization with itself or a heterodimerization with IL-17F, their complex binds to a dimerized receptor consisting of IL-17RA and IL-17RC and subsequently transmits signals to downstream pathways through traf3-interacting protein 2 (TRAF3IP2), which share intracellular signal transduction molecules, such as I-κB and NF-κB, with the TNF-α signaling pathway [8–10]. Moreover, upregulation of parallel signaling pathways, including HGF and MET, to bypass the inhibited EGFR signaling pathway is known as one of the resistance mechanisms to gefitinib for patients with lung adenocarcinoma [11]. Thus, we speculate that the same resistance mechanism may occur to the second loss of response to IFX after 1 year of treatment. Indeed, IL-17A is overexpressed in inflammatory lesions and in the blood of patients with CD, multiple sclerosis, or systemic lupus erythematosus [12–14]. Furthermore, a correlation between the therapeutic effect of IFX and a decrease in the expression of IL-17RA after IFX administration has been observed in patients with rheumatoid arthritis [15]. Thus, IL-17 and its intracellular signaling pathways play a pivotal role not only in the pathogenesis of immune diseases including CD, but also in the response to IFX treatment.

Here, to assess as putative genes related to response to IFX, we examined a candidate gene-based association study by selecting several target genes involved in the IL-17 signaling pathway and investigated whether polymorphisms of these target genes are associated with the therapeutic effect of IFX for Japanese CD patients. We further investigated whether such polymorphisms could be used as new genetic biomarkers to identify Japanese CD patients showing response to IFX after the long-term treatment of 1 year.

2. Subjects and Methods

2.1. Subjects. The present study consisted of 113 unrelated Japanese CD patients treated with IFX in Oita Red Cross Hospital or Nagasaki University Hospital from 2004 to 2011.

The study protocol was approved by the Ethics Committee dealing with Human Genome and Gene Analysis at Oita Red Cross Hospital as well as at Nagasaki University. Written informed consent was obtained from each patient.

The diagnosis of CD was made based on the endoscopic, radiological, histological, and clinical criteria established by both the World Health Organization Council for International Organizations of Medical Sciences and the International Organization for the Study of Inflammatory Bowel Disease [16, 17]. Patients with indeterminate colitis, multiple sclerosis, systemic lupus erythematosus, or any other diagnosed autoimmune diseases were excluded from this study.

2.2. Definition of the Therapeutic Effect of IFX. Since Crohn's disease activity index (CDAI) of more than 150 [18] is regarded as active-phase CD, responders to IFX were defined as those showing a decrease in CDAI of less than 150 and an improvement in clinical manifestations, laboratory data, and/or endoscopic findings. Nonresponders to IFX were defined as those showing no change in the CDAI value or exacerbation of disease activity.

2.3. Study Design. Of the 113 CD patients enrolled in this study, 103 patients who had shown response to IFX after 10 weeks of IFX treatment were subjected to this association study (Figure 1). These 103 responders to IFX at the end of the 10-week treatment were then divided into two groups, responders ($n = 89$) and nonresponders ($n = 14$), based on the presence or absence of IFX effect after the long-term IFX treatment of 1 year as shown in Figure 1. In addition, Table 1 shows the clinical characteristics including mean age, gender, smoking, concomitant use of immunomodulators, colonic location, and disease behavior, of both responders and nonresponders after 1 year of treatment (Table 1).

2.4. Selection of Tag Single Nucleotide Polymorphisms in Candidate Genes. All candidate genes selected in this study are involved in the IL-17 signaling pathway, including the genes encoding interleukin-17A (IL17A/*IL17A*; OMIM #603149); interleukin-17F (IL17F/*IL17F*; OMIM #606496); interleukin-17 receptor A (IL17RA/*IL17RA*; OMIM #605461); interleukin-17 receptor C (IL17RC/*IL17RC*; OMIM #610925); and traf3-interacting protein 2 (TRAF3IP2/*TRAF3IP2*; OMIM #1607043).

All of the information regarding single nucleotide polymorphisms (SNPs) in the candidate genes was obtained from Japanese data in Tokyo (Rel 24/phase II Nov. 08, on NCBI B36 assembly, dbSNP b126), which are available on the International HapMap website [19]. Candidate tag SNPs were selected from all SNPs in each chromosomal region including 2 kb upstream of the gene with priority for a minor allele frequency of more than 20% in the International HapMap

Table 1: Comparison of the clinical characteristics of responders and nonresponders to IFX after 1 year of treatment of CD patients.

Characteristics	CD patients		P value
	Responders	Nonresponders	
Number	89	14	
Age, mean ± SD (years)	35.4 ± 12.9	37.8 ± 10.3	0.368
Male/female (%)	50/39 (56.2/43.8)	9/5 (64.3/35.7)	0.773
Smoking (%)	17 (19.1)	5 (35.7)	0.172
Concomitant use of immunomodulators (%)	20 (22.5)	8 (57.1)	0.007
Colonic location (%)*			
Ilium	16 (18.0)	2 (14.3)	1.000
Colon	17 (19.1)	3 (21.4)	1.000
Ileocolon	56 (62.9)	9 (64.3)	1.000
Disease behavior (%)*			
Nonstricturing/penetrating	43 (48.3)	5 (35.7)	0.409
Stricturing	37 (41.6)	3 (21.4)	0.238
Penetrating	18 (20.2)	8 (57.1)	0.006

*Classification according to Montreal Classification for CD.
IFX: infliximab; CD: Crohn's disease; SD: standard deviation.

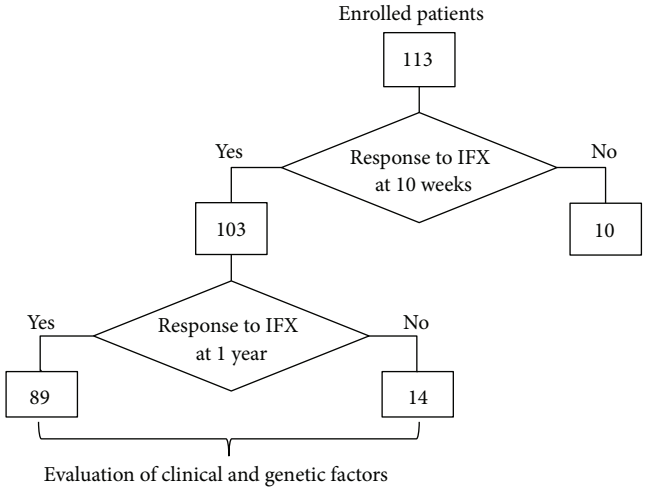


Figure 1: Flowchart of study design. A total of 113 CD patients were enrolled in this study. Of these patients, at the end of the 10-week IFX treatment, 103 patients showed response to IFX, 8 patients indicated loss of response to IFX, and IFX treatments of 2 patients were stopped due to side reactions of IFX. After 1 year of IFX treatment, 89 of the 103 patients still showed response to IFX, but the other 14 patients showed loss of response to IFX. CD: Crohn's disease; IFX: infliximab.

data. Subsequently, genotyped tag SNPs among the candidate tag SNPs were determined based on linkage disequilibrium (LD) tagging using the Haploview 4.1 software program ($r^2 > 0.8$) [20].

Twenty-eight tag SNPs, five in *IL17A*, seven in *IL17F*, eight in *IL17RA*, two in *IL17RC*, and six in *TRAF3IP2*, were selected as the genotyped tag SNPs. Information regarding the structure of the gene and the positions of the genotyped tag SNP sites in each candidate gene is shown in Figure 2.

2.5. Genotyping of Tag SNPs in Each Gene. Genomic DNA was extracted from whole blood samples using a DNA Extractor WB-Rapid Kit (Wako, Osaka, Japan) or a Quick Gene DNA Whole Blood Kit S (Fujifilm, Tokyo, Japan) with a Quick Gene-800 (Fujifilm) according to the manufacturer's protocol.

A total of 28 tag SNPs in 5 candidate genes were genotyped by polymerase chain reaction- (PCR-) restriction fragment length polymorphism (RFLP) or PCR-direct DNA sequencing method (Table 2) [21]. The polymorphic region was amplified by PCR with a GeneAmp PCR System 9700 thermal cycler (Life Technologies, Carlsbad, CA, USA) using 20 ng genomic DNA in a 25 μL reaction mixture containing 1x GoTaq Green master mix (Promega, Madison, WI, USA) and 15 pmol each of forward and reverse primers (Table 2). The amplification protocol consisted of initial denaturation at 95°C for 2 minutes, followed by 30 or 35 cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds at the appropriate temperature for the primer pair (Table 2), extension at 72°C for 30 seconds, and final extension at 72°C for 5 minutes.

For the RFLP method, the PCR products were digested with the relevant restriction enzyme (Table 2), separated by electrophoresis on a 6% to 12% polyacrylamide gel (Nacalai Tesque, Kyoto, Japan) or a 2% ME-agarose gel (Nacalai Tesque), stained with ethidium bromide, and visualized using an ultraviolet transilluminator (Alpha Innotech Co., San Leandro, CA, USA).

For the direct DNA sequencing method, the PCR products were treated with ExoSAP-IT (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and cycle sequenced using a BigDye Terminator v3.1 Cycle Sequencing FS Ready Reaction Kit (Life Technologies). The cycle sequencing was hot-started at 96°C for 30 seconds, followed by 25 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds, and extension at 60°C for 4 minutes using 1 pmol PCR forward or reverse primer. After the sequencing reaction solutions were

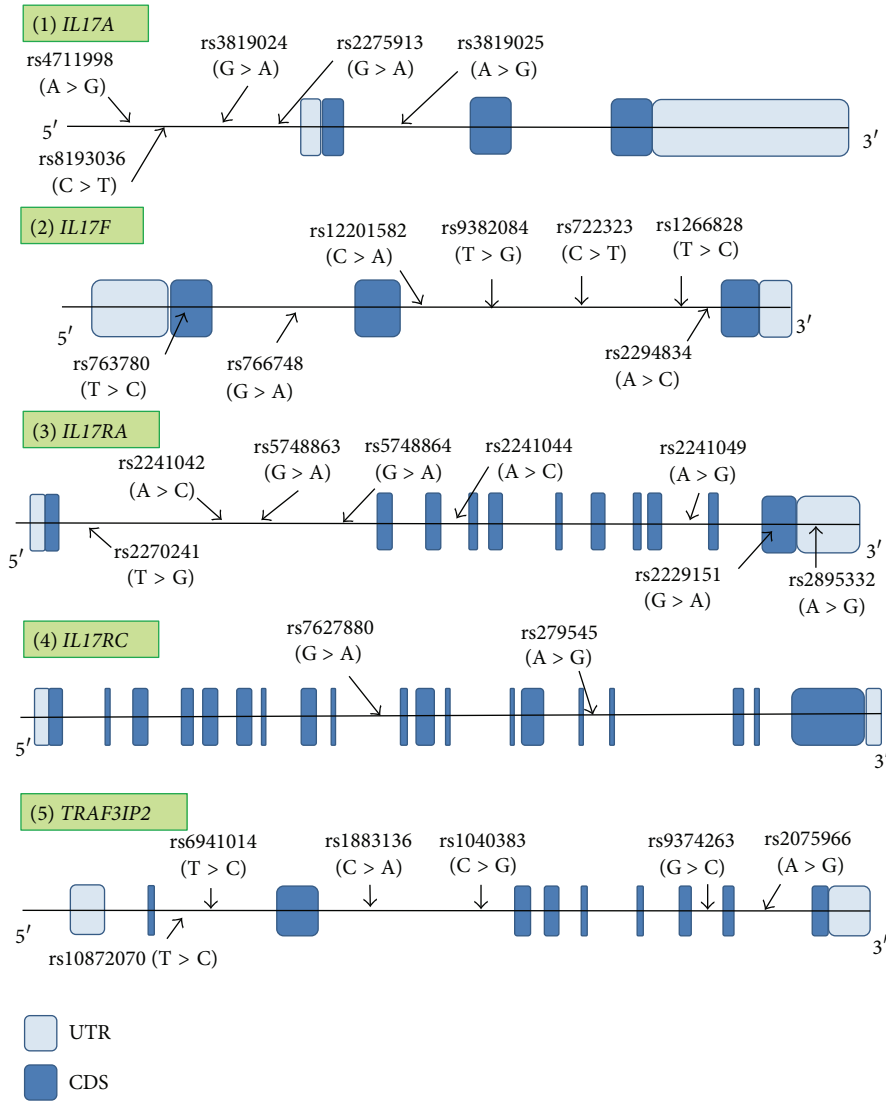


Figure 2: Gene structures and locations of genotyped tag SNP sites in each gene. The horizontal bars indicate the genomic sequences of candidate genes. Full boxes represent exons in each gene, and open boxes show the untranslated regions. The arrows indicate the positions of the genotyped tag SNP sites in this study and their names are presented above each site. SNPs: single nucleotide polymorphisms; CDS: coding sequence; UTR: untranslated region.

purified using Sephadex G-50 superfine columns (Amersham Pharmacia Biotech), the samples were dried and sequenced with an ABI Prism 3100 Genetic Analyzer (Life Technologies).

2.6. Statistical Analyses. The mean age of IFX-responder CD patients and nonresponder CD patients after 1 year of treatment was presented as means ± standard deviation and was compared using the Mann-Whitney U test. The other clinical characteristics were compared using a chi-square or Fisher's exact test. The above statistical analyses were performed using the IBM SPSS Statistics 20 software package (IBM Japan, Tokyo, Japan) or Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

The significance of deviation from the Hardy-Weinberg equilibrium (HWE) and LD between pairs of SNPs was analyzed using a chi-square test based on the expectation-maximization algorithm using the SNPAlyze 7.0 standard software package (Dynacom Inc., Chiba, Japan). The frequencies of alleles and genotypes were compared between responders and nonresponders after 1 year of treatment using the chi-squared or Fisher's exact test with odds ratio (OR) and 95% confidence interval in three different inheritance models: the allele, the minor allele dominant, and the minor allele recessive, using the SNPAlyze 7.0 standard software package.

In addition to univariate analyses, multivariate logistic regression analysis was carried out for analysis of the interaction of clinical environmental factors and putative genetic

Table 2: Information regarding the genotyping of tag SNPs in candidate genes.

Gene	Tag SNP	Major > minor	Sequence of primers (5′ to 3′)		Annealing temperature (°C)	Cycle number	Analytical method (restriction enzyme)
			Forward	Reverse			
IL17A	rs4711998	A > G	TGTCCTCCAATTCCCTTTTG	GAGACATGATGGGGGAAAGA	60	30	PCR-RFLP (*Bpu* E I)
	rs8193036	C > T	TCTTTCCCCCATCATGTCTC	GTTCCAACCCTGCATGCTAC	60	30	PCR-direct DNA sequencing
	rs3819024	G > A	AGGCACAAACTCATCCATCC	GTCAGAACCCAGCGTTTCAT	60	30	PCR-direct DNA sequencing
	rs2275913	G > A	GTTTCCGGAATTGTCTCCAC	CCCAGGAGTCATCGTTGTTT	60	30	PCR-RFLP (*Bsl* I)
	rs3819025	A > G	AGTTTCCGGAATTGTCTCCA	CAGCTGCCAGAAGAGTTATGC	60	30	PCR-direct DNA sequencing
IL17F	rs763780	T > C	CACTGGTGCTCTGATGAGGA	CTGCATCAATGCTCAAGGAA	60	35	PCR-RFLP (*Hsp* 92 II)
	rs766748	G > A	GTGGGAATGAGTGGAGGAGA	TCCACTCGCTAAGCTGGACT	60	30	PCR-RFLP (*Afa* I)
	rs12201582	C > A	ACATTACGCAAAACCAACGA	TCATCCCCAGTAAGGGTCAG	60	30	PCR-direct DNA sequencing
	rs9382084	T > G	ATCAACTTTCATCCCCCACA	CGCCATAGCAGTTTGTCAAG	60	35	PCR-RFLP (*Alu* I)
	rs722323	C > T	GGATTTCTCTGAGAGGTGCTG	GGAAATCAGATGATGTCTGCAA	60	30	PCR-direct DNA sequencing
	rs1266828	T > C	CCCTGGATGGAAGAAATGAA	CATCAAAGCCTATGCCCCTA	60	30	PCR-RFLP (*Hpy* CH4 V)
	rs2294834	A > C	TTTGATTGGGGTCTTTTTGG	GGGATTACAGGCAACTGACC	60	30	PCR-direct DNA sequencing
IL17RA	rs2270241	T > G	CTGCGACTCCTGGACCAC	GTATTCCACACCGCAACTCC	60	35	PCR-direct DNA sequencing
	rs2241042	A > C	AGCTGCTTGCACAACTGCTA	CGCTTCTGGCATCTTTCTC	60	30	PCR-RFLP (*Hha* I)
	rs5748863	G > A	CAGGCGTGAGCCATGAAATT	GGCGCAGGGATCTACTGTTT	61	35	PCR-RFLP (*Bsa* A I)
	rs5748864	G > A	CTTGGTCTGGGTCTTCGTTG	GGAACCTCCACATGTTCCAC	60	30	PCR-RFLP (*Hpy* CH4 IV)
	rs2241044	A > C	TATGGGAACCAGAGCACCTC	GGTCCCAGGATGAAGAAGGT	60	35	PCR-RFLP (*Tsp* R I)
	rs2241049	A > G	ATGACCCTAGGCTGCTCCTT	GCGGGGGTTAACTCCTTAGT	60	30	PCR-RFLP (*Hpy* 188 I)
	rs2229151	G > A	TCATCTACTCAGCCGACCAC	GGAGCACAGGACGATGATCT	60	30	PCR-RFLP (*Hpy* 188 III)
	rs2895332	A > G	GCGTCCTTGAGGCTCCATTA	CTGGCCCATTCAGCGTTTAC	60	30	PCR-RFLP (*Hpy* CH4 IV)
IL17RC	rs7627880	G > A	CAAGGTCTCTTGTGCTTGC	GGATGCACTCATTCAGCAG	58	30	PCR-RFLP (*Mse* I)
	rs279545	A > G	CAGCCCTGGGAAAGTTAAG	CTGTCAAGATCCCCACTCC	60	30	PCR-RFLP (*Bse* Y I)
TRAF3IP2	rs10872070	T > C	CAAGCCTAGGCCATAAGCAG	TCCCACGTAGTCACCATTCA	60	30	PCR-RFLP (*Hsp* 92 II)
	rs6941014	T > C	AGCAGAGGGTGAGAGCATGT	TTGCTGATGAGCCTGAGATG	60	30	PCR-RFLP (*Hae* III)
	rs1883136	C > A	AATAGCTTCCCTGCGGACTT	GTGGGAGTTCCTGCAACAGT	60	30	PCR-RFLP (*Ssp* I)
	rs1040383	C > G	AGGCAACCAACTGGCAATAC	CTTTCTCCCAGGTTGCACAT	60	30	PCR-RFLP (*Xsp* I)
	rs9374263	G > C	TTTAACCAGGCCCACATGAT	TCAGGAGAGGAGCTGTTGGT	60	30	PCR-RFLP (*Hae* III)
	rs2075966	A > G	GAAATTGGCGATGGTATTGG	TCACACCTCCAGACATTTGC	60	30	PCR-RFLP (*Xsp* I)

SNP: single nucleotide polymorphism; 3′-UTR: 3′ untranslated region; PCR: polymerase chain reaction; RFLP: restriction fragment length polymorphism.

factors with the therapeutic effect of IFX after 1 year of treatment using JMP Pro 11 (SAS Institute Inc., Tokyo, Japan). A P value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Comparison of the Clinical Characteristics of Responders and Nonresponders to IFX. When the clinical characteristics of responders and nonresponders to IFX after 1 year of treatment were compared, there were significant differences in the concomitant use of immunomodulators as well as in the presence of penetrating disease behavior between the two groups (Table 1). Thus, the percentage of nonresponder CD patients concomitantly treated with immunomodulators was higher than that of the responders (57.1% versus 22.5%, $P = 0.007$). The percentage of nonresponder CD patients with penetrating disease was also higher than that of the responders (57.1% versus 20.2%, $P = 0.006$).

3.2. Association between Tag SNPs and Response to IFX after 1 Year of Treatment. Comparison of the distribution of alleles and genotypes of tag SNPs in each gene between responders and nonresponders to IFX after 1 year of treatment is shown in Table 3. The three tag SNPs, rs5748863 in *IL17RA* and rs10872070 and rs2075966 in *TRAF3IP2*, were excluded from the subsequent analyses because they were not in HWE.

Chi-square or Fisher's exact test in three different inheritance models indicated that the frequency of a heterozygous G/A genotype or a minor homozygous A/A genotype of rs766748 in *IL17F* in the minor allele dominant model was significantly decreased in responders as compared to that in nonresponders ($P = 0.019$, OR = 0.203; Table 3). This result implies that there was a ~4.9-fold loss of response to IFX in the nonresponders with these genotypes after 1 year of treatment as compared to the responders. Conversely, possessing a major homozygous G/G genotype of rs766748 in *IL17F* indicated that there was a ~4.9-fold response to IFX in the responders with this genotype as compared to the nonresponders.

Moreover, the frequency of a minor homozygous A/A genotype of rs1883136 in *TRAF3IP2* in the minor allele recessive model was significantly lower in responders in comparison to that in nonresponders ($P = 0.041$, OR = 0.213; Table 3), indicating that this genotype is associated with a ~4.7-fold loss of response to IFX. Conversely, possessing a major homozygous C/C genotype or a heterozygous C/A genotype of rs1883136 in *TRAF3IP2* indicated a ~4.7-fold increase in response to IFX after 1 year of treatment.

3.3. The Interaction of Genetic and Environmental Factors on Response to IFX after 1 Year of Treatment. Univariate analyses of the differences between responders and nonresponders indicated that the environmental factors of nonconcomitant use of immunomodulators or nonpenetrating disease and the genetic factors of the G/G genotype of rs766748 in *IL17F* or the C/C or C/A genotype of rs1883136 in *TRAF3IP2* showed response to IFX after 1 year of treatment. We then performed multivariate logistic regression analysis of the influence of the interaction of these factors on response to IFX after 1 year of treatment. This analysis revealed that these four variable factors independently contributed to response to IFX ($P = 0.0162$, OR = 5.281, $P = 0.0073$, OR = 6.529, $P = 0.0213$, OR = 5.123, and $P = 0.0149$, OR = 10.43, resp.; Table 4).

3.4. Verification of Genetic Test for Prediction of Response to IFX. In order to better predict the response to IFX for CD patients after 1 year of treatment, we carried out a genetic test using a combination of the two independent genetic factors (*IL17F* and *TRAF3IP2* genotypes) with or without the concomitant use of immunomodulators and/or penetrating disease (Tables 5 and 6). With regard to the patients concomitantly treated with immunomodulators, the prediction panel indicated that both the G/G genotype of rs766748 in *IL17F* and the C/C or C/A genotype of rs1883136 in *TRAF3IP2* were strongly associated with response to IFX in the CD patients with use of immunomodulators ($P = 0.0019$, OR = 37.92; Table 5). In this genetic test, the sensitivity, specificity, positive predictive value, and negative predictive value were estimated at 70.0%, 100%, 100%, and 57.1%, respectively.

Moreover, when the CD patients with both the concomitant use of immunomodulators and penetrating disease were considered, the above described polymorphisms showed a close association with response to IFX with the sensitivity, specificity, positive predictive value, and negative predictive value all estimated as 100% (Table 6).

On the other hand, there were no significant associations with response to IFX after 1 year of treatment in the patients with other combinations of with or without the concomitant use of immunomodulators and/or penetrating disease (Supplemental Tables 1–6 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/416838).

4. Discussion

The present study is the first report to show that each of the genetic factors, polymorphisms of *IL-17F* and *TRAF3IP2*, and the clinical risk factors, concomitant use of immunomodulators and penetrating disease, independently contributed to the therapeutic effect of IFX after the long-term (1 year) IFX treatment of Japanese CD patients. The CD patients possessing the G/G genotype of rs766748 in *IL-17F* or the C/C or C/A genotype of rs1883136 in *TRAF3IP2*, or having nonuse of immunomodulators or nonpenetrating disease behavior, showed good response to IFX after 1 year of treatment. Conversely, the patients possessing the G/A or A/A genotype of rs766748 in *IL-17F* or the A/A genotype of rs1883136 in *TRAF3IP2*, or having the concomitant use of immunomodulators or penetrating disease behavior, displayed the secondary loss of response to IFX after 1 year of treatment although they had presented good response to IFX at the end of the 10-week IFX treatment.

TRAF3IP2 is an IL-17R adaptor protein that is also referred to as NF-κB activator 1. After a heterodimer of IL-17A and IL-17F binds to their receptor, that is, comprised of IL-17RA and IL-17RC, the IL17-IL17R complex signals induce

Table 3: Allele and genotype comparisons in three inheritance models between responders and nonresponders to IFX after 1 year of treatment of CD patients.

Gene symbol	Tag SNP (Major > minor)	Genotype	Number of genotypes (%) Responders $n = 89$	Nonresponders $n = 14$	Inheritance model*	P value	OR	95% CI
IL17A	rs4711998 A > G	MAF	0.337	0.321	Allele	0.870	1.073	0.458–2.517
		A/A	39 (43.8)	6 (42.9)				
		A/G	40 (44.9)	7 (50.0)	Dominant	0.946	0.962	0.308–3.002
		G/G	10 (11.2)	1 (7.1)	Recessive	1.000	1.646	0.194–13.96
	rs8193036 C > T	MAF	0.438	0.393	Allele	0.653	1.205	0.534–2.722
		C/C	32 (36.0)	5 (35.7)				
		C/T	36 (40.4)	7 (50.0)	Dominant	1.000	0.990	0.305–3.208
		T/T	21 (23.6)	2 (14.3)	Recessive	0.730	1.853	0.384–8.954
	rs3819024 G > A	MAF	0.421	0.423	Allele	0.669	0.840	0.377–1.870
		G/G	33 (37.1)	5 (35.7)				
		G/A	37 (41.6)	5 (35.7)	Dominant	1.000	0.943	0.291–3.053
		A/A	19 (21.3)	4 (28.6)	Recessive	0.509	0.679	0.191–2.406
	rs2275913 G > A	MAF	0.343	0.393	Allele	0.605	0.806	0.355–1.828
		G/G	40 (44.9)	5 (35.7)				
		G/A	37 (41.6)	7 (50.0)	Dominant	0.575	0.681	0.211–2.194
		A/A	12 (13.5)	2 (14.3)	Recessive	1.000	0.935	0.186–4.707
	rs3819025 G > A	MAF	0.292	0.321	Allele	0.752	0.871	0.370–2.052
		G/G	47 (52.8)	7 (50.0)				
		G/A	32 (36.0)	5 (35.7)	Dominant	0.845	0.894	0.289–2.760
		A/A	10 (11.2)	2 (14.3)	Recessive	0.666	0.759	0.148–3.897
IL17F	rs763780 T > C	MAF	0.157	0.071	Allele	0.385	2.427	0.545–10.81
		T/T	63 (70.8)	12 (85.7)				
		T/C	24 (27.0)	2 (14.3)	Dominant	0.341	2.476	0.518–11.85
		C/C	2 (2.2)	0 (0)	Recessive	1.000	0.829	0.038–18.17
	rs766748 G > A	MAF	0.247	0.429	Allele	0.045	0.438	0.192–0.997
		G/G	51 (57.3)	3 (21.4)				
		G/A	32 (36.0)	10 (71.4)	Dominant	0.019	0.203	0.053–0.779
		A/A	6 (6.7)	1 (7.1)	Recessive	1.000	0.940	0.105–8.454
	rs12201582 C > A	MAF	0.112	0.143	Allele	0.750	0.760	0.239–2.414
		C/C	70 (78.7)	10 (71.4)				
		C/A	18 (20.2)	4 (28.6)	Dominant	0.509	0.679	0.191–2.406
		A/A	1 (1.1)	0 (0)	Recessive	1.000	0.554	0.021–14.29
	rs9382084 T > G	MAF	0.433	0.357	Allele	0.453	1.372	0.600–3.141
		T/T	34 (38.2)	4 (28.6)				
		T/G	33 (37.1)	10 (71.4)	Dominant	0.564	0.647	0.188–2.227
		G/G	22 (24.7)	0 (0)	Recessive	0.037	9.671	0.554–168.8
	rs722323 C > T	MAF	0.438	0.429	Allele	0.924	1.040	0.465–2.326
		C/C	31 (34.8)	4 (28.6)				
		C/T	38 (42.7)	8 (57.1)	Dominant	0.768	0.748	0.217–2.584
		T/T	20 (22.5)	2 (14.3)	Recessive	0.729	1.739	0.359–8.425
	rs1266828 T > C	MAF	0.146	0.143	Allele	1.000	1.026	0.329–3.201
		T/T	66 (74.2)	10 (71.4)				
		T/C	20 (22.5)	4 (28.6)	Dominant	0.757	0.871	0.249–3.051
		C/C	3 (3.4)	0 (0)	Recessive	1.000	1.173	0.058–23.94
	rs2294834 A > C	MAF	0.157	0.179	Allele	0.783	0.859	0.301–2.449
		A/A	65 (73.0)	9 (64.3)				
		A/C	20 (22.5)	5 (35.7)	Dominant	0.530	0.665	0.202–2.184
		C/C	4 (4.5)	0 (0)	Recessive	1.000	1.526	0.078–29.90

Table 3: Continued.

Gene symbol	Tag SNP (Major > minor)	Genotype	Number of genotypes (%) Responders $n = 89$	Nonresponders $n = 14$	Inheritance model*	P value	OR	95% CI
		MAF	0.365	0.429	Allele	0.519	0.767	0.342–1.721
	rs2270241 T > G	T/T	34 (38.2)	5 (35.7)				
		T/G	45 (50.6)	6 (42.9)	Dominant	1.000	0.899	0.278–2.908
		G/G	10 (11.2)	3 (21.4)	Recessive	0.379	0.464	0.110–1.952
		MAF	0.225	0.286	Allele	0.478	0.725	0.297–1.769
	rs2241042 A > C	A/A	52 (58.4)	6 (42.9)				
		A/C	34 (38.2)	8 (57.1)	Dominant	0.275	0.534	0.171–1.668
		C/C	3 (3.4)	0 (0)	Recessive	1.000	1.173	0.058–23.94
		MAF	0.478	0.536	Allele	0.567	0.792	0.356–1.761
	rs5748864 G > A	G/G	24 (27.0)	3 (21.4)				
		G/A	45 (50.6)	7 (50.0)	Dominant	0.757	0.739	0.190–2.878
		A/A	20 (22.5)	4 (28.6)	Recessive	0.734	0.725	0.205–2.560
		MAF	0.292	0.357	Allele	0.486	0.743	0.321–1.717
IL17RA	rs2241044 A > C	A/A	43 (48.3)	5 (35.7)				
		A/C	40 (44.9)	8 (57.1)	Dominant	0.407	0.594	0.185–1.915
		C/C	6 (6.7)	1 (7.1)	Recessive	1.000	0.940	0.105–8.454
		MAF	0.326	0.429	Allele	0.286	0.644	0.286–1.451
	rs2241049 A > G	A/A	43 (48.3)	6 (42.9)				
		A/G	34 (38.2)	4 (28.6)	Dominant	0.704	0.802	0.257–2.502
		G/G	12 (13.5)	4 (28.6)	Recessive	0.225	0.390	0.105–1.444
		MAF	0.287	0.321	Allele	0.706	0.849	0.360–1.998
	rs2229151 G > A	G/G	44 (49.4)	7 (50.0)				
		G/A	39 (43.8)	5 (35.7)	Dominant	0.969	1.023	0.331–3.158
		A/A	6 (6.7)	2 (14.3)	Recessive	0.297	0.434	0.078–2.401
		MAF	0.348	0.286	Allele	0.517	1.336	0.556–3.209
	rs2895332 A > G	A/A	37 (41.6)	6 (42.9)				
		A/G	42 (47.2)	8 (57.1)	Dominant	0.928	1.054	0.337–3.295
		G/G	10 (11.2)	0 (0)	Recessive	0.350	3.830	0.212–69.09
		MAF	0.112	0	Allele	0.082	7.372	0.433–125.5
	rs7627880 G > A	G/G	71 (79.8)	14 (100)				
		G/A	16 (18.0)	0 (0)	Dominant	0.122	7.503	0.427–131.8
IL17RC		A/A	2 (2.2)	0 (0)	Recessive	1.000	0.829	0.038–18.17
		MAF	0.213	0.179	Allele	0.806	1.249	0.445–3.503
	rs279545 A > G	A/A	55 (61.8)	9 (64.3)				
		A/G	30 (33.7)	5 (35.7)	Dominant	1.000	1.113	0.344–3.600
		G/G	4 (4.5)	0 (0)	Recessive	1.000	1.526	0.078–29.90
		MAF	0.365	0.500	Allele	0.173	0.575	0.258–1.282
	rs6941014 T > C	T/T	35 (39.3)	4 (28.6)				
		T/C	43 (48.3)	6 (42.9)	Dominant	0.560	0.617	0.179–2.123
		C/C	11 (12.4)	4 (28.6)	Recessive	0.120	0.353	0.094–1.321
		MAF	0.292	0.464	Allele	0.068	0.476	0.212–1.071
	rs1883136 C > A	C/C	44 (49.4)	5 (35.7)				
		C/A	38 (42.7)	5 (35.7)	Dominant	0.398	0.568	0.176–1.830
TRAF3IP2		A/A	7 (7.9)	4 (28.6)	Recessive	0.041	0.213	0.053–0.860
		MAF	0.528	0.357	Allele	0.257	1.609	0.703–3.679
	rs1040383 C > G	C/C	24 (27.0)	6 (42.9)				
		C/G	46 (51.7)	6 (42.9)	Dominant	0.224	2.031	0.638–6.465
		G/G	19 (21.3)	2 (14.3)	Recessive	0.729	1.629	0.335–7.914

Table 3: Continued.

Gene symbol	Tag SNP (Major > minor)	Genotype	Number of genotypes (%) Responders $n = 89$	Nonresponders $n = 14$	Inheritance model*	*P* value	OR	95% CI
		MAF	0.331	0.429	Allele	0.315	0.661	0.294–1.488
	rs9374263 G > C	G/G	40 (44.9)	6 (42.9)				
		G/C	39 (43.8)	4 (28.6)	Dominant	0.884	0.919	0.294–2.868
		C/C	10 (11.2)	4 (28.6)	Recessive	0.096	0.317	0.083–1.201

*Allele: allele model; Dominant: the minor allele dominant model; Recessive: the minor allele recessive model.
IFX: infliximab; CD: Crohn's disease; SNP: single nucleotide polymorphism; MAF: minor allele frequency; OR: odds ratio; CI: confidence interval.

Table 4: The interaction of genetic and environmental factors for response to IFX after 1 year of treatment of CD patients.

Factor	OR (95% CI)	*P* value*
Nonconcomitant use of immunomodulators	5.281 (1.360–23.09)	0.0162
Nonpenetrating	6.529 (1.651–30.41)	0.0073
G/G genotype of rs766748 in *IL17F*	5.123 (1.261–27.77)	0.0213
C/C or C/A genotype of rs1883136 in *TRAF3IP2*	10.43 (1.603–77.68)	0.0149

*Factors were statistically analyzed by multivariate logistic regression analysis.
IFX: infliximab; CD: Crohn's disease; OR: odds ratio; CI: confidence interval.

Table 5: Evaluation of a genetic test for response to IFX after 1 year of treatment of the CD patients with concomitant use of immunomodulators.

Factor	Number of CD patients (%) Responders $n = 20$	Nonresponders $n = 8$	OR (95% CI)	*P* value*
Both G/G genotype of rs766748 in *IL17F* and C/C and C/A genotype of rs1883136 in *TRAF3IP2*	14 (70.0)	0 (0)	37.92 (1.890–761.1)	0.0019
Others	6 (30.0)	8 (100)		

*Factors were statistically analyzed by Fisher's exact test.
IFX: infliximab; CD: Crohn's disease; OR: odds ratio; CI: confidence interval.

the activation of TRAF3IP2 and TNF receptor-associated factor 6. This signaling pathway eventually leads to the production of proinflammatory cytokines through NF-κB [8–10, 22–24]. Moreover, TRAF3IP2 interacts with other TRAF proteins such as TRAF3 and TRAF6 that play multiple signaling roles in the IL-17 and TNF-α signaling pathways [8, 22, 25, 26]. For these reasons, it seems reasonable to speculate that the polymorphisms in *IL-17F* and *TRAF3IP2* identified in this study may affect these signaling pathways. Although we did not functionally analyze these SNPs, it is possible that in the intestines of nonresponder CD patients with genetic backgrounds of the G/A or A/A genotype of rs766748 in *IL-17F* or of the A/A genotype of rs1883136 in *TRAF3IP2* may affect the slight gain-of-function of both IL-17F and TRAF3IP2, thereby leading to a certain level of activation of both the IL-17 and TNF-α signaling pathways. It is known that (1) downstream signal transduction molecules, including I-κB and NF-κB, are shared by the IL-17 and TNF-α signaling pathways [8–10], (2) IL-17R knock-out mice are protected from inflammatory diseases including CD [27], and (3) *TRAF3IP2*-deficient mice are protected against dextran sodium sulfate-induced colitis, which presents with IBD-like manifestations [28]. Thus, elevated production of proinflammatory cytokines through the activation of both the IL-17 and TNF-α signaling pathways, which may occur in the IFX nonresponders, can lead to perpetuation of the chronic intestinal inflammatory process and might thereby result in the secondary loss of response to IFX after 1 year of treatment. Since these patients showed good response to IFX at the end of the 10-week treatment, IFX treatment can inhibit the weak activation of the IL-17 and TNF-α signaling pathways that might occur due to these polymorphisms of *IL-17F* and *TRAF3IP2* until the 10-week IFX treatment of these patients. However, the secondary loss of response to IFX, which occurs in these CD patients in the period from the end of the 10 weeks to the end of the 1 year of IFX treatment, may be due to a number of factors. Thus, during this period, the diminution of IFX-induced inhibition due to other environmental and genetic factors may lead to activation of the IL-17 and TNF-α signaling pathways, thereby resulting in the secondary loss of response to IFX. For example, various clinical risk factors, including smoking, enhancement of IFX clearance, new production of anti-IFX antibodies, a decrease in the blood concentration of IFX, and other unknown environmental factors and host genetic variations, can also contribute to the secondary loss of response to IFX [29–35]. In this study,

Table 6: Evaluation of a genetic test for response to IFX after 1 year of treatment of the CD patients with both concomitant use of immunomodulators and penetrating disease.

Factor	Number of CD patients (%)		OR (95% CI)	P value*
	Responders $n = 4$	Nonresponders $n = 5$		
Both G/G genotype of rs766748 in *IL17F* and C/C and C/A genotype of rs1883136 in *TRAF3IP2*	4 (100)	0 (0)	99.00 (1.618–6059)	0.0079
Others	0 (0)	5 (100)		

*Factors were statistically analyzed by Fisher's exact test.
IFX: infliximab; CD: Crohn's disease; OR: odds ratio; CI: confidence interval.

among the clinical factors such as smoking, concomitant use of immunomodulators, colonic location, and disease behavior that we analyzed, two factors, the concomitant use of immunomodulators and penetrating disease, independently contributed to response to IFX after 1 year of treatment of the CD patient population.

With regard to this association, even if the effect of IL-17F is weaker than that of IL-17A, the polymorphisms of *IL-17F* were associated with the therapeutic effect of IFX after 1 year of treatment, but not *IL-17A*. Although we did not carry out protein analysis of IL-17, we guess the possibilities that (1) the percentage of homodimerization of IL-17F protein may be higher in the responder CD patients, but lower in the nonresponders, (2) the level of the gain-of-function of the IL-17 signaling pathway due to the G/A or A/A genotype of rs766748 in *IL-17F* may be accelerated in comparison to other polymorphisms of *IL-17F*, and (3) the activation of the IL-17 signaling pathway may be independent of the polymorphisms of IL-17A. Additional studies are needed to elucidate this mechanism.

On the other hand, the production of proinflammatory cytokines through the IL-17 and TNF-α signaling pathways may be downregulated in the CD patients possessing the G/G genotype of rs766748 in *IL-17F* and/or the C/C or C/A genotype of rs1883136 in *TRAF3IP2*. Therefore, IFX-induced inhibition of the TNF-α signals may keep stronger effect on IFX response than various risk factors, thereby resulting in a good response to IFX after 1 year of treatment of these patients.

With regard to other host genetic variations, the previously reported association studies of rheumatoid arthritis (RA) have shown that some SNPs in genes encoding proteins, such as p38 mitogen-activated protein kinase, AF4/FMR2 family, member 3, CD226, protein tyrosine phosphatase receptor type C, Fc gamma receptors IIA and IIIA, and tumor necrosis factor receptor superfamily member 1B, are associated with response to anti-TNF treatment [36–40]. It has been suggested that these SNPs are also involved in the therapeutic effect of IFX in CD patients. It is necessary to confirm that these RA-responsible SNPs do contribute to the effect of IFX in CD patients.

From the perspective of using genetic biomarkers to predict response to IFX for CD patients after 1 year of treatment, the present genetic test using the associated SNPs as biomarkers for the CD patients with the concomitant use of immunomodulators showed the highest specificity as well as the positive predictive value of 100% with significant differences. In addition, when this test was limited to the CD patients with both concomitant use of immunomodulators and penetrating disease, each of the sensitivity, specificity, positive predictive value, and negative predictive value was 100% with significant differences. These data suggest that, even though the CD patients possessing these polymorphisms of *IL-17F* and *TRAF3IP2* have clinical risk factors including the concomitant use of immunomodulators and penetrating disease, they should be treated with IFX because of the 100% sensitivity and 100% positive predictive value of the test. Conversely, the patients not possessing these polymorphisms of *IL-17F* and *TRAF3IP2* should not be treated with IFX based on the 100% test value for the specificity and negative predictive value. Therefore, the CD patients possessing these polymorphisms of *IL-17F* and *TRAF3IP2* together with both the concomitant use of immunomodulators and penetrating disease can perfectly indicate the responders and nonresponders to IFX after 1 year of IFX treatment.

5. Conclusion

This is the first report that *IL-17F* and *TRAF3IP2* are IFX-related genes in Japanese CD patients. The combination of the polymorphisms in these two genes is useful as a new biomarker to predict response to IFX after 1 year of treatment. Since the activation of the IL-17 signaling pathway may be associated with the secondary loss of response to IFX, the signal transduction molecules including IL-17F and TRAF3IP2 in the IL-17 signaling pathway might be possible new therapeutic targets for a part of CD and/or therapeutic agents to overcome the secondary loss of response to IFX treatment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] K. L. Isaacs, J. D. Lewis, W. J. Sandborn, B. E. Sands, and S. R. Targan, "State of the art: IBD therapy and clinical trials in IBD," *Inflammatory Bowel Diseases*, vol. 11, no. 1, pp. S3–S12, 2005.

[2] T. Hibi and H. Ogata, "Novel pathophysiological concepts of inflammatory bowel disease," *Journal of Gastroenterology*, vol. 41, no. 1, pp. 10–16, 2006.

[3] M. J. Carter, A. J. Lobo, and S. P. L. Travis, "Guidelines for the management of inflammatory bowel disease in adults," *Gut*, vol. 53, no. 5, pp. v1–v16, 2004.

[4] F. Ueno, T. Matsui, T. Matsumoto, K. Matsuoka, M. Watanabe, and T. Hibi, "Evidence-based clinical practice guidelines for Crohn's disease, integrated with formal consensus of experts in Japan," *Journal of Gastroenterology*, vol. 48, no. 1, pp. 31–72, 2013.

[5] C. P. Braegger, S. Nicholls, S. H. Murch, T. T. MacDonald, and S. Stephens, "Tumour necrosis factor alpha in stool as a marker of intestinal inflammation," *The Lancet*, vol. 339, no. 8785, pp. 89–91, 1992.

[6] S. B. Hanauer, B. G. Feagan, G. R. Lichtenstein et al., "Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial," *The Lancet*, vol. 359, no. 9317, pp. 1541–1549, 2002.

[7] D. J. Cua and C. M. Tato, "Innate IL-17-producing cells: the sentinels of the immune system," *Nature Reviews Immunology*, vol. 10, no. 7, pp. 479–489, 2010.

[8] Y. Iwakura, H. Ishigame, S. Saijo, and S. Nakae, "Functional specialization of interleukin-17 family members," *Immunity*, vol. 34, no. 2, pp. 149–162, 2011.

[9] S. L. Gaffen, "Structure and signalling in the IL-17 receptor family," *Nature Reviews Immunology*, vol. 9, no. 8, pp. 556–567, 2009.

[10] S. Zhu and Y. Qian, "IL-17/IL-17 receptor system in autoimmune disease: mechanisms and therapeutic potential," *Clinical Science*, vol. 122, no. 11, pp. 487–511, 2012.

[11] H. A. Yu, G. J. Riely, and C. M. Lovly, "Therapeutic strategies utilized in the setting of acquired resistance to EGFR tyrosine kinase inhibitors," *Clinical Cancer Research*, vol. 20, no. 23, pp. 5898–5907, 2014.

[12] L. Rovedatti, T. Kudo, P. Biancheri et al., "Differential regulation of interleukin 17 and interferon γ production in inflammatory bowel disease," *Gut*, vol. 58, no. 12, pp. 1629–1636, 2009.

[13] C. Liu, X. Xia, W. Wu et al., "Anti-tumour necrosis factor therapy enhances mucosal healing through down-regulation of interleukin-21 expression and T helper type 17 cell infiltration in Crohn's disease," *Clinical and Experimental Immunology*, vol. 173, no. 1, pp. 102–111, 2013.

[14] R. M. Onishi and S. L. Gaffen, "Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease," *Immunology*, vol. 129, no. 3, pp. 311–321, 2010.

[15] S. Alzabin, S. M. Abraham, T. E. Taher et al., "Incomplete response of inflammatory arthritis to TNFα blockade is associated with the Th17 pathway," *Annals of the Rheumatic Diseases*, vol. 71, no. 10, pp. 1741–1748, 2012.

[16] D. K. Podolsky, "Inflammatory bowel disease," *The New England Journal of Medicine*, vol. 325, no. 13, pp. 928–937, 1991.

[17] D. K. Podolsky, "Inflammatory bowel disease (2)," *The New England Journal of Medicine*, vol. 325, no. 14, pp. 1008–1016, 1991.

[18] W. R. Best, J. M. Becktel, J. W. Singleton, and F. Kern Jr., "Development of a Crohn's disease activity index. National cooperative Crohn's disease study," *Gastroenterology*, vol. 70, no. 3, pp. 439–444, 1976.

[19] The International HapMap Consortium, "A haplotype map of the human genome," *Nature*, vol. 437, no. 7063, pp. 1299–1320, 2005.

[20] J. C. Barrett, B. Fry, J. Maller, and M. J. Daly, "Haploview: analysis and visualization of LD and haplotype maps," *Bioinformatics*, vol. 21, no. 2, pp. 263–265, 2005.

[21] K. Sato, M. Shiota, S. Fukuda et al., "Strong evidence of a combination polymorphism of the tyrosine kinase 2 gene and the signal transducer and activator of transcription 3 gene as a DNA-based biomarker for susceptibility to Crohn's disease in the Japanese population," *Journal of Clinical Immunology*, vol. 29, no. 6, pp. 815–825, 2009.

[22] H. C. Seon, H. Park, and C. Dong, "Act1 adaptor protein is an immediate and essential signaling component of interleukin-17 receptor," *The Journal of Biological Chemistry*, vol. 281, no. 47, pp. 35603–35607, 2006.

[23] Y. Qian, C. Liu, J. Hartupee et al., "The adaptor Act1 is required for interleukin 17-dependent signaling associated with autoimmune and inflammatory disease," *Nature Immunology*, vol. 8, no. 3, pp. 247–256, 2007.

[24] M. S. Doyle, E. S. Collins, O. M. FitzGerald, and S. R. Pennington, "New insight into the functions of the interleukin-17 receptor adaptor protein Act1 in psoriatic arthritis," *Arthritis Research & Therapy*, vol. 14, no. 5, article 226, 2012.

[25] U. Hüffmeier, S. Uebe, A. B. Ekici et al., "Common variants at TRAF3IP2 are associated with susceptibility to psoriatic arthritis and psoriasis," *Nature Genetics*, vol. 42, no. 11, pp. 996–999, 2010.

[26] X. Song and Y. Qian, "The activation and regulation of IL-17 receptor mediated signaling," *Cytokine*, vol. 62, no. 2, pp. 175–182, 2013.

[27] S. Brand, "Crohn's disease: Th1, Th17 or both? The change of a paradigm: new immunological and genetic insights implicate Th17 cells in the pathogenesis of Crohn's disease," *Gut*, vol. 58, no. 8, pp. 1152–1167, 2009.

[28] Y. Qian, C. Liu, J. Hartupee et al., "The adaptor Act1 is required for interleukin 17–dependent signaling associated with autoimmune and inflammatory disease," *Nature Immunology*, vol. 8, no. 3, pp. 247–256, 2007.

[29] N. Maharshak and S. E. Plevy, "A Crohn's disease patient who does not respond to infliximab: what is next?" *Clinical Gastroenterology and Hepatology*, vol. 9, no. 12, pp. 1033–1038, 2011.

[30] I. D. R. Arnott, G. McNeill, and J. Satsangi, "An analysis of factors influencing short-term and sustained response to infliximab treatment for Crohn's disease," *Alimentary Pharmacology and Therapeutics*, vol. 17, no. 12, pp. 1451–1457, 2003.

[31] P. Lionetti, F. Bronzini, C. Salvestrini et al., "Response to infliximab is related to disease duration in paediatric Crohn's disease," *Alimentary Pharmacology and Therapeutics*, vol. 18, no. 4, pp. 425–431, 2003.

[32] N. V. Casteele, A. Gils, S. Singh et al., "Antibody response to infliximab and its impact on pharmacokinetics can be transient," *The American Journal of Gastroenterology*, vol. 108, no. 6, pp. 962–971, 2013.

[33] H. Yanai and S. B. Hanauer, "Assessing response and loss of response to biological therapies in IBD," *The American Journal of Gastroenterology*, vol. 106, no. 4, pp. 685–698, 2011.

[34] S. Mascheretti, J. Hampe, T. Kühbacher et al., "Pharmacogenetic investigation of the TNF/TNF-receptor system in patients with chronic active Crohn's disease treated with infliximab," *The Pharmacogenomics Journal*, vol. 2, no. 2, pp. 127–136, 2002.

[35] T. Hlavaty, M. Pierik, L. Henckaerts et al., "Polymorphisms in apoptosis genes predict response to infliximab therapy in luminal and fistulizing Crohn's disease," *Alimentary Pharmacology and Therapeutics*, vol. 22, no. 7, pp. 613–626, 2005.

[36] L. R. Coulthard, J. C. Taylor, S. Eyre et al., "Genetic variants within the MAP kinase signalling network and anti-TNF

treatment response in rheumatoid arthritis patients," *Annals of the Rheumatic Diseases*, vol. 70, no. 1, pp. 98–103, 2011.

[37] R. J. L. Tan, L. J. Gibbons, C. Potter et al., "Investigation of rheumatoid arthritis susceptibility genes identifies association of AFF3 and CD226 variants with response to anti-tumour necrosis factor treatment," *Annals of the Rheumatic Diseases*, vol. 69, no. 6, pp. 1029–1035, 2010.

[38] J. Cui, S. Saevarsdottir, B. Thomson et al., "PTPRC rheumatoid arthritis risk allele is also associated with response to anti-TNF therapy," *Arthritis & Rheumatism*, vol. 62, no. 7, pp. 1849–1861, 2010.

[39] J. D. Cañete, B. Suárez, M. V. Hernández et al., "Influence of variants of Fcγ receptors IIA and IIIA on the American College of Rheumatology and European League Against Rheumatism responses to anti-tumour necrosis factor α therapy in rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 68, no. 10, pp. 1547–1552, 2009.

[40] C. Rooryck, T. Barnetche, C. Richez, A. Laleye, B. Arveiler, and T. Schaeverbeke, "Influence of FCGR3A-V212F and TNFRSF1B-M196R genotypes in patients with rheumatoid arthritis treated with infliximab therapy," *Clinical and Experimental Rheumatology*, vol. 26, no. 2, pp. 340–342, 2008.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 172756, 8 pages
http://dx.doi.org/10.1155/2015/172756

Research Article

Immunohistochemical Expression of Ornithine Decarboxylase, Diamine Oxidase, Putrescine, and Spermine in Normal Canine Enterocolic Mucosa, in Chronic Colitis, and in Colorectal Cancer

Giacomo Rossi,[1] Matteo Cerquetella,[1] Graziano Pengo,[2] Subeide Mari,[1] Emilia Balint,[3] Gabrio Bassotti,[4] and Nicolae Manolescu[3]

[1] *School of Biosciences and Veterinary Medicine, University of Camerino, Via Circonvallazione 93/95, 62024 Matelica, Italy*
[2] *S. Antonio Clinic, 26020 Madignano, Italy*
[3] *Faculty of Veterinary Medicine, 050097 Bucharest, Romania*
[4] *Gastroenterology and Hepatology Section, Department of Medicine, Santa Maria della Misericordia Hospital, Piazzale Menghini 1, San Sisto, 06153 Perugia, Italy*

Correspondence should be addressed to Giacomo Rossi; giacomo.rossi@unicam.it

Received 29 May 2015; Revised 3 August 2015; Accepted 10 September 2015

Academic Editor: Atsushi Sakuraba

We compared the immunohistochemical expression of putrescine (PUT), spermine (SPM), ornithine decarboxylase (ODC), and diamine oxidase (DAO) in bioptic samples of canine colonic mucosa with chronic inflammation (i.e., granulomatous colitis and lymphoplasmacytic colitis) or neoplasia. Single and total polyamines levels were significantly higher in neoplastic tissue than in normal samples. Samples with different degrees of inflammation showed a general decrease expression of ODC if compared to controls; SPM was practically not expressed in control samples and very low in samples with chronic-granulomatous inflammation. In carcinomatous samples, the ODC activity was higher with respect to controls and samples with inflammation. This is the first description of polyamines expression in dog colonic mucosa in normal and in different pathological conditions, suggesting that the balance between polyamine degradation and biosynthesis is evidently disengaged during neoplasia.

1. Introduction

The polyamines (PAs) spermine (SPM) and putrescine (PUT) are intimately involved in regulation of DNA, RNA, and protein synthesis; therefore, they are essential for proliferation of both normal and neoplastic cells. Dysregulation of cellular polyamines is associated with various pathological conditions, including inflammation, and cancer; for this latter association, polyamine pathways have been explored as targets for cancer chemotherapy and chemoprevention [1, 2]. Fujiwara et al. [3] reported the first immunohistochemical demonstration of PAs distribution in different portions of healthy gut mucosa of rats and mice using specific monoclonal antibodies and observed that PAs are well expressed in gastrointestinal tract according to the rapid turnover of gastrointestinal epithelium. The intracellular amount of PAs is the result of the biosynthetic activity of the key-enzyme ornithine decarboxylase (ODC) and of the uptaking from extracellular environment. In human beings several studies reported an increased expression of ODC in neoplastic colorectal tissue versus normal-appearing mucosa [4, 5].

The enzyme diamine oxidase (DAO) inactivates histamine and other biogenic amines, such as PAs by a reaction of oxidative deamination. DAO is mainly expressed in the intestine, located almost exclusively in the villus tip enterocytes of mammals [6]. Decreased levels of DAO were found in various bowel diseases both in dogs and in humans [6, 7]. Although polyamines are critical for optimal cell growth, excessive accumulation may interfere directly with normal cell function, and they have been implicated recently in the control of the apoptotic response, inflammation, and cancerogenesis [8, 9].

The present study was undertaken with an objective to evaluate, by means of immunohistochemistry, the localization, the pattern, and the levels of expression of polyamines SPM, PUT, ODC, and DAO in dog. For this purpose, we have compared results obtained from biopsy samples taken from clinically healthy dogs, and dogs with clinical and histological signs of large bowel pathology (ranging from chronic inflammation to neoplasia), to evaluate the significance of polyamines within the proliferative process and their possible role as prognostic markers and target of antiproliferative drugs.

2. Materials and Methods

2.1. Animals. Forty dogs participated in the study, after histological evaluation and inclusion in four different groups (control (group 1), granulomatous colitis (group 2), lymphoplasmacytic colitis (group 3), and colonic adenocarcinoma (group 4) consisting of 10 dogs each) (Table 1).

Dogs with histological diagnosis of intestinal lymphoma were excluded from the study, because the completely flat mucosa, showing crypt atrophy or hypertrophy and reduced epithelial cell number and height, with important alterations of epithelium showing colonocytes loss related to lymphocytes and neutrophils infiltration, could reduce the degree of polyamines expression. All enrolled dogs, excluding control animals, showed a long-time diagnosis of IBD according to published criteria [10] and were evaluated at the Veterinary Teaching Hospital, Camerino University, for chronic gastroenteritis. Inclusion criteria included recurrence of clinical signs and absence of any immunomodulating drug therapy (e.g., corticosteroids, metronidazole, and sulfasalazine) within a month before referral. Furthermore, for dogs of group 3 the diagnostic plan was integrated as expected while diagnosing inflammatory bowel diseases [11]. Diagnostic criteria for IBD included persistent (>3 weeks) gastrointestinal signs, failed responses to dietary (hydrolysate or commercial intact protein elimination diet) or symptomatic therapies (anthelmintics, antibiotics, anticholinergics, and gastrointestinal protectants) alone, a thorough diagnostic evaluation with failure to document other causes for gastroenteritis, and histopathologic evidence of intestinal inflammation. The minimum diagnostic evaluation in all dogs included a complete blood count, serum biochemistry, urinalysis, direct (wet mount) and indirect (flotation) examination of feces for endoparasites, and survey abdominal radiographs. In some instances, additional tests including contrast radiography, abdominal ultrasound (performed in 22 of the 30 pathologic dogs), and measurement of serum concentrations of trypsin-like immunoreactivity and/or folate and cobalamin were performed. Additional inclusion criteria were the absence of extra-alimentary tract inflammation based on results obtained from initial diagnostic testing. Dogs with hypoproteinemia or a suspicion of intestinal lymphangiectasia were excluded from the study. The clinical disease activity (CIBDAI score) was assessed at the enrollment. The CIBDAI is based on 6 criteria, each scored on a scale from 0 to 3: attitude/activity, appetite, vomiting, stool consistency, stool frequency, and weight loss. After summation, the total composite score is determined to be clinically insignificant (scores 0–3), mild (scores 4-5), moderate (scores 6–8), or severe (score 9 or greater) [12].

Table 1: Summary characteristics of enrolled dogs.

Group and race	Sex	Median age (range) in years
Group 1 (CTR) = 10 dogs Labradors (1) Mix (4) Cocker (1) Beagle (2) German sheph. (2)	m = 3, mn = 1, f = 2, fs = 4	6.3 (3–11)
Group 2 (GC) = 10 dogs Schnauzers (1) Cocker (1) Yorkie (1) Mix (1) Rottweiler (1) Collie (1) Pekinese (1) Shih Tzu (1) Boxer (1) German sheph. (1)	m = 2, mn = 3, f = 1, fs = 4	6.4 (1–12)
Group 3 (LPC) = 10 dogs Labradors (1) Mix (2) Boxers (1) Rottweiler (1) Shih Tzu (1) Boxer (1) Siberian Husky (1) Basset-hound (1) German sheph. (1)	m = 3, mn = 1, f = 1, fs = 5	4.9 (1–10)
Group 4 (Carc) = 10 dogs Shar pei (1) Chow chow (2) Labrador (1) Boxer (1) Belgian sheph. (1) *Staffordshire bull terrier* (1) Mix (3)	m = 4, mn = 1, f = 2, fs = 3	9.9 (6–14)

m = male, mn = neutered male, f = female, fs = spayed female; mix = not pure breed.

The control group was composed of dogs hospitalized in urgency and that died for different causes (mainly for severe trauma) but that were free of gastrointestinal signs for at least four months, not presenting gastrointestinal signs before death and in which necropsy did not reveal neoplastic conditions. Control dogs were considered "to be healthy," based on clinical history, normal results on physical examination (excluding lesions due to trauma), serum biochemistry, urinalysis, fecal examinations, and *Dirofilaria* antigen assay performed immediately before or after death.

All the owners of the IBD dogs gave informed written consent before enrollment.

2.1.1. Tissue Sampling. After enrollment, multiple (10–15 specimens) mucosal biopsy specimens were procured endoscopically from the large intestine of all dogs ($n = 30$, 10 dogs per group of disease). Naturally deceased or humanely suppressed dogs (for the severity of the condition and independent of the study) were sampled directly, during the necropsy procedures, performed immediately after death. In dogs undergoing endoscopy, food administration was suspended around 36–48 hours before endoscopy, and dogs were prepared following standard protocols. Dogs of groups 2, 3, and 4 underwent a colonoscopy under general anesthesia (video-endoscope (Olympus EVIS GIF-100, Tokyo, Japan) (outer diameter, 9.5 mm; biopsy channel diameter, 2.8 mm)); a set of a minimum of 8 biopsies was taken from each enterocolic tract. In dogs of group 3, additional bioptic samples were taken in neoplastic areas. All dogs showed predominantly mixed signs of enterocolitis (i.e., GI signs associated with tenesmus, hematochezia, mucoid feces, and/or frequent defecation), and upper and lower endoscopic examinations were performed. Biopsy specimens were obtained directly from mucosal lesions of increased granularity, friability, or erosions as well as areas of normal-appearing mucosa. Tissues for histopathology were fixed in 4% buffered formaldehyde and then paraffin embedded and serial 4 μm thick sections were prepared. Sections were cut, dewaxed, and stained with hematoxylin-eosin (H&E). Adjacent sections were subjected to immunohistochemical analysis (IHC) using a set of polyclonal antibodies. Histopathology was performed by a single pathologist, who was blinded regarding history, clinical signs, or endoscopic observations. A severity score was assigned for each dog, by using a standardized and previously described histologic grading system, based on the extent of architectural disruption and mucosal epithelial changes [12, 13], as has recently been proposed by the WSAVA for diagnosis of gastrointestinal inflammation [14].

2.2. Histopathology, Histochemistry, and Immunohistochemistry. Paraffin sections were rehydrated and neutralized for endogenous peroxidases with 3% hydrogen peroxide for 5 minutes followed by rinsing for 5 minutes in distilled water. For antigen retrieval, slides were incubated in EDTA buffer, pH 9.0, and processed in a microwave oven at 650 W for two cycles of 10 minutes each to unmask antigens. Slides were then allowed to cool at room temperature for at least 20 minutes before being processed for immunostaining by standard procedures. Tissue sections were incubated overnight in a moist chamber at 4°C with different primary antibodies (Abs): rabbit polyclonal Ab against ODC (*Bioss antibodies*, pAb rb-anti ODC antibody, *bs-1294R*; diluted 1 : 50), DAO (*Biorbyt*; pAb rb-anti DAO antibody, *orb192676*; diluted 1 : 100), PUT (*Thermo Fisher Scientific*; pAb rb-anti Pentane-1,5-diamine, #PA1-86537, diluted 1 : 20), and SPM (*Abcam*, ab7318, diluted 1 : 20) antigens. All primary antibodies were diluted in Tris-buffered solution (TBS) containing 0.1% crystalline bovine serum albumin (BSA). Tissue sections were incubated overnight in a moist chamber at 4°C with different primary antibodies, diluted (1 : 50). The antigens-antibodies complex was detected by ABC-peroxidase technique using 3-3′-diaminobenzidine-hydrochloride (Vector Laboratories, Inc., Burlingame, CA) as chromogen substrate to reveal the immunoreaction, with Meyer Haematoxylin as nuclear counterstain.

Nonspecific binding was blocked by incubation of slides for 10 minutes with a protein-blocking agent (protein-blocking agent, Dako, Carpinteria, CA, USA) before application of the primary antibody. Specific primary antibodies substituted with TBS or nonimmune sera were used as negative controls in immunohistochemical techniques.

The antibodies used were not validated for canine tissue, but the specificity of the reaction was assured by the fact that polyamines, consisting in polycationic molecules with multiple amino groups, and their cellular receptor are identical in both prokaryotic and eukaryotic cells [15]. Canine polyamines present the same structure of man polyamines and this reinforces the specificity of reaction. Finally, to test the specificity of antibodies used in our experiments, and the possibility of cross-reaction to each other, a western blot was performed as standardized by Kurien et al., 2011 [16]. In brief, different polyamines (Sigma) were loaded in equal amounts of samples onto 3–8% Tris-acetate gels and separated by electrophoresis (100 V for 1.5 h). The proteins were transferred to a Hybond-ECL Nitrocellulose membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and then immersed in a block solution with 5% dry milk in PBS for 1.5 h at room temperature. The different molecules were detected with the specific antibody employed also in IHC tests, used at a concentration of 1 : 5000 in 5% milk solution, and incubated overnight at 4°C. After washing in a Tris-buffered saline with 0.1% Tween (TBS-T) buffer and incubating for 45 min with a secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG; 1 : 5000), positively stained bands were detected by a chemiluminescent blot assay with the ECL Plus western blot reagent.

Histopathologic examination of all biopsies was performed by a single pathologist, who graded endoscopic specimens and assigned a lesion severity score for each dog by using a standardized and previously described histologic grading system, based on the extent of architectural disruption and mucosal epithelial changes [12, 13, 17, 18]. Briefly, the histologic examination of H&E-stained sections included the assessment of the number of inflammatory cells, using a visual analogue scale modified for canine specimens [19]. Number of inflammatory cells (mononuclear cells such as lymphocytes, plasma cells, macrophages, and neutrophils) was assessed at 400x magnification (high-power field (HPF)). Number of lymphocyte aggregates was assessed at 100x magnification. The number of inflammatory cells was recorded and results were reported as the mean for the entire specimen.

Histologic criteria for normal colonic mucosa included detection of none or only a few mononuclear cells scattered in the chorion per HPF, absence of lymphoid aggregates, and none or only a few scattered neutrophils across the intestinal epithelium.

In pathological samples, neutrophils were considered as absent (score 0) when there was none or only single sporadic cells per high-power field (HPF, using 400x magnification), mild (score 1) for a few cells (5 to 10 cells) per HPF, moderate (score 2) for several cells (11 to 50 cells) per HPF, or severe (score 3) (50 to 200 cells or more) per HPF. Number of mononuclear cells was considered to be normal when none or only few cells (<5 cells) were visible in each HPF among intestinal glands (score 0). Increasing in number of cells was considered mild for specimens with several cells per HPF (score 1 = 5 to 10 cells), moderate (score 2 = 11 to 30) for specimens with many cells for HPF, or severe (score 3 > 30 cells) for specimens with conspicuous and very conspicuous number of cells per HPF, respectively. Number of lymphocytic aggregates in a specimen was counted.

Finally colonic adenocarcinomas were described according to Head et al. [20, 21] on the basis of the current WHO's classification for tumours in domestic animals. The morphological types of neoplastic samples we examined were classifiable as tubular, well, middle, and poorly differentiated and mucinous, middle differentiated.

2.2.1. Histochemistry. In the second step, a histochemical staining was performed on the same sections previously subjected to the histological and immunohistochemical assay. The histochemical staining was performed with only minor modifications according to Poletti et al. [22]. For Alcian blue staining, the sections were stained with Alcian blue solution (pH 2.5) for 30 min at room temperature, washed in running tap water for 10 min, rinsed in DI water, counterstained in Mayer's hematoxylin for 5 min, and washed in DI water. The acetic acid as mordant was not used because it has no effect on the final results. For PAS staining, sections were oxidized in 0,5% periodic acid solution for 5 min, rinsed in DI water, placed in Schiff reagent for 15 min, washed in tap water for 5 min, counterstained in Mayer's hematoxylin for 1 min, and washed in DI water. Finally, the slides were dehydrated, cleared, and mounted after differentiation with hydrochloric acid. To evaluate the localization and intensity of Alcian/PAS stain, the same method described also for immunohistochemical evaluation was used (see below).

2.2.2. Immunohistochemistry. In immunohistochemical essays, the number of immunoreactive cells for each antibody observed in normal or pathological colonic samples was calculated using a light microscope (Carl Zeiss), a ×40 objective, a ×10 eyepiece, and a square eyepiece graticule (10 × 10 squares, with a total area of 62 500 μm^2). Ten appropriate sites were chosen for each colonic biopsy and arithmetic means were calculated for each colonic area. Results were expressed as IHC positive cells per 62 500 μm^2.Cells on the margins of the tissue sections were not considered for evaluation to avoid inflation of positive cell numbers.

To assess the intensity of polyamines expression (ODC, DAO, PUT, and SPM) in biopsies sampled of all groups and to compare data to the polyamines expression in healthy control dogs (group 1) stained tissue sections, ×250 photos were used. Photographs were evaluated by two blinded investigators who scored both the extent and intensity of staining in the lining and crypts epithelium of each picture on a scale of 0–3 (0 = absent and 3 = severe). The score of extent and intensity of staining were added and the mean of the total scores was calculated and used for analysis. The ×400 photographs were used to calculate the amount of immunostaining present in each section. One examiner opened each photograph in ImageJ (http://rsb.info.nih.gov/ij/) and a pixel intensity threshold was determined to include only those image pixels in immunopositive areas. For evaluation, colonic epithelium was divided into luminal, proximal, and distal gland/crypt regions. Finally, the scoring of colonic molecules expression was calculated as previously described.

2.3. Statistical Analysis. Differences between groups were assessed by nonparametric tests, using the Friedman test to evaluate variance and then the Wilcoxon test for paired samples. Values of $p < 0.05$ were chosen for rejection of the null hypothesis.

3. Results

3.1. Histopathology and Histochemistry. In chronic-granulomatous colitis (group 2), a general altered morphological status of the epithelial cells was observed, with flattened aspect of some areas of epithelium and epithelial cells loss, particularly in lining superficial epithelium; additionally an evident decrease in mucous goblet cells was found, comparing samples with normal dogs or dogs with LPC. Additionally, more pyknotic and karyorrhectic epithelial cells occurred both in superficial lining and in crypts of granulomatous colitis cases than in LPC. Lymphoplasmacytic colitis (group 3) still manifested histologic evidence of chronic inflammation, with a significant increased number of mononuclear cells in the mucosal chorion of bioptic samples ($p < 0.001$), while granulomatous colitis biopsies showed significantly increased neutrophils and macrophages ($p < 0.001$) interspersed in the lamina propria (Figure 2).

The Alcian PAS stain revealed an alteration of the chemical composition of mucin limited to the groups of adenocarcinoma and granulomatous colitis; a strong cytosolic PAS positivity was in fact observable, indicating a remarkable increase in the mucopolysaccharides amount if compared to controls or LPC samples ($p < 0.001$).

3.1.1. Immunohistochemistry. Changes in polyamines cellular expression between groups were compared using Wilcoxon matched pairs tests. Resulting p values were corrected for multiple comparisons using the false discovery rate as described by Benjamini and Hochberg, and a $p < 0.05$ was considered significant. The positivity for the antigens ODC, PUT, and SPM had a cytosolic paranuclear localisation, while for the DAO antigen a basolateral positivity was appreciable according to what was observed by Oliva et al. [6]. No nuclear positivity was detectable for polyamines. The statistical elaboration of cellular counts for each polyamine in all the examined cases, compared to values obtained in control healthy dog, revealed a significant ($p < 0.01$) difference

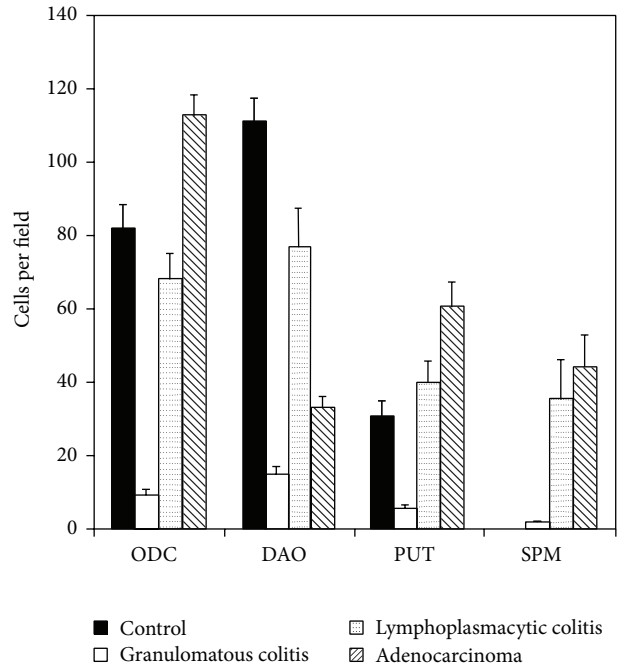


Figure 1

and a characteristic trend of the expression for every kind of lesion considered.

In both colitides (groups 2 and 3) the ODC antigen was "downexpressed" if compared to controls ($p < 0.01$; for both groups) (Figure 2(f)). In samples of adenocarcinoma the tendency was opposite (Figure 2(c)). The expression of PUT and SPM followed almost the same trend as ODC, both in neoplastic and in inflammatory lesions (data not shown). DAO antigen showed a significant lower expression ($p < 0.001$) in all kinds of lesions (Figures 2(b) and 2(e)), without specificity, than in controls as shown in Figure 1.

4. Discussion

The gastrointestinal (GI) tract is lined by a continuous layer ofepithelial cells which maintain the physical and functional barrier to undesirable luminal antigens [23]. Epithelial cells of mammalian GI mucosa rapidly proliferate and turn over approximately every three days under biological conditions [23, 24]. This process balances cell proliferation, differentiation, migration, and apoptosis. Polyamines (putrescine, spermidine, and spermine) have low molecular weight and are highly charged aliphatic polycations which are intimately involved in many distinct cellular functions. An increasing body of evidence has advanced our understanding of the cellular and molecular functions of polyamines especially in man and in mice/rats, used as principal animal models of GI inflammation and tumorigenesis. Until now, a substantial lack of information is observed regarding dogs. The goal of the present study was the evaluation, for the first time, of the presence and the pattern of expression of polyamines, their precursor and suppressor, in normal colonic conditions and in some pathological conditions frequently observed in this species. This preliminary study represents the first step to highlight the roles and mechanisms of cellular polyamines in dog GI mucosal pathology and also point out their potential clinical regulation in animals with mucosal injury-associated disorders.

Polyamine metabolism is involved with a wide variety of GI diseases. Numerous factors may influence polyamine homeostasis; however, the changes seem to be tissue-specific. The altered polyamine metabolism contributes to epithelial cell proliferation and esophageal carcinogenesis in experimental animals [25, 26]. In man, ODC activity is upregulated in Barrett's esophagus, a premalignant lesion, and correlated with the degree of dysplasia [27]. The relationship between polyamines and inflammatory bowel diseases (IBD) is extensively studied. Conflicting results are reported in different studies regarding the ODC activity and polyamine content in IBD patients. As observed in our data, in which decreased ODC levels are observed in both groups of dogs with different forms of colitis, it has been found that ODC activity was decreased in IBD patients in both involved and in uninvolved mucosal tissues [28]. This decrease was related to the severity of disease [28]. In contrast, ODC was found to be elevated both in human and in animal studies [29–31]. The discrepancy may be due to the content of tissue sample or, as in our cases of granulomatous colitis, due to the dramatic reduction/loss of epithelial cells in damaged areas, with a consequence of relative lowered levels of PAs expression [32, 33]. A few studies indicate that increased levels of spermidine and polyamine catabolism in IBD patients and in experimental models may relate to the accelerated proliferation of injured tissues [33, 34]. Spermine exerts an inhibitory role in the inflammatory reaction and is downregulated in severe ulcerative colitis patients and in chronic colitis experimental models [33, 35]. Our results are in agreement with these observations, demonstrating a significant and dramatic decrease of SPM in group 2, with respect to the levels of expression observed in group 3. It has been noticed that the decrease of spermine content may further aggravate the disease [33]. The role of polyamine metabolism in IBD is further supported by the fact that L-arginine improves colitis by enhancing the formation of polyamines in animal models while DFMO, an irreversible inhibitor of ODC, worsens the disease [33]. Pathological conditions outside the GI tract also closely relate to polyamine metabolism and interfere with internal polyamine pool. In colon cancer, the activities of polyamine-synthesizing enzymes and polyamine content are increased 3-4-fold compared to the equivalent normal colonic mucosa, and polyamines have even been attributed as markers of neoplastic proliferation in the colon [36]. However, the exact mechanisms on the role of internal polyamine pool affecting GI mucosal homeostasis are not yet clearly understood.

Our results suggest that, in the adenocarcinoma affected colonic dog mucosa, the balance between biosynthesis and degradation of polyamines may be disengaged. In fact, while ODC is strongly upregulated, DAO follows both an absolute and relative decrease, from a quantitative point of view, if compared, respectively, to control's levels of the same enzyme and to the levels of the other PAs.

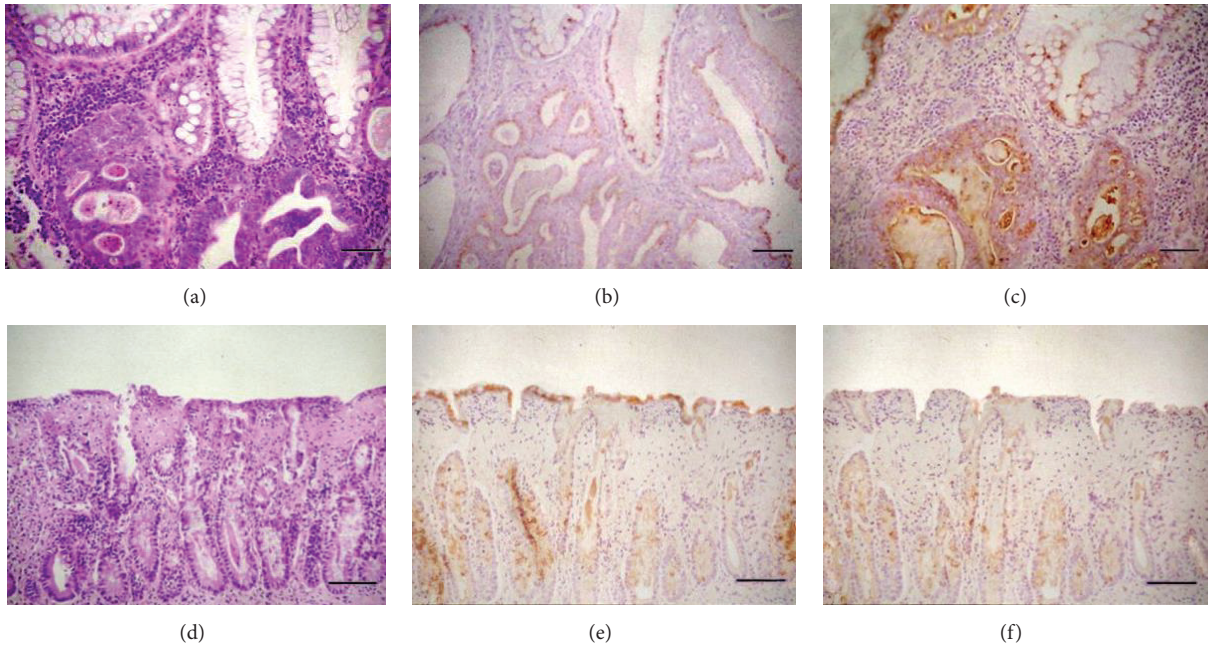


Figure 2: Expression of DAO and ODC in different colonic pathological conditions. (a) Morphological aspect of colonic carcinoma with areas of squamous metaplasia. (b) The same section stained with anti-DAO polyclonal antibody; note the weak positivity diffused particularly in well-preserved mucosal epithelium. (c) In a consequent section stained with anti-ODC pAb the strong expression of the enzyme is observed in metaplastic undifferentiated areas. (d) Morphology of granulomatous colitis with severe crypts involvement and areas of subepithelial fibrosis. (e) The expression of DAO in a consequent section shows a continuous and strong expression in the superficial epithelium, but only occasional and spotted strong stain in epithelium lining the crypts. (f) Weak and focal expression of ODC in a successive section evidences the low concentration of the enzyme in colonic epithelium during the granulomatous phlogosis. ((a) and (d) H&E; (b), (c), (e), and (f) IHC stain, Meyer's haematoxylin counterstain; (a), (b), and (c) bar = 300 μm; (d), (e), and (f) bar = 600 μm).

On the other hand, the balance is conserved in inflammatory disease. Our main hypothesis about the trend of that balance in different class of diseases is based on the pathogenesis of the epithelial Noxa. In case of colitis both the upper and lower part of the cryptae are affected by the damage. The reduction in number of the mature enterocytes justifies the decrease of DAO which is an enzyme usually expressed in well-differentiated cells. The downexpression of ODC and PAs could be explained by the fact that the hyperplasia of the proliferative section does not supply from a quali-quantitative point of view the lack of cells, even if lymphoplasmacytic colitis PAs are higher than controls.

The neoplastic tissue is characterized by a population of immature cells, which produces a lower amount of DAO; at the same time, the exceptional rate of proliferation produces a large amount of newborn and highly immature cells that are able to express PAs. This evidence could explain the remarkable overexpression of ODC and PAs. Interestingly, almost all our markers, except SPM, have been found to be downregulated in IBD with respect to controls. Despite the fact that PAs and their acetylated derivatives are a prerequisite for cellular metabolism and considered to be essential for proliferation and differentiation of the rapidly renewing intestinal mucosa, their role during intestinal mucosal inflammation is less clear [33]. In some studies, a correlation of polyamine levels of patients with IBD with their corresponding inflammatory index revealed that increased concentrations of PAs were found in CECs from the most severe inflamed mucosal areas [33]. Using acute and chronic DSS colitis as a model of mucosal inflammation, the same authors found enhanced levels of PAs in acute forms, whereas in chronic inflammation, PAs concentrations were decreased [33]. Also our data indicate a lack of the anti-inflammatory PAs, especially spermine, in chronic colitis, which may aggravate the disease. In our opinion, the PAs biosynthetic pathway overexpression, reported in acute or chronic-active forms of intestinal inflammation, is one of the steps in the cancerogenic process; anyway, several investigations are requested.

The immunohistochemical evaluation of the polyamine's cycle in bioptic samples could be therefore useful in detecting the severity of some "borderline" lesions. A predictive value of these markers could be assessed by evaluating the group of dysplastic lesions, looking for a "cut-off" point in the quantitative expression between preneoplastic and nonneoplastic lesions.

The coherence of our results with the findings reported in human beings [37] could suggest a possible role of the dog with spontaneous disease as a model, especially in the evaluation of the efficacy of new therapeutic trials and protocols for adenocarcinoma.

5. Conclusion

Similar to humans, dogs express different intestinal levels of PAs, relative to different pathological conditions. We can

hypothesize that contextual evaluation of ODC, DAO, and PAs could be suggestive of the severity of the lesion and in cases of overexpression a possible predictive factor of malignancy. While such a high polyamine supply may be of benefit in nonneoplastic colonic mucosal growth, the role of tissutal and luminal polyamines in colon cancer is a clear concern. The pool of PAs is taken up by neoplastic colonocytes, they are utilized in full to support neoplastic growth, and their uptake is strongly upregulated by the mitogens known to play an important role in colonic carcinogenesis. Inhibition of polyamine synthesis and their uptake, impaired utilization of exogenous polyamines, and enhanced catabolism of polyamines in neoplastic colonocytes may be therefore conceivable future approaches also in the chemoprevention of dog colorectal cancer.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] A. E. Pegg, "Mammalian polyamine metabolism and function," *IUBMB Life*, vol. 61, no. 9, pp. 880–894, 2009.

[2] N. S. Rial, F. L. Meyskens Jr., and E. W. Gerner, "Polyamines as mediators of APC-dependent intestinal carcinogenesis and cancer chemoprevention," *Essays in Biochemistry*, vol. 46, no. 1, pp. 111–124, 2009.

[3] K. Fujiwara, Y. Masuyama, and T. Kitagawa, "Immunocytochemical localization of polyamines in the gastrointestinal tracts of rats and mice," *Histochemistry and Cell Biology*, vol. 106, no. 5, pp. 465–471, 1996.

[4] M. Linsalata, F. Russo, A. Cavallini, P. Berloco, and A. Di Leo, "Polyamines, diamine oxidase, and ornithine decarboxylase activity in colorectal cancer and in normal surrounding mucosa," *Diseases of the Colon & Rectum*, vol. 36, no. 7, pp. 662–667, 1993.

[5] N. Matsubara, O. A. Hietala, S. K. Gilmour et al., "Association between high levels of ornithine decarboxylase activity and favorable prognosis in human colorectal carcinoma," *Clinical Cancer Research*, vol. 1, no. 6, pp. 665–671, 1995.

[6] F. Oliva, L. Cortese, S. Papparella, L. D'Agostino, B. Daniele, and A. Perschino, "Postheparin plasma diamine oxidase and its intestinal location in dog," *The European Journal of Comparative Gastroenterology*, vol. 2, pp. 3–6, 1997.

[7] M. Raithel, P. Ulrich, J. Hochberger, and E. G. Hahn, "Measurement of gut diamine oxidase activity: diamine oxidase as a new biologic marker of colorectal proliferation?" *Annals of the New York Academy of Sciences*, vol. 859, pp. 262–266, 1998.

[8] E. W. Gerner and F. L. Meyskens Jr., "Polyamines and cancer: old molecules, new understanding," *Nature Reviews Cancer*, vol. 4, no. 10, pp. 781–792, 2004.

[9] S. Zaletok, N. Alexandrova, N. Berdynskykh et al., "Role of polyamines in the function of nuclear transcription factor NF-kappaB in breast cancer cells," *Experimental Oncology*, vol. 26, no. 3, pp. 221–225, 2004.

[10] K. W. Simpson and A. E. Jergens, "Pitfalls and progress in the diagnosis and management of canine inflammatory bowel disease," *Veterinary Clinics of North America—Small Animal Practice*, vol. 41, no. 2, pp. 381–398, 2011.

[11] M. Cerquetella, A. Spaterna, F. Laus et al., "Inflammatory bowel disease in the dog: differences and similarities with humans," *World Journal of Gastroenterology*, vol. 16, no. 9, pp. 1050–1056, 2010.

[12] A. E. Jergens, C. A. Schreiner, D. E. Frank et al., "A scoring index for disease activity in canine inflammatory bowel disease," *Journal of Veterinary Internal Medicine*, vol. 17, no. 3, pp. 291–297, 2003.

[13] A. J. German, C. R. Helps, E. J. Hall, and M. J. Day, "Cytokine mRNA expression in mucosal biopsies from German shepherd dogs with small intestinal enteropathies," *Digestive Diseases and Sciences*, vol. 45, no. 1, pp. 7–17, 2000.

[14] M. J. Day, T. Bilzer, J. Mansell et al., "International standards for the histopathological diagnosis of gastrointestinal inflammation in the dog and cat: a report from the World Small Animal Veterinary Association Gastrointestinal Standardization Group," *Journal of Comparative Pathology*, vol. 138, no. 1, pp. S1–S43, 2008.

[15] S. S. Cohen, *A Guide to the Polyamines*, Oxford University Press, New York, NY, USA, 1997.

[16] B. T. Kurien, Y. Dorri, S. Dillon, A. Dsouza, and R. H. Scofield, "An overview of western blotting for determining antibody specificities for immunohistochemistry," *Methods in Molecular Biology*, vol. 717, pp. 55–67, 2011.

[17] A. E. Jergens, "Inflammatory bowel disease: current perspectives," *Veterinary Clinics of North America—Small Animal Practice*, vol. 29, no. 2, pp. 501–521, 1999.

[18] K. Allenspach, B. Wieland, A. Gröne, and F. Gaschen, "Chronic enteropathies in dogs: evaluation of risk factors for negative outcome," *Journal of Veterinary Internal Medicine*, vol. 21, no. 4, pp. 700–708, 2007.

[19] M. F. Dixon, R. M. Genta, J. H. Yardley et al., "Classification and grading of gastritis: the updated Sydney system," *The American Journal of Surgical Pathology*, vol. 20, no. 10, pp. 1161–1181, 1996.

[20] K. W. Head, R. W. Else, and R. R. Dubielzig, "Tumors of the alimentary tract," in *Tumors in Domestic Animals*, D. J. Meuten, Ed., pp. 451–477, Iowa State Press, Ames, Iowa, USA, 2002.

[21] K. W. Head, J. M. Cullen, R. R. Dubielzig et al., *Histological Classification of Tumors of the Alimentary System of Domestic Animals*, Armed Forces Institute of Pathology, Washington, DC, USA, 2003.

[22] A. Poletti, C. Giacon, and N. Pennelli, "Simultaneous visualization of immunodetected antigens and tissue components revealed by non-enzymatic histochemical stains," *Journal of Histochemistry and Cytochemistry*, vol. 40, no. 12, pp. 1965–1970, 1992.

[23] R.-M. Catalioto, C. A. Maggi, and S. Giuliani, "Intestinal epithelial barrier dysfunction in disease and possible therapeutical interventions," *Current Medicinal Chemistry*, vol. 18, no. 3, pp. 398–426, 2011.

[24] J. Timmons, E. T. Chang, J.-Y. Wang, and J. N. Rao, "Polyamines and gut mucosal homeostasis," *Journal of Gastrointestinal & Digestive System*, vol. 2, supplement 7, article 001, 9 pages, 2012.

[25] M. Pera, L. Grande, M. Gelabert et al., "Epithelial cell hyperproliferation after biliopancreatic reflux into the esophagus of rats," *Annals of Thoracic Surgery*, vol. 65, no. 3, pp. 779–786, 1998.

[26] E. W. Gerner, H. S. Garewal, S. S. Emerson, and R. E. Sampliner, "Gastrointestinal tissue polyamine contents of patients with Barrett's esophagus treated with alphadifluoromethylornithine," *Cancer Epidemiology Biomarkers and Prevention*, vol. 3, no. 4, pp. 325–330, 1994.

[27] H. S. Garewal and R. Sampliner, "Barrett's esophagus: a model premalignant lesion for adenocarcinoma," *Preventive Medicine*, vol. 18, no. 5, pp. 749–756, 1989.

[28] G. Ricci, G. Stabellini, G. Bersani et al., "Ornithine decarboxylase in colonic mucosa from patients with moderate or severe Crohn's disease and ulcerative colitis," *European Journal of Gastroenterology and Hepatology*, vol. 11, no. 8, pp. 903–904, 1999.

[29] R. B. Pillai, V. Tolia, R. Rabah, P. M. Simpson, R. Vijesurier, and C.-H. Lin, "Increased colonic ornithine decarboxylase activity in inflammatory bowel disease in children," *Digestive Diseases and Sciences*, vol. 44, no. 8, pp. 1565–1570, 1999.

[30] A. E. Pegg, R. Wechter, R. Pakala, and R. J. Bergeron, "Effect of N1,N12-bis(ethyl)spermine and related compounds on growth and polyamine acetylation, content, and excretion in human colon tumor cells," *The Journal of Biological Chemistry*, vol. 264, no. 20, pp. 11744–11749, 1989.

[31] A. P. Gobert, Y. Cheng, M. Akhtar et al., "Protective role of arginase in a mouse model of colitis," *Journal of Immunology*, vol. 173, no. 3, pp. 2109–2117, 2004.

[32] S. P. Marcuard, J. F. Silverman, J. L. Finley, and E. R. Seidel, "Ornithine decarboxylase activity during gastric ulcer healing in dogs," *Digestive Diseases and Sciences*, vol. 37, no. 7, pp. 1015–1019, 1992.

[33] T. S. Weiss, H. Herfarth, F. Obermeier et al., "Intracellular polyamine levels of intestinal epithelial cells in inflammatory bowel disease," *Inflammatory Bowel Diseases*, vol. 10, no. 5, pp. 529–535, 2004.

[34] M. Obayashi, I. Matsui-Yuasa, T. Matsumoto, A. Kitano, K. Kobayashi, and S. Otani, "Polyamine metabolism in colonic mucosa from patients with ulcerative colitis," *American Journal of Gastroenterology*, vol. 87, no. 6, pp. 736–740, 1992.

[35] J. Mössner, R. Hammermann, and K. Racké, "Concomitant down-regulation of L-arginine transport and nitric oxide (NO) synthesis in rat alveolar macrophages by the polyamine spermine," *Pulmonary Pharmacology & Therapeutics*, vol. 14, no. 4, pp. 297–305, 2001.

[36] V. Milovic and L. Turchanowa, "Polyamines and colon cancer," *Biochemical Society Transactions*, vol. 31, no. 2, pp. 381–383, 2003.

[37] N. Seiler, C. L. Atanassov, and F. Raul, "Polyamine metabolism as target for cancer chemoprevention," *International Journal of Oncology*, vol. 13, no. 5, pp. 993–1006, 1998.

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 528523, 8 pages
http://dx.doi.org/10.1155/2015/528523

Research Article

Actions of Probiotics on Trinitrobenzenesulfonic Acid-Induced Colitis in Rats

Takahiko Shiina,[1] Takeshi Shima,[1] Kiyotada Naitou,[1] Hiroyuki Nakamori,[1] Yuuki Sano,[1] Kazuhiro Horii,[1] Masaki Shimakawa,[2] Hiroshi Ohno,[2] and Yasutake Shimizu[1]

[1]*Department of Basic Veterinary Science, Laboratory of Physiology, The United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan*
[2]*Biofermin Kobe Research Institute, Biofermin Pharmaceutical Co. Ltd., 7-3-4 Ibukidai-Higashimachi, Nishi-ku, Kobe 651-2242, Japan*

Correspondence should be addressed to Takahiko Shiina; tshiina@gifu-u.ac.jp

Received 23 July 2015; Revised 25 September 2015; Accepted 27 September 2015

Academic Editor: Atsushi Sakuraba

We investigated the actions of probiotics, *Streptococcus faecalis* 129 BIO 3B (SF3B), in a trinitrobenzenesulfonic acid- (TNBS-) induced colitis model in rats. After TNBS was administered into the colons of rats for induction of colitis, the rats were divided into two groups: one group was given a control diet and the other group was given a diet containing SF3B for 14 days. There were no apparent differences in body weight, diarrhea period, macroscopic colitis score, and colonic weight/length ratio between the control group and SF3B group, suggesting that induction of colitis was not prevented by SF3B. Next, we investigated whether SF3B-containing diet intake affects the restoration of enteric neurotransmissions being damaged during induction of colitis by TNBS using isolated colonic preparations. Recovery of the nitrergic component was greater in the SF3B group than in the control group. A compensatory appearance of nontachykininergic and noncholinergic excitatory components was less in the SF3B group than in the control group. In conclusion, the present study suggests that SF3B-containing diet intake can partially prevent disruptions of enteric neurotransmissions induced after onset of TNBS-induced colitis, suggesting that SF3B has therapeutic potential.

1. Introduction

Inflammatory bowel disease (IBD) is a group of chronic, incurable inflammatory disorders of the gastrointestinal tract, including Crohn's disease and ulcerative colitis [1, 2]. IBD has become almost a global disease affecting people of almost all ages including the pediatric population [3, 4]. A convenient approach to study the pathogenesis of human IBD is to use animal models of IBD [4, 5]. Various animal models have been established to study IBD including chemical-induced colitis models such as trinitrobenzenesulfonic acid (TNBS), dextran sodium sulphate (DSS), and oxazolone-induced colitis models [1, 4].

Intracolonic application of TNBS induces colonic inflammation characterized by increased leukocyte infiltration, edema, and ulceration [4]. In addition, TNBS-induced colitis leads to alterations in enteric neuronal transmission regulating gastrointestinal motility, which is commonly found in IBD [6–10]. For instance, we previously demonstrated by using a TNBS-induced colitis model that the colonic inflammation causes indiscriminate damage to enteric neurons and that noncholinergic nontachykininergic excitatory neural components appear during restoration of inflammation probably as a result of a compensatory neurogenesis [11]. TNBS-induced colitis model animals have been widely used for identifying anti-inflammatory components such as plant extracts [12], seed oils [13], and probiotics [2, 14].

Probiotics are organisms that provide a desired and beneficial effect on human health. The probiotic *Streptococcus faecalis* 129 BIO 3B (SF3B: strain currently classified as *Enterococcus faecium*), which is a normal bowel commensal and lactic acid bacterium, has been reported to have various actions such as intestinal regulation [15, 16]. SF3B attenuated abdominal pain in an experimental rat model of TNBS-induced visceral hypersensitivity [16], suggesting that SF3B might affect the pathogenesis of TNBS-induced dysfunctions

Table 1: Compositions of experimental diets (%).

	Control diet	SF3B diet
CE-2*	90	90
SF3B powder	—	10
Dextrin	10	—

*CE-2: standard diet (CLEA Japan, Tokyo, Japan).

including colitis. In addition, growing evidence for a role of intestinal microflora in the development of IBD has led to studies on the therapeutic potential of modifying the composition of intestinal microbiota using probiotics [14]. In accordance with this, Yoshimatsu et al. recently reported that probiotics might be effective for maintaining clinical remission in patients with quiescent ulcerative colitis [17]. Hence, in the present study, we studied actions of probiotics on TNBS-induced colitis with focus on the alteration/recovery of neural components of the enteric nervous system that regulates intestinal motility.

2. Materials and Methods

2.1. Animals. Male Wistar rats (8–11 weeks of age, 200–300 g in weight) were obtained from Japan SLC (Shizuoka, Japan). They were maintained in plastic cages at 24 ± 2°C with a 12:12-hour light-dark cycle (light on at 08:00–20:00 h) and given free access to food and water. The Animal Care and Use Committee of Gifu University approved the animal experiments (experiment numbers: 12114, 13086, and 14108).

2.2. Preparation of Bacterial Powder and Bacterial Powder-Containing Diet. Dry powder consisting of SF3B (5×10^{11} cfu/g) isolated from human feces and a vehicle composed mainly of dextrin were used. This powder was supplied by Biofermin Pharmaceutical Co. Ltd. (Kobe, Japan). The compositions of diets used in this experiment are shown in Table 1.

2.3. Induction of Colitis and Feeding Program. After overnight fasting, animals were anesthetized with isoflurane. Then, they were given 1 mg of TNBS dissolved in 0.8 mL of 40% ethanol (v/v) by means of a silicon catheter inserted 7 cm through the anus. The animals were maintained in a head-down position for about 1 min to prevent leakage of the intracolonic solution. Animals were divided into two groups: one group was given a control diet (CONT group) and the other group was given a diet containing SF3B (SF3B group). The animals were maintained in their plastic cages and used at 7 days or 14 days after receiving TNBS. Macroscopic colitis score was calculated according to the criteria shown in Table 2.

2.4. Tissue Preparation. Animals were anesthetized by isoflurane and exsanguinated via the carotid artery. The abdominal cavity was opened immediately, and a 3-4 cm long segment of the distal colon (2 cm from the anus) was dissected out and immersed in modified Tyrode's solution (see below) at room temperature. The intraluminal contents were flushed using a small cannula filled with modified Tyrode's solution.

Table 2: Criteria for scoring of colitis by macroscopic assessment.

Feature graded	Grade	Description
Feces	0	Normal
	1	Loose
Mucosa damage	0	None
	1	Mild
	2	Moderate
	3	Severe
Adhesion	0	None
	1	Mild
	2	Severe

2.5. Mechanical Recordings. A segment of the distal colon of 3-4 cm length was mounted in a Magnus tube (20 mL in capacity) filled with modified Tyrode's solution (pH 7.4). The solution was continuously bubbled with 95% O_2 + 5% CO_2 gas mixture and maintained at 37°C. The distal end of each segment was tied to organ holders and the proximal end was secured with a silk thread to an isometric force transducer. The preparation was stimulated electrically by means of two platinum electrodes, one of which was placed in the lumen of the preparation and the other in the bathing solution (coaxial stimulation). Supramaximal rectangular pulses of 80 V in intensity and 0.5 msec in duration were delivered by using an electrical stimulator (model SEN-3301, Nihon Kohden, Tokyo, Japan) with frequency spectra of 20 Hz for 1 sec. Longitudinal contractile activity was recorded isometrically with a force transducer (T7-8-240, Orientec, Tokyo, Japan). An initial tension of 1.0 g was applied to the colonic preparations, which were subsequently allowed to equilibrate for 30 min. At the end of this period, the tension created by the segment was considered as the resting tension and no further mechanical adjustment was made during experimentation. Isometric responses were filtered and amplified by an amplifier (NEC, AS1202, Tokyo, Japan) and recorded using a PowerLab system (AD Instruments, Bella Vista, NSW, Australia).

2.6. Solutions and Drugs. During experiments for recording mechanical responses, tissues were maintained in modified Tyrode's solution consisting of (in mM) NaCl 136.9, KCl 2.68, $CaCl_2$ 1.80, $MgCl_2$ 1.0, NaH_2PO_4 0.41, $NaHCO_3$ 11.90, and glucose 5.55. Tetrodotoxin was used as a blocker of voltage-dependent sodium channels on neurons. Atropine was used to block muscarinic acetylcholine receptors on smooth muscle cells. N-Acetyl-L-tryptophan 3,5-bis (trifluoromethyl) benzyl ester (L-732,138) and (S)-N-methyl-N[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl) butyl] benzamide (SR48968) were used to block tachykinin NK_1 and tachykinin NK_2 receptors, respectively. Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) was used as a nitric oxide (NO) synthase (NOS) inhibitor. L-732,138 and L-NAME were obtained from Sigma (St. Louis, MO, USA). Atropine sulfate salt monohydrate, TNBS, and tetrodotoxin were obtained from Wako (Osaka, Japan). SR48968 was a gift from Sanofi-Synthelabo (Montpellier, France). L-732,138

and SR48968 were dissolved in ethyl acetate and ethanol, respectively. The vehicles (ethanol and ethyl acetate) for the drugs alone had no effect on basal tone. Other drugs were dissolved in distilled water. The drug concentrations given in the test were final concentrations in the bath solution.

2.7. RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). The expression of mRNA was assessed by RT-PCR. Total cellular RNA was extracted from tissue homogenates of the rat colon using TRIzol reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA). First-strand cDNA was synthesized from 2 μg of total RNA by using SuperScript III Reverse Transcriptase (Fisher Scientific Inc.) and Random Primers (Fisher Scientific Inc.). The absence of PCR-amplified DNA fragments in the samples without reverse transcription indicated the isolation of RNA free from genomic DNA contamination. The PCR was performed with platinum Taq DNA polymerase (Fisher Scientific Inc.). The primer sets were as follows: inducible NOS (iNOS) sense 5′-TAG AAA CAA CAG GAA CCT AC-3′ and antisense 5′-AAC ATC TCC TGG TGG AAC A-3′ (predicted size = 925 bp); neuronal NOS (nNOS) sense 5′-CCG GAA TTC GAA TAC CAG CCT GAT CCA TGG AA-3′ and antisense 5′-CCG AAT TCC TCC AGG AGG GTG TCC ACC GCA TG-3′ (predicted size = 617 bp); c-Myc sense 5′-AAG AGA ATT TCT ATC ACC AG-3′ and antisense 5′-TTG ATG GGG ATG ACC CTG AC-3′ (predicted size = 771 bp); CyclinD1 sense 5′-AGG AGC AGA AGT GCG AAG AG-3′ and antisense 5′-TAG CAG GAG AGG AAG TTG TT-3′ (predicted size = 475 bp); leucine-rich repeat containing G protein-coupled receptor 5 (Lgr5) sense 5′-GCG GGG CTG CCC ATC ATA CT-3′ and antisense 5′-TTG CTG TGG AAT CCT AGT TC-3′ (predicted size = 708 bp); hairy and enhancer of split-1 (Hes1) sense 5′-AGA AAA ATT CCT CGT CCC CG-3′ and antisense 5′-GGA AGC CGC CAA AAA CCT TG-3′ (predicted size = 642 bp); atonal protein homolog 1 (Atoh1) sense 5′-ACC ACC ATC GCC ATC CCC AG-3′ and antisense 5′-CGC CTG CTC CTC CTT CAT AA-3′ (predicted size = 687 bp); Notch1 sense 5′-GAG GTG CGA AGT GGC CAA CG-3′ and antisense 5′-AGT CCA GCC ATT GAC ACA CA-3′ (predicted size = 880 bp); and β-actin sense 5′-TGA CCC TGA AGT ACC CCA TTG-3′ and antisense 5′-TCA GGA TCT TCA TGA GGT AG-3′ (predicted size = 387 bp). All primers were purchased from Eurofins Genomics (Tokyo, Japan). Amplifications were performed by 25 or 30 cycles. The reaction products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide (0.4 μg/mL). The gels were exposed to UV light with a UV transilluminator (UVP Laboratory Products, Upland, CA, USA) and photographed. Densitometry analyses of the results were performed with ImageJ software. Relative values of mRNA expression were calculated by taking the value of the respective β-actin mRNA as unity (1.0).

2.8. Macroscopic Assessment of Colitis. The isolated colon segments were examined visually, and their damage was scored. Macroscopic colitis scores were assigned values of 0–6 as shown in Table 2. In addition, colonic weight/length ratio was calculated as a parameter for evaluating inflammation damage, since colonic inflammation can result in an increase in weight of the colon and decrease in length of the colon [18].

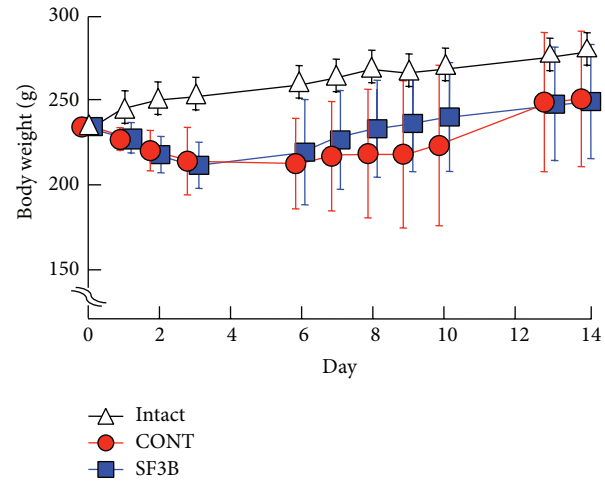


Figure 1: Body weight changes in TNBS-treated rats taking the control diet (CONT group; $n = 8$) or SF3B-containing diet (SF3B group; $n = 10$) and intact rats ($n = 4$). At day 0, TNBS was injected into the distal colon. Each value represents the mean ± S.D.

2.9. Data Analysis. Data are presented as means ± standard deviation (S.D.). n indicates the number of experiments performed using different tissue preparations from different animals. The significance of differences between mean values was determined by one-way or two-way analysis of variance followed by the Turkey-Kramer test for comparison of multiple groups or by Student's t-test or Mann-Whitney U test for comparison of two groups. A P value less than 0.05 denotes the presence of a statistically significant difference.

3. Results

3.1. Effects of SF3B-Containing Diet Intake on TNBS-Induced Colitis in Rats. TNBS was injected into the distal colon of each rat to induce colitis, and then a control diet or a diet containing SF3B was given for 14 days. Body weights in both the CONT group and SF3B group decreased within the initial 3 days after treatment with TNBS but recovered gradually as days passed (Figure 1). There was no significant difference in body weight changes between the groups. In contrast, the weight of intact animals increased during the same period. Diarrhea occurred 1–3 days after TNBS treatment and persisted for about 10–12 days in both groups (Figure 2(a)). Macroscopic observation showed that treatment with TNBS induced visible inflammation in the colon, as indicated by the macroscopic colitis score (Table 2): close to 0 in intact rats versus about 2-3 in the CONT group and SF3B group at 14 days after TNBS treatment (Figure 2(b)). Colonic weight/length ratios in the CONT group and SF3B group at 14 days after TNBS treatment were higher than the ratio in the intact group, while there was no significant difference between the CONT group and SF3B group (Figure 2(c)).

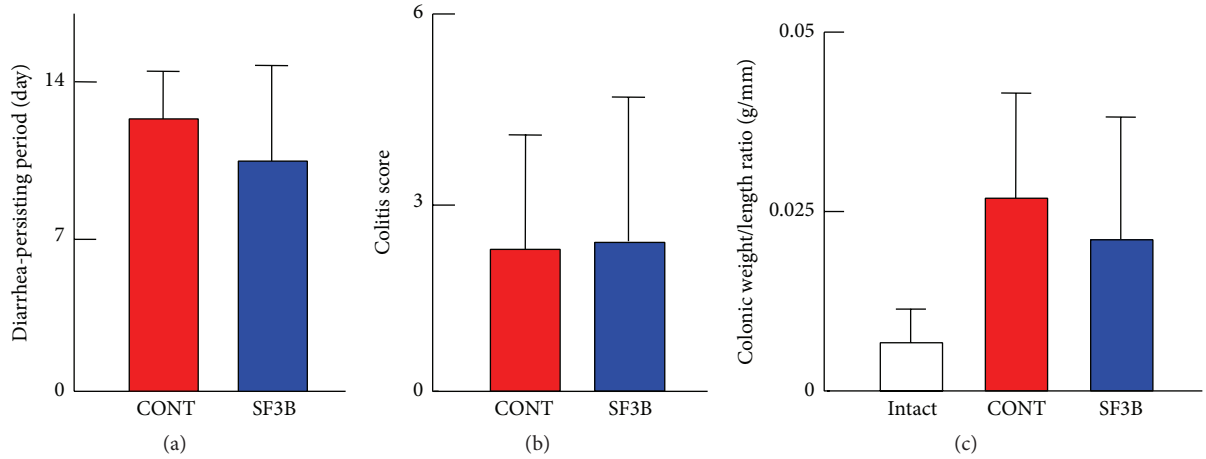


Figure 2: Effects of SF3B-containing diet intake on TNBS-induced colitis in rats. (a) Summary graphs showing diarrhea-persisting periods (days) in the CONT group ($n = 9$) and SF3B group ($n = 9$). (b) Summary graphs showing macroscopic colitis scores in the CONT group ($n = 7$) and SF3B group ($n = 10$) at 14 days after TNBS treatment (see Table 2). (c) Summary graphs showing colonic weight/length ratios in the CONT group ($n = 7$) and SF3B group ($n = 10$) at 14 days after TNBS treatment and in the intact group ($n = 4$). Each bar represents the mean ± S.D.

3.2. Effects of SF3B-Containing Diet Intake on Inflammation-Induced Changes in Nitrergic Responses in Colonic Motor Functions. At 14 days after TNBS treatment, we investigated functions of enteric neurons in motility of the inflamed colon using an *in vitro* system. In segments of the distal colon from the intact group, spontaneous contractions were observed (Figure 3(a)). Application of EFS induced contractile responses in the rat colon (Figure 3(a)). The EFS-induced contractile response was abolished by application of tetrodotoxin (1 μM), a blocker of voltage-dependent sodium channels on neurons (data not shown). To determine whether NO influences mechanical responses in the rat colon, L-NAME (200 μM), a NOS inhibitor, was applied. In all of the preparations from the intact group ($n = 7$), as shown in Figure 3(a), L-NAME application increased the frequency and amplitude of spontaneous contractions, suggesting that the colonic contractile activity is subject to tonic nitrergic inhibitory control. In agreement with this, amplitude of the EFS-induced contraction was increased after application of L-NAME (Figure 3(a)). In contrast, 36.4% of the preparations from the CONT group ($n = 11$) were resistant to L-NAME: spontaneous contractions and EFS-induced contractions were not enhanced even in the presence of L-NAME (Figure 3(b)), indicating reduction of the tonic nitrergic inhibition. However, all of the preparations from the SF3B group ($n = 9$) were sensitive to L-NAME application as were the preparations from the intact group (Figure 3(c)).

3.3. Effects of SF3B-Containing Diet Intake on Inflammation-Induced Changes in Excitatory Responses in Colonic Motor Functions. Next, we examined the effect of SF3B-containing diet on neuronal components regulating EFS-induced contractile responses in the inflamed colon at 14 days after TNBS treatment. To simplify the interpretation of results of pharmacological experiments, blockers were applied under the condition in which the inhibitory components were eliminated by L-NAME (200 μM). In preparations of the intact group ($n = 7$), as shown in Figure 4(a), combined application of blockers for tachykinin NK_1 receptors and tachykinin NK_2 receptors (L-732,132 and SR48968, 2 μM, resp.) significantly diminished the EFS-evoked contractile responses (Figure 4(a)). The contraction being resistant to tachykininergic antagonists was blocked by the cumulative application of atropine (5 μM), a blocker for muscarinic cholinoceptors (Figure 4(a)). In contrast, in the majority of preparations from the CONT group ($n = 11$), EFS-induced contractile response was not completely blocked by combined application of tachykininergic and cholinergic antagonists (Figure 4(b)). The noncholinergic/nontachykininergic response was abolished by tetrodotoxin (1 μM), an inhibitor of neurogenic responses (data not shown). In contrast, in half of the preparations from the SF3B group ($n = 9$), combined application of tachykininergic and cholinergic antagonists almost totally abolished EFS-induced contraction in a manner similar to that in the intact group (Figure 4(c)).

3.4. Effects of SF3B-Containing Diet Intake on Expression of mRNAs of Inflammation-Related and Tissue Regeneration-Related Genes and an Enteric Neuron-Expressing Gene in the Inflamed Colon. Expression of iNOS, Atoh1, CyclinD1, c-Myc, Lgr5, Hes-1, Notch1, and nNOS genes in the inflamed colon was analyzed by RT-PCR. After treatment with TNBS, the expression of iNOS mRNA was increased, while the expressions of other inflammation-related and tissue regeneration-related gene mRNAs were decreased (Figures 5(a), 5(b), and 5(c)). Expression levels of iNOS and CyclinD1 mRNAs in the SF3B group were lower than those in the CONT group at 7 days after treatment with TNBS (Figures 5(a) and 5(b)). On the other hand, expression level of Atoh1 mRNA in the SF3B group was higher than that in the CONT group at 7 days after TNBS application (Figure 5(c)). In addition, expression level of nNOS mRNA in the SF3B group

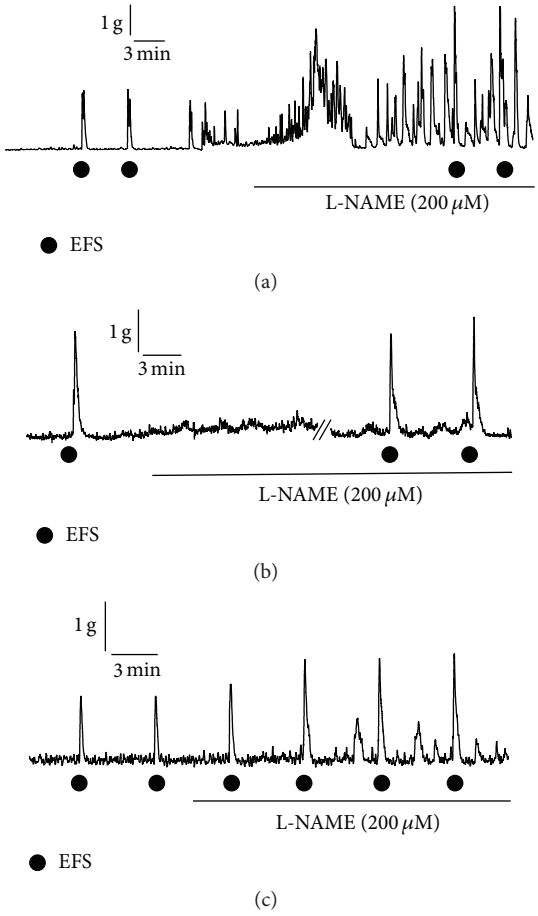


Figure 3: Effects of a NOS inhibitor on spontaneous contractions and electrical field stimulation- (EFS-) induced contractions in isolated colonic preparations from rats. Representative tracings of mechanical responses in the absence and presence of a NOS inhibitor, L-NAME (200 μM), in an intact rat (a), a TNBS-treated rat with the control diet at 14 days after TNBS treatment (b), and a TNBS-treated rat with the SF3B-containing diet at 14 days after TNBS treatment (c) are shown. Circles indicate the points of EFS.

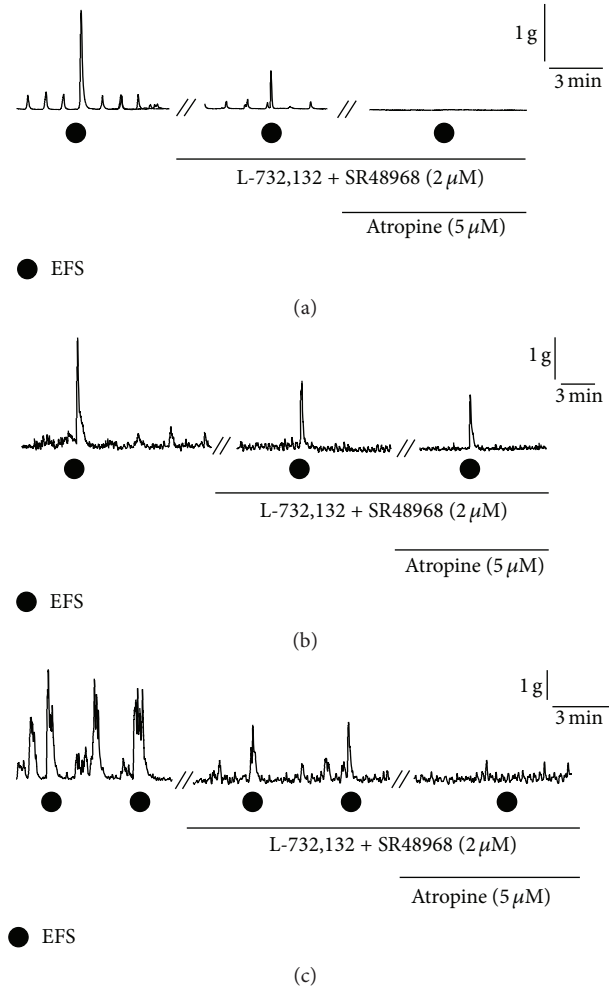


Figure 4: Effects of tachykinergic and cholinergic inhibitors on EFS-induced contractions in isolated colonic preparations from rats. Representative tracings of mechanical responses in the absence and presence of blockers for tachykinin NK_1 receptors and tachykinin NK_2 receptors (L-732,132 and SR48968, 2 μM, resp.) and atropine, a blocker for muscarinic cholinoceptors (5 μM), in an intact rat (a), a TNBS-treated rat with the control diet at 14 days after TNBS treatment (b), and a TNBS-treated rat with the SF3B-containing diet at 14 days after TNBS treatment (c), are shown. Circles indicate the points of EFS. To simplify the interpretation of results of pharmacological experiments, blockers were applied under the condition in which the inhibitory components were eliminated by L-NAME (200 μM).

at 14 days after treatment with TNBS was higher than that in the SF3B group at 7 days after TNBS application (Figure 5(d)). There was no significant difference in expression levels of other genes between the SF3B group and CONT group.

4. Discussion

In the present study, we tried to determine whether SF3B has beneficial actions on IBD, especially the recovery phase of IBD, by using a TNBS-induced colitis model in rats. We found no apparent differences in body weight, diarrhea period, macroscopic colitis score, and colonic weight/length ratio between the control diet group and SF3B-containing diet group. On the basis of these results, induction and progression of colitis itself did not seem to be prevented even by taking SF3B-containing diet at least in this experimental condition. However, a remarkable finding of our study is that enteric neural components, which are impaired by an acute inflammatory process, are normally restored by ingestion of an SF3B-containing diet. Considering that alterations in enteric neural components being promoted following transient intestinal inflammation, as manifested in the CONT group in this study, can be a cause of abnormal intestinal motility [11, 19–21], the action of SF3B to bring about normal restoration would be beneficial for treatment of IBD.

Disruption of enteric neurotransmissions is one of the principle causes of colitis-induced abnormal intestinal motility [7–11, 22, 23]. In the present study, we investigated whether ingestion of an SF3B-containing diet affects restoration of

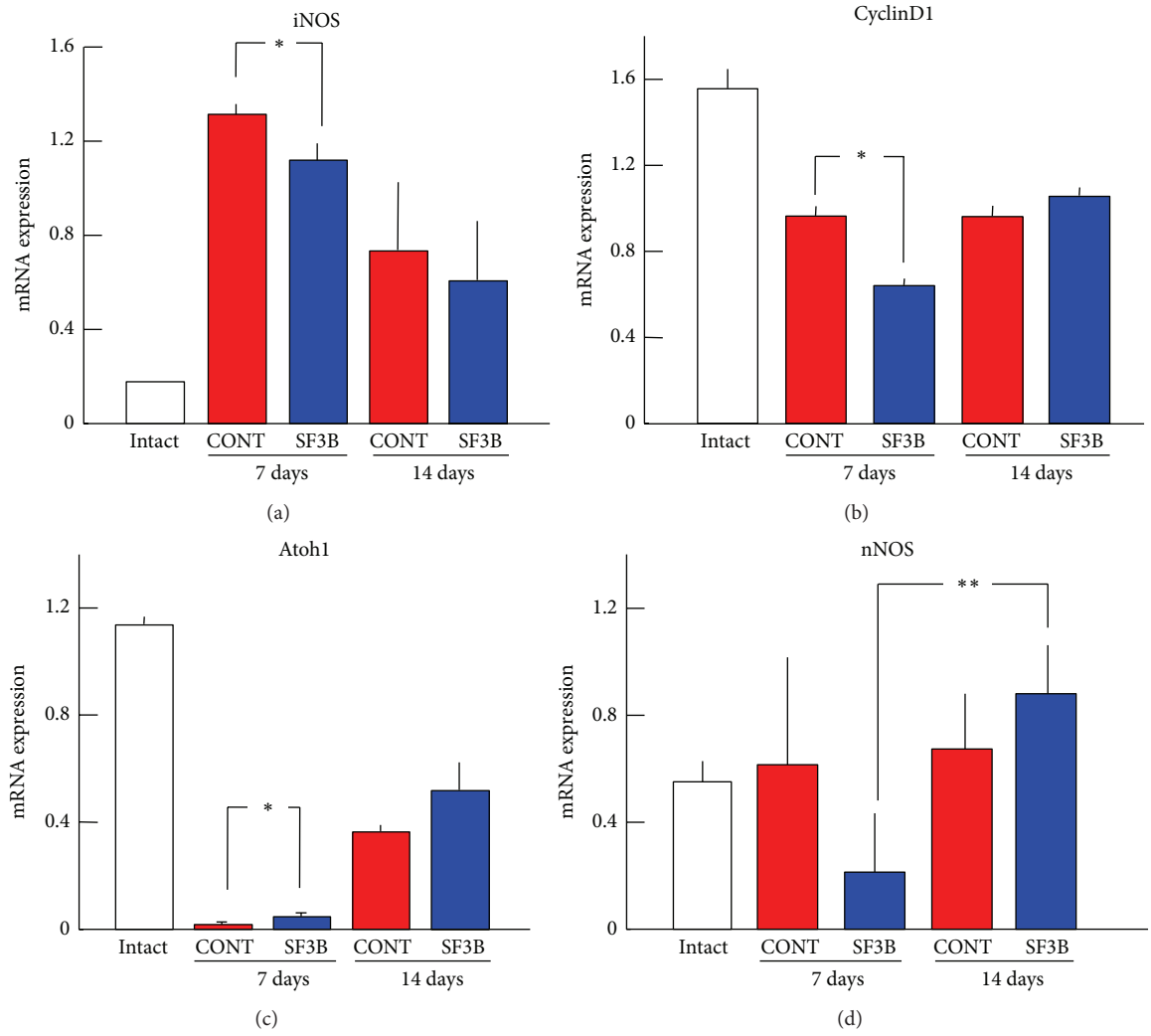


Figure 5: Effects of SF3B-containing diet intake on expression of mRNAs of inflammation-related and tissue regeneration-related genes in the inflamed colon. Expression levels of iNOS (a), CyclinD1 (b), Atoh1 (c), and nNOS (d) genes in colons of the CONT group ($n = 5$) and SF3B group ($n = 5$) at 7 or 14 days after TNBS treatment and of the intact group ($n = 3$) were analyzed by RT-PCR. Summary graphs showing relative values of mRNA expression calculated by taking the value of the respective β-actin mRNA as unity (1.0). Each bar represents the mean ± S.D. $^{*}P < 0.05$ and $^{**}P < 0.01$, comparison between the CONT group and SF3B group at the same day after TNBS treatment or between the SF3B groups at different day after TNBS treatment.

nitrergic, tachykininergic, and cholinergic neurotransmissions being damaged during induction of colitis by TNBS. Recovery of the nitrergic component as assessed by increases in frequency and amplitude of spontaneous contractions after application of L-NAME was greater in the SF3B group than in the CONT group. This suggests that ingestion of SF3B accelerates restoration of the nitrergic component. In accordance with this, the expression level of nNOS gene at 14 days after treatment with TNBS was higher than that at 7 days after treatment with TNBS in the SF3B group but not in the CONT group. Since the inhibitory nitrergic component of the enteric nervous system plays an important role in the regulation of intestinal motility, the effect of SF3B would be accessible to avoid persistent alteration of motility patterns commonly observed after the resolution of intestinal inflammation [19, 21]. In addition, appearance of nontachykininergic and noncholinergic excitatory components was less in the SF3B group than in the CONT group. The appearance of unusual neural components can be considered to be a compensatory mechanism to maintain intestinal motility [11]. However, excessive compensation might not always be beneficial for normal functions. Thus, less expression of the nontachykininergic and noncholinergic excitatory components can be appreciated as a valuable action of SF3B.

IBD is an important etiologic factor in the development of colorectal cancer [24]. It has been pointed out that several molecular alterations associated with IBD are related to colorectal cancer. For instance, activation of CyclinD1-mediated cell proliferation and high activity of iNOS might contribute to colon oncogenesis [20, 25]. Interestingly, SF3B-containing diet intake reduced the expression of iNOS and

CyclinD1 mRNA compared to that in rats fed the control diet. It is therefore expected that the risk for IBD-associated colorectal cancer would be alleviated by the action of SF3B.

Although probiotics have been reported to exert various actions in humans and animals, the mechanisms of their actions are not completely understood [14]. Probiotics can occupy the physical space and interact with intestinal tissue or release bioactive products/metabolites, resulting in the blocking of pathogenic bacteria, enhancement of barrier function, and alteration of the mucosal immune system [14]. In fact, it had been reported that ingestion of *Streptococcus faecalis* resulted in increasing bifidobacteria and enterococci of the intestinal flora in rats [26]. Moreover, probiotics can exert anti-inflammatory action by reducing the production of proinflammatory cytokines [14]. In accordance with this, SF3B-containing diet intake inhibited expression of a proinflammation marker gene iNOS. Considering that enteric neuroplasticity evoked by inflammation such as that in IBD might be caused by proinflammatory cytokines from activated immune cells [6, 7, 14], SF3B might prevent colitis-associated disruptions of enteric neurons by blocking activation of the inflammatory response at least partially.

In conclusion, the present study suggests that ingestion of an SF3B-containing diet can partially prevent disruptions of enteric neurotransmissions induced after onset of TNBS-induced colitis and inhibit the expression of genes involved in oncogenesis, suggesting that SF3B has therapeutic potential.

Disclosure

This study was a part of studies on actions of *Streptococcus faecalis* 129 BIO 3B. Biofermin Pharmaceutical Co. Ltd. had no role in the study design, conduct of the study, data collection, data interpretation, or preparation of the report.

Conflict of Interests

M. Shimakawa and H. Ohno are employees of Biofermin Pharmaceutical Co. Ltd. (Kobe, Japan).

Acknowledgments

The authors are indebted to Ms. Ayako Takahashi and Noriko Endo (Gifu University, Japan) for their technical support. This study was partially funded by Biofermin Pharmaceutical Co. Ltd.

References

[1] D. Corridoni, K. O. Arseneau, and F. Cominelli, "Inflammatory bowel disease," *Immunology Letters*, vol. 161, no. 2, pp. 231–235, 2014.

[2] S. Guandalini, "Are probiotics or prebiotics useful in pediatric irritable bowel syndrome or inflammatory bowel disease?" *Frontiers of Medicine*, vol. 1, article 23, 2014.

[3] N. A. Molodecky, I. S. Soon, D. M. Rabi et al., "Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review," *Gastroenterology*, vol. 142, no. 1, pp. 46.e42–54.e42, 2012.

[4] P. K. Randhawa, K. Singh, N. Singh, and A. S. Jaggi, "A review on chemical-induced inflammatory bowel disease models in rodents," *Korean Journal of Physiology and Pharmacology*, vol. 18, no. 4, pp. 279–288, 2014.

[5] N. Goyal, A. Rana, A. Ahlawat, K. R. V. Bijjem, and P. Kumar, "Animal models of inflammatory bowel disease: a review," *Inflammopharmacology*, vol. 22, no. 4, pp. 219–233, 2014.

[6] C. Giaroni, F. De Ponti, M. Cosentino, S. Lecchini, and G. Frigo, "Plasticity in the enteric nervous system," *Gastroenterology*, vol. 117, no. 6, pp. 1438–1458, 1999.

[7] A. E. Lomax, E. Fernández, and K. A. Sharkey, "Plasticity of the enteric nervous system during intestinal inflammation," *Neurogastroenterology and Motility*, vol. 17, no. 1, pp. 4–15, 2005.

[8] G. M. Mawe, S. M. Collins, and T. Shea-Donohue, "Changes in enteric neural circuitry and smooth muscle in the inflamed and infected gut," *Neurogastroenterology and Motility*, vol. 16, no. 1, pp. 133–136, 2004.

[9] D. M. Moynes, G. H. Lucas, M. J. Beyak, and A. E. Lomax, "Effects of inflammation on the innervation of the colon," *Toxicologic Pathology*, vol. 42, no. 1, pp. 111–117, 2014.

[10] V. Vasina, G. Barbara, L. Talamonti et al., "Enteric neuroplasticity evoked by inflammation," *Autonomic Neuroscience: Basic & Clinical*, vol. 126-127, pp. 264–272, 2006.

[11] T. Shiina, Y. B. Gurung, Y. Suzuki, T. Takewaki, and Y. Shimizu, "Alteration of neuromuscular transmissions in the hamster colon following the resolution of TNBS-induced colitis," *Journal of Physiological Sciences*, vol. 63, no. 4, pp. 241–249, 2013.

[12] X. Liu and J. Wang, "Anti-inflammatory effects of iridoid glycosides fraction of Folium syringae leaves on TNBS-induced colitis in rats," *Journal of Ethnopharmacology*, vol. 133, no. 2, pp. 780–787, 2011.

[13] F. Isik, T. Tunali Akbay, A. Yarat et al., "Protective effects of black cumin (*Nigella sativa*) oil on TNBS-induced experimental colitis in rats," *Digestive Diseases and Sciences*, vol. 56, no. 3, pp. 721–730, 2011.

[14] G. R. Veerappan, J. Betteridge, and P. E. Young, "Probiotics for the treatment of inflammatory bowel disease," *Current Gastroenterology Reports*, vol. 14, no. 4, pp. 324–333, 2012.

[15] M. Kumada, M. Motegi, R. Nakao et al., "Inhibiting effects of *Enterococcus faecium* non-biofilm strain on *Streptococcus mutans* biofilm formation," *Journal of Microbiology, Immunology and Infection*, vol. 42, no. 3, pp. 188–196, 2009.

[16] M. Shimakawa, S. Tsunemine, Y. Makizaki, T. Komada, H. Ohno, and H. Yamamura, "Effects of *Streptococcus faecalis* 129 BIO 3B on abdominal pain in a rat model," *Journal of Pharmacological Sciences*, vol. 121, supplement 1, p. 114, 2013.

[17] Y. Yoshimatsu, A. Yamada, R. Furukawa et al., "Effectiveness of probiotic therapy for the prevention of relapse in patients with inactive ulcerative colitis," *World Journal of Gastroenterology*, vol. 21, no. 19, pp. 5985–5994, 2015.

[18] X. Lei, M. Liu, Z. Yang, M. Ji, X. Guo, and W. Dong, "Thymoquinone prevents and ameliorates dextran sulfate sodium-induced colitis in mice," *Digestive Diseases and Sciences*, vol. 57, no. 9, pp. 2296–2303, 2012.

[19] G. Barbara, B. A. Vallance, and S. M. Collins, "Persistent intestinal neuromuscular dysfunction after acute nematode infection in mice," *Gastroenterology*, vol. 113, no. 4, pp. 1224–1232, 1997.

[20] Y. B. Gurung, Y. Shimizu, T. Shiina, M. E. Mahmoud, S. Saito, and T. Takewaki, "Impairment and restoration of spontaneous contractile activity of longitudinal smooth muscles in the TNBS-inflamed hamster distal colon," *Biomedical Research*, vol. 28, no. 6, pp. 301–308, 2007.

[21] A. E. Lomax, J. R. O'Hara, N. P. Hyland, G. M. Mawe, and K. A. Sharkey, "Persistent alterations to enteric neural signaling in the guinea pig colon following the resolution of colitis," *American Journal of Physiology: Gastrointestinal and Liver Physiology*, vol. 292, no. 2, pp. G482–G491, 2007.

[22] K. J. Gross and C. Pothoulakis, "Role of neuropeptides in inflammatory bowel disease," *Inflammatory Bowel Diseases*, vol. 13, no. 7, pp. 918–932, 2007.

[23] T.-S. Sung, J.-H. La, T.-W. Kim, and I.-S. Yang, "Alteration of nitrergic neuromuscular transmission as a result of acute experimental colitis in rat," *Journal of Veterinary Science*, vol. 7, no. 2, pp. 143–150, 2006.

[24] M. Yashiro, "Molecular alterations of colorectal cancer with inflammatory bowel disease," *Digestive Diseases and Sciences*, vol. 60, no. 8, pp. 2251–2263, 2015.

[25] N. Gopalakrishnan, M. Saravanakumar, P. Madankumar, M. Thiyagu, and H. Devaraj, "Colocalization of β-catenin with Notch intracellular domain in colon cancer: a possible role of Notch1 signaling in activation of CyclinD1-mediated cell proliferation," *Molecular and Cellular Biochemistry*, vol. 396, no. 1-2, pp. 281–293, 2014.

[26] S. Katsumata, "Effects of oral administration of lactic acid bacteria preparation on enterobacterial flora in rats," *Journal of the Japan Pediatric Society*, vol. 58, no. 10, pp. 893–899, 1954 (Japanese).

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 359130, 8 pages
http://dx.doi.org/10.1155/2015/359130

Research Article

The Demographic and Clinical Characteristics of Ulcerative Colitis in a Northeast Brazilian Population

Bruno César da Silva,[1,2] Andre Castro Lyra,[1,3] Carlos Maurício Cardeal Mendes,[4] Camila Paula Oliveira Ribeiro,[5] Sonyara Rauedys Oliveira Lisboa,[5] Mariana Tinoco Lordello de Souza,[5] Renata Cavalcanti Portela,[5] and Genoile Oliveira Santana[1,3]

[1] *Postgraduate Program in Medicine and Health, Federal University of Bahia, Rua Augusto Viana s/n, 5 Andar, Canela, 40110-060 Salvador, BA, Brazil*
[2] *Gastroenterology Department, Hospital da Bahia, Avenida Professor Magalhães Neto 1541, Pituba, 41820-011 Salvador, BA, Brazil*
[3] *Gastroenterology and Hepatology Discipline, Department of Medicine, Federal University of Bahia, Rua Augusto Viana s/n, 5° Andar, Canela, 40110-060 Salvador, BA, Brazil*
[4] *Institute of Health Sciences, Federal University of Bahia, Avenida Sete de Setembro, s/n, Canela, 40110-060 Salvador, BA, Brazil*
[5] *Bahiana School of Medicine and Public Health, Rua Silveira Martins, 3386 - Cabula, 41150-100 Salvador, BA, Brazil*

Correspondence should be addressed to Bruno César da Silva; bcesars@hotmail.com

Received 9 June 2015; Revised 9 August 2015; Accepted 9 September 2015

Academic Editor: Kian Keyashian

Introduction. The purpose of this study was to describe the clinical and demographic characteristics of UC in Bahia, a Brazilian state, and to identify the variables associated with extensive colitis, steroid therapy, immunosuppression, and colectomy. *Methods*. In this cross-sectional study UC patients were interviewed, and additional information was collected from the medical records. Descriptive statistics and multivariate Poisson regression analysis were used. *Results*. This study included 267 individuals, the mean age of whom was 39.4 years at diagnosis. There was a predominance of females and left-side colitis. Extensive colitis was positively associated with male gender, diarrhea, weight loss, and a younger age at diagnosis. In contrast, active smoking and a family history of IBD were negatively associated with extensive colitis. Positive associations were observed between steroid therapy and diarrhea, weight loss, urban patients, extraintestinal manifestations (EIMs), and hospitalization. Younger age and weight loss at diagnosis, a family history of IBD, extensive colitis, EIMs, hospitalization, and steroid therapy were all positively associated with immunosuppression. In contrast, Caucasian individuals, smokers, patients with rectal bleeding, and rural patients areas were all observed to have a decreased likelihood of immunosuppression. *Conclusions*. Our results corroborate the association between higher prevalence of extensive colitis and younger age at diagnosis. An association between steroid therapy and clinical presentation at diagnosis was observed. The observation that white individuals and rural patients use less immunosuppressive drugs highlights the need to study the influence of environmental and genetic factors on the behavior of UC in this population.

1. Introduction

In recent decades, the incidence of inflammatory bowel disease (IBD) has increased in several regions of the world, especially in developing countries [1, 2]. Although nationwide data on the incidence and prevalence of ulcerative colitis (UC) in Brazil are lacking, research results from several regions of the southeast have shown that the incidence of UC and the number of hospitalizations have been increasing over the past several years [3, 4].

Most publications that describe the demographic and clinical characteristics of patients with UC have been conducted in Europe or North America. However, even these studies from developed countries have been characterized by variable methods and usually have been conducted with specific populations within certain regions at medical

reference centers [5]. Studies examining the demographic and clinical data of patients with UC are scarce in Latin America, including Brazil.

UC presents variable demographic and clinical characteristics in different regions of the world. Environmental and genetic factors appear to be related to this phenotypic heterogeneity [6]. For example, research has shown that the evolution of UC is usually less severe (i.e., there is a decreased need for surgery, a lower incidence of colorectal cancer, and fewer intestinal manifestations) in Asian countries than in developed Western countries [7, 8].

The Brazilian population is comprised of individuals from several different ethnic backgrounds, including those of European descents, Brazilian Indigenous descents, and African descents. Bahia is a state in which the population is composed primarily of individuals of African descent. This population results from Brazil's unique history since the sixteenth century. Knowing the characteristics of UC in the population of northeastern Brazil, especially in Bahia, is important because most prior publications regarding UC have been performed with predominantly Caucasian or Asian populations.

Our purpose was to describe the demographic and clinical characteristics of patients with UC from two IBD treatment referral centers in Bahia (a state in northeastern Brazil) and to identify the presence of factors associated with the extent of disease, the use of corticosteroids, the use of immunosuppressive therapy, and colectomy. Such studies are useful to evaluate the phenotypic differences in various regions of the world. In addition, this information may be able to guide research that could potentially elucidate the role of genetic and environmental factors in the etiology and natural history of the disease.

2. Methods

This cross-sectional study was performed between January 2011 and September 2012 with UC patients of the Professor Edgard Santos University Hospital and of the Roberto Santos General Hospital, the only two IBD treatment referral centers in Bahia, both of which are located in the city of Salvador. The patients were interviewed, and additional information was collected from medical records immediately following each interview. Individuals included in the study were diagnosed with UC based on clinical, endoscopic, radiological, and histological findings [9]. Patients with indeterminate colitis, Crohn's disease, or infectious and parasitic colitis were not included.

The following clinical and demographic characteristics were analyzed: gender, skin color, origin (urban or rural), the year when the diagnosis occurred, the age at the time of diagnosis, disease duration, the extent of UC according to the Montreal classification [10], symptoms (diarrhea, weight loss, abdominal pain, and blood in the stool), family history of IBD, smoking history, hospital admission, extraintestinal manifestations (EIMs), colectomy, and the use of corticosteroids, azathioprine, or antibodies against tumor necrosis factor (anti-TNF). For our multivariate regression models, the extent of disease was classified into two categories (i.e., with and without extensive colitis).

To classify patients according to skin color, the method adopted by the Brazilian Institute of Geography and Statistics (IBGE) was used [11], which allows individuals to declare themselves as black, pardo (mixed-race), white, yellow, or indigenous. In multivariate regression models, individuals identified as black, mixed-race, and Indian were grouped together to form the category of nonwhites.

In Bahia, the treatment of IBD is part of a special program of the government. For patients with UC, the first choice of aminosalicylate is sulfasalazine. If the patients progress with adverse events, it is recommended to use mesalazine. Patients with mild to moderate disease activity were treated with aminosalicylates (i.e., sulfasalazine—3.0 to 6.0 g/day; mesalazine—2.4 to 4.8 g/day; or suppository mesalazine—0.5 to 1.5 g/day). Individuals refractory to initial therapy were treated with systemic steroids orally (prednisone 40–60 mg/day), with a weaning regimen lasting approximately two months. Azathioprine was used for steroid dependent patients (2.0 to 2.5 mg/kg/day). Individuals with severe UC were hospitalized and treated with intravenous steroids (hydrocortisone 300–400 mg/day) [12]. Colectomy was indicated when there was no response to intravenous corticosteroid therapy after one week of treatment. Infliximab is not part of the UC treatment protocol adopted by the Brazilian government. However, in recent years, specific cases have been individually assessed by a technical chamber that has the prerogative to approve the use of infliximab.

Data were analyzed using the Statistical Package for the Social Sciences (version 21.0, SPSS, Chicago, IL) and the statistical package R (version 3.0.1, 2013). The population was characterized using descriptive statistics. Because the entire population of the two reference centers was examined (rather than a random sample) statistical inference was not performed [13]. The selection of variables to be included in each multivariate regression model to analyze the associations between variables accounted for the importance of each variable according to evidence from the literature and the authors' own experience. An association with a prevalence ratio equal to or greater than 20% was adopted as the criterion for keeping variables in the final model. Akaike information criterion (AIC) and residual analyses were used as criteria for the goodness of fit. Collinearity and overdispersed variance were also evaluated.

Written informed consent was obtained from each patient. The study was conducted in accordance with the Declaration of Helsinki and it was approved by the ethics committee of the University Hospital Professor Edgard Santos.

3. Results

A total of 267 patients were included in the study, of whom 180 (67.4%) were female and 218 (83.8%) were nonwhite. The majority of patients resided in urban areas, and the average age at the time of diagnosis was 39.4 years (with a range of 11–78 years). The mean duration of disease was 7.56 years (with a range of 0–34 years). The demographic characteristics of

Table 1: Demographic characteristics of patients with ulcerative colitis in two reference centers in Salvador, Bahia, Brazil (n = 267).

Variables	n (%)
Gender	
Male	87 (32.6)
Female	180 (67.4)
Skin color	
Blacks	69 (26.5)
Mixed-race	147 (56.5)
Whites	42 (16.2)
Yellows	—
Indigenous	2 (0.8)
Origin	
Urban areas	234 (87.6)
Rural areas	33 (12.4)
Age at diagnosis (in years)	
Average	39.4; SD = 13.5
Range	11–78
Interval between symptom onset and diagnosis (in months)	
Average	20.36; SD = 31.3
Range	0–180
Mean disease duration (in years)	
Average	7.56; SD = 6.3
Range	0–34
Smoking	
Active	8 (3.0)
Former	92 (34.5)
No	167 (62.5)
Family history of IBD	
Yes	21 (7.9)
No	245 (92.1)

SD = standard deviation. IBD = inflammatory bowel disease.

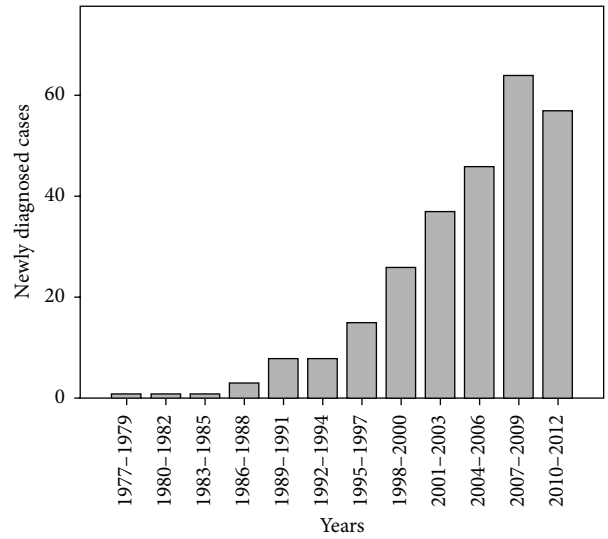


Figure 1: Distribution of patients according to the year of diagnosis of ulcerative colitis in two reference centers in Salvador, Bahia, Brazil (1977–2012).

the patients are shown in Table 1. Figure 1 shows the distribution of patients according to the year of diagnosis of ulcerative colitis in two reference centers in Salvador, Brazil.

Because colonoscopies were not performed, it was not possible to determine the extent of disease of 14 (5.2%) patients. There was a slight predominance of left colitis in the study population (42.7%). Among the symptoms presented at the time of diagnosis, the elimination of blood in the stool was the most frequent (74.8%). The clinical characteristics of the patients are shown in Table 2.

The Poisson regression model revealed a positive association between extensive colitis and the following factors: age at the time of diagnosis less than 30 years, male, diarrhea, and weight loss at the time of diagnosis. Individuals who had been diagnosed with UC prior to the age of 30 years had a prevalence of extensive colitis that was 58% higher than that observed in individuals diagnosed with UC when they were over 30 years of age. Male patients had a prevalence of extensive colitis that was 33% higher than females. The group that exhibited diarrhea at the time of diagnosis had a 67% increased prevalence of extensive colitis, and patients exhibiting weight loss had a prevalence of extensive colitis that was 51% higher than patients without this symptom. In contrast, active smoking and a family history of IBD were negatively associated with the occurrence of extensive colitis (Table 3).

In the Poisson regression model, systemic corticosteroid therapy was positively associated with the following variables: diarrhea and weight loss at the time of diagnosis, the occurrence of EIMs, and the need for hospitalization. Conversely, a smaller proportion of patients from rural areas used steroids (Table 4).

The factors associated with azathioprine usage were also evaluated using multivariate Poisson regression analysis. Being under 30 at the time of diagnosis, weight loss early in the disease, family history of IBD, extensive colitis, EIMs, previous hospitalization, and the use of corticosteroids were all shown to be positively associated with the use of azathioprine. In contrast, living in rural areas, active smoking, identifying as white, and rectal bleeding at the time of diagnosis were all shown to be protective factors against the use of immunosuppressive drugs (Table 5). On account of the numerical imbalance caused by the existence of only two cases of azathioprine usage in patients with diarrhea who had not previously used corticosteroids and due to a lack of individuals who used both azathioprine and corticosteroids but did not have diarrhea, diarrhea was not included as a variable in the Poisson regression model.

Due to the small number of patients treated surgically, it was not possible to perform a multivariate regression analysis to assess the factors associated with colectomy.

Table 2: Clinical characteristics of patients with ulcerative colitis in two reference centers in Salvador, Bahia, Brazil (n = 267).

Variables	n (%)
Extension according to the Montreal classification	
Proctitis	41 (16.2)
Left colitis	108 (42.7)
Extensive colitis	104 (41.1)
Predominant symptoms at diagnosis	
Blood in stool	199 (74.8)
Diarrhea	143 (53.8)
Abdominal pain	97 (36.5)
Weight loss	26 (9.8)
Extraintestinal manifestations	
Joint	93 (34.8)
Cutaneous	9 (3.4)
Hepatobiliary	6 (2.2)
Eye	2 (0.7)
Need for hospitalization	
Yes	117 (43.8)
No	150 (56.2)
Use of azathioprine	
Yes	52 (19.5)
No	215 (80.5)
Use of corticosteroids	
Yes	167 (62.8)
No	99 (37.2)
Biological therapy	
Yes	4 (1.5)
No	263 (98.5)
Colectomy	
Yes	9 (3.4)
No	258 (96.6)

Table 3: Poisson regression model of the factors associated with extensive colitis in ulcerative colitis patients in two reference centers in Salvador, Brazil.

Variables	Initial model		Final model	
	PRadj**	Coefficent*	PRadj**	Coefficent*
(Intercept)		−1.17		−1.39
Age (years)		0.45		0.45
<30	1.56		1.58	
≥30	1		1	
Gender		0.29		0.28
Male	1.34		1.33	
Female	1		1	
Family history of IBD		−0.33		−0.33
Yes	0.71		0.71	
No	1		1	
Weight loss		0.49		0.51
Yes	1.64		1.67	
No	1		1	
Diarrhea		0.38		0.41
Yes	1.46		1.51	
No	1		1	
Active smoking		−1.20		−1.17
Yes	0.29		0.30	
No	1		1	
Origin		−0.03		—
Rural areas	0.96		—	
Urban areas	1		—	
White		−0.04		—
Yes	0.95		—	
No	1		—	
Blood in stools		−0.07		—
Yes	0.93		—	
No	1		—	
EIMs		−0.01		—
Yes	0.99		—	
No	1		—	

Note: Akaike information criterion (AIC): 383.
*Coefficent from Poisson regression model.
**Adjusted prevalence ratio from the Poisson regression model.
EIMs = extraintestinal manifestations.
IBD = inflammatory bowel disease.

4. Discussion

This is the first study to evaluate clinical and demographic characteristics in patients with UC in Bahia, a state in northeastern Brazil. In general, the phenotypic characteristics of UC observed in our population are similar to those of other published studies. Our results help to reinforce concepts already well established, such as the association between extensive colitis and the diagnosis of UC at younger age and the protection conferred by smoking against more severe forms of UC. Because of the importance that is currently attributed to the influence of environment and genetics on the pathogenesis of IBD and the relationships of these factors with the natural course of the disease, we emphasize that our results reveal a lower prevalence of the use of steroids and immunosuppressants in patients from rural areas and a lower use of immunosuppressants by the white population. These results encourage us to attempt to discover the precise genetic and environmental factors that impact UC in these populations.

Although some studies, especially those conducted in high incidence areas of IBD, have reported a higher prevalence of IBD in men or an equal distribution between the genders [14–17], approximately two-thirds of our patients were female. Similar results have also been observed in studies of Brazilian and Portuguese patients [4, 18].

Our study population was primarily composed of nonwhites. It was observed that the prevalence of azathioprine usage in white patients was 44% lower than in nonwhites. This

Table 4: Poisson regression model of the factors associated with systemic corticosteroid therapy in patients with UC in two reference centers in Salvador, Brazil.

Variables	Initial model		Final model	
	PRadj**	Coefficent*	PRadj**	Coefficent*
(Intercept)				−0.98
Weight loss		0.26		0.25
Yes	1.30		1.29	
No	1		1	
Diarrhea		0.20		0.24
Yes	1.23		1.27	
No	1		1	
EIMs		0.29		0.28
Yes	1.34		1.32	
No	1		1	
Origin		−0.28		−0.29
Rural areas	0.75		0.74	
Urban areas	1		1	
Need for hospitalization		0.57		0.51
Yes	1.78		1.67	
No	1		1	
Age (years)		0.11		—
<30	1.12		—	
≥30	1		—	
Gender		−0.19		—
Male	0.82		—	
Female	1		—	
Blood in stool		0.03		—
Yes	1.04		—	
No	1		—	
Active smoking		0.18		—
Yes	1.21		—	
No	1		—	
Family history of IBD		0.002		—
Yes	1.0		—	
No	1		—	
White		0.06		—
Yes	1.06		—	
No	1		—	
Extensive colitis		0.16		—
Yes	1.18		—	
No	1		—	

Note: Akaike information criterion (AIC): 479.2.
*Coefficent from Poisson regression model.
**Adjusted prevalence ratio from the Poisson regression model.
EIMs = extraintestinal manifestations.
IBD = inflammatory bowel disease.

Table 5: Poisson regression model of the factors associated with the use of azathioprine in patients with UC in two reference centers in Salvador, Brazil.

Variables	Initial model		Final model	
	PRadj**	Coefficient*	PRadj**	Coefficient*
(Intercept)		−3.34		−3.92
Age (years)		0.54		0.55
<30	1.72		1.73	
≥30	1		1	
Origin		−0.69		−0.66
Rural areas	0.50		0.51	
Urban areas	1		1	
Family history of IBD		0.24		0.23
Yes	1.27		1.26	
No	1		1	
White		−0.58		−0.56
Yes	0.55		0.56	
No	1		1	
Active smoking		−0.26		−0.28
Yes	0.76		0.75	
No	1		1	
Blood in stool		−0.32		−0.33
Yes	0.72		0.71	
No	1		1	
Weight loss		0.37		0.37
Yes	1.45		1.45	
No	1		1	
EIM		0.24		0.22
Yes	1.27		1.25	
No	1		1	
Extensive colitis		0.33		0.34
Yes	1.40		1.41	
No	1		1	
Need for hospitalization		0.53		0.56
Yes	1.71		1.75	
No	1		1	
Use of corticosteroids		2.09		2.07
Yes	8.10		7.96	
No	1		1	
Gender		0.12		—
Male	1.13		—	
Female	1		—	
Diarrhea†		—		—
Yes	1.37		—	
No	1		—	

Note: Akaike information criterion (AIC): 221.6.
†Gross prevalence ratio.
*Coefficient from the Poisson regression model.
**Adjusted prevalence ratio from the Poisson regression model.
EIM = extraintestinal manifestations.
IBD = inflammatory bowel disease.

result suggests that nonwhite patients require more intensive therapy over time, at least in our environment. In contrast, a systematic review by Mahid and colleagues revealed no significant differences in the phenotypic presentation and

severity of IBD between groups of white and black Americans [19].

The percentage of UC patients with a family history of IBD is variable and appears to be higher in Caucasians and in countries with the highest incidence of UC. In a study of the US population, 14.6% of UC patients had a family history of IBD [14]. In contrast, this rate is less than 2% in eastern populations [20]. Compared to these studies, our study identified an intermediate percentage of UC patients with a family history of IBD (7.9%), which is similar to the results described by Portela et al. for the Portuguese population (7.2%) [21]. The lower occurrence of family history and increased incidence of UC in less developed countries in recent decades suggests that the populations in these countries may have increased exposure to environmental triggers of IBD. Our results indicate that the use of immunosuppression is 26% higher in patients with a family history of IBD. In a prospective study, Henriksen et al. found no significant differences in drug therapy or indications for colectomy in the groups with or without a family history of IBD [22].

Extensive colitis and a greater need for azathioprine were both more common in individuals who were less than 30 years old at the time of disease diagnosis. In an Italian study of 1705 UC patients, younger patients were observed to present with more severe disease evolution and a greater need for more intensive treatment [23]. The IBSEN study showed that the risk of colectomy was higher in individuals diagnosed with UC before the age of 50 [24]. Despite these publications, there is no consensus regarding the influence of age at the time of diagnosis in the natural history of UC.

The extent of UC is a factor that influences disease progression and the long-term prognosis for patients [14, 25]. Solberg et al. followed patients with UC for 10 years and noted that individuals with extensive colitis showed an increased need for colectomy [24]. Barreiro-de Acosta et al. demonstrated that extensive colitis is a factor associated with an increased need for immunosuppression and biological therapy [18]. In our study, the use of azathioprine was 41% higher in patients with extensive colitis.

In our study, the occurrence of weight loss, diarrhea, and EIMs at the time of diagnosis and a greater need for hospitalization were variables significantly associated with the use of corticosteroids. However, there have been no publications with the main objective of describing the factors that are associated with the use of corticosteroids in UC patients.

The use of azathioprine in UC is indicated in patients who are steroid dependent and in patients who are refractory to aminosalicylates [12]. The identification of factors associated with the use of thiopurines has not been extensively explored, but it has the potential to help predict the patients who are likely to develop severe UC and thus require more intensive therapy. In our study, a positive association was observed between the use of azathioprine and several other factors, including receiving an initial UC diagnosis when under the age of 30, weight loss at the time of diagnosis, EIMs, a family history of IBD, extensive colitis, hospitalization, and a history of systemic corticosteroid therapy. In contrast, living in rural areas was negatively associated with azathioprine use. In our state, patients living in rural and urban areas have similar access to thiopurines through a government program. A Canadian study published in 2008 also revealed an association between the use of azathioprine and the extent of UC [26].

Unlike Crohn's disease, smoking has been recognized as a protective factor against the development of UC [27]. Moreover, among patients with UC, those who do not smoke exhibit a worse disease prognosis [28]. Our results demonstrate that active smoking is associated with a lower prevalence of extensive colitis (PR: 0.30) and less use of immunosuppression (PR: 0.75) in our study population.

Individuals residing in urban areas have a higher risk of developing UC compared to those living in rural areas [29]. In our study, 87.6% of the study sample lived in urban areas and only 12.4% lived in rural areas. In contrast, according to IBGE data, approximately 28% of the population of Bahia lives in rural areas [30]. It is possible that this lower percentage of UC patients from rural areas compared to the total percentage of individuals from rural areas in the general population may result from decreased exposure to environmental factors involved in the pathophysiology of IBD. Furthermore, our results showed that living in a rural area was associated with a decreased need for corticosteroids and immunosuppression. Consistent with this, a study by Ananthakrishnan et al. concluded that UC patients from areas with high levels of pollutant emissions have an increased risk of hospitalization (RR 1.48; 95% CI: 1.27–1.73) [31].

In our study, the presence of EIMs was associated with an increased use of steroids (PR: 1.32) and azathioprine (PR: 1.25). At least one previous study concluded that the presence of EIMs is associated with a greater extent of disease [32]. In addition, a recent study described an association between the presence of EIMs and an increased rate of colectomy in the pediatric population [33].

The colectomy rates reported in published studies have been highly variable, mainly due to the year and the geographical area in which they were performed. In Western countries, colectomy rate has been reduced over time. Recent studies indicate a colectomy rate around 10% [24, 34, 35]. The increased use of immunosuppressants and anti-TNF has been implicated as responsible for this decline [36]. The colectomy rate found in our population was 3.4%. In a retrospective study, a similar result was described by Park et al., who reported cumulative colectomy rates of 2%, 2.8%, and 3.3% at 1, 3, and 5–15 years, respectively [37]. In our study, environmental factors and a higher rate of using immunosuppressive drugs may have contributed to this low colectomy rate. However, because this is a cross-sectional study, it is possible that this rate may be underestimated due to the loss of follow-up of patients who may have eventually undergone colectomies.

In conclusion, the occurrence of UC in patients who were receiving specialized IBD treatment at centers in the state of Bahia increased over time. There was a predominance of females and left-side colitis in the present sample, but in general, the clinical characteristics of UC patients in Bahia are similar to those reported in the literature. Our results also corroborate the higher prevalence of extensive

colitis in patients who were diagnosed at a younger age. Association between steroid therapy and clinical presentation at diagnosis was observed. Several of our results, including the negative association between living in rural areas and the use of azathioprine or systemic corticosteroids and the reduced incidence of azathioprine use by the white subgroup, highlight the need for additional studies examining the effects of environmental and genetic factors on the progression of UC in our study population.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] C. G. Loftus, E. V. Loftus Jr., W. S. Harmsen et al., "Update on the incidence and prevalence of Crohn's disease and ulcerative colitis in Olmsted County, Minnesota, 1940–2000," *Inflammatory Bowel Diseases*, vol. 13, no. 3, pp. 254–261, 2007.

[2] E. V. Loftus Jr., "Clinical epidemiology of inflammatory bowel disease: incidence, prevalence, and environmental influences," *Gastroenterology*, vol. 126, no. 6, pp. 1504–1517, 2004.

[3] C. R. Victoria, L. Y. Sassaki, and H. R. D. C. Nunes, "Incidence and prevalence rates of inflammatory bowel diseases, in midwestern of São Paulo State, Brazil," *Arquivos de Gastroenterologia*, vol. 46, no. 1, pp. 20–25, 2009.

[4] M. H. L. P. Souza, L. E. D. A. Troncon, C. M. Rodrigues et al., "Trends in the occurrence (1980–1999) and clinical features of Crohn's disease and ulcerative colitis in a university hospital in southeastern Brazil," *Arquivos de Gastroenterologia*, vol. 39, no. 2, pp. 98–105, 2002.

[5] C. S. Gismera and B. S. Aladrén, "Inflammatory bowel diseases: a disease (s) of modern times? Is incidence still increasing?" *World Journal of Gastroenterology*, vol. 14, no. 36, pp. 5491–5498, 2008.

[6] S. H. Wong and S. C. Ng, "What can we learn from inflammatory bowel disease in developing countries?" *Current Gastroenterology Reports*, vol. 15, no. 3, 2013.

[7] X.-L. Jiang and H.-F. Cui, "An analysis of 10218 ulcerative colitis cases in China," *World Journal of Gastroenterology*, vol. 8, no. 1, pp. 158–161, 2002.

[8] W. Gong, N. Lv, B. Wang et al., "Risk of ulcerative colitis-associated colorectal cancer in China: a multi-center retrospective study," *Digestive Diseases and Sciences*, vol. 57, no. 2, pp. 503–507, 2012.

[9] A. Dignass, R. Eliakim, F. Magro et al., "Second European evidence-based consensus on the diagnosis and management of ulcerative colitis part 1: definitions and diagnosis," *Journal of Crohn's and Colitis*, vol. 6, no. 10, pp. 965–990, 2012.

[10] M. S. Silverberg, J. Satsangi, T. Ahmad et al., "Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: report of a Working Party of the 2005 Montreal World Congress of Gastroenterology," *Canadian Journal of Gastroenterology*, vol. 19, pp. 5A–36A, 2005.

[11] Instituto Brasileiro de Geografia e Estatística, *Características Étnico-Raciais da População*, Instituto Brasileiro de Geografia e Estatística, 2008, http://www.ibge.gov.br/home/estatistica/populacao/caracteristicas_raciais/notas_tecnicas.pdf.

[12] A. Dignass, J. O. Lindsay, A. Sturm et al., "Second European evidence-based consensus on the diagnosis and management of ulcerative colitis part 2: current management," *Journal of Crohn's and Colitis*, vol. 6, no. 10, pp. 991–1030, 2012.

[13] D. A. Ludwig, "Use and misuse of p-values in designed and observational studies: guide for researchers and reviewers," *Aviation Space and Environmental Medicine*, vol. 76, no. 7, pp. 675–680, 2005.

[14] R. G. Farmer, K. A. Easley, and G. B. Rankin, "Clinical patterns, natural history, and progression of ulcerative colitis. a long-term follow-up of 1116 patients," *Digestive Diseases and Sciences*, vol. 38, no. 6, pp. 1137–1146, 1993.

[15] F. Molinié, C. Gower-Rousseau, T. Yzet et al., "Opposite evolution in incidence of Crohn's disease and ulcerative colitis in Northern France (1988–1999)," *Gut*, vol. 53, no. 6, pp. 843–848, 2004.

[16] C. N. Bernstein, A. Wajda, L. W. Svenson et al., "The epidemiology of inflammatory bowel disease in Canada: a population-based study," *The American Journal of Gastroenterology*, vol. 101, no. 7, pp. 1559–1568, 2006.

[17] C. Gower-Rousseau, F. Vasseur, M. Fumery et al., "Epidemiology of inflammatory bowel diseases: new insights from a French population-based registry (EPIMAD)," *Digestive and Liver Disease*, vol. 45, no. 2, pp. 89–94, 2013.

[18] M. Barreiro-de Acosta, F. Magro, D. Carpio et al., "Ulcerative colitis in northern Portugal and Galicia in Spain," *Inflammatory Bowel Diseases*, vol. 16, no. 7, pp. 1227–1238, 2010.

[19] S. S. Mahid, A. M. Mulhall, R. D. Gholson, M. R. Eichenberger, and S. Galandiuk, "Inflammatory bowel disease and African Americans: a systematic review," *Inflammatory Bowel Diseases*, vol. 14, no. 7, pp. 960–967, 2008.

[20] S. Wei, M. Shieh, M. Chang, Y. Chang, C. Wang, and J. Wong, "Long-term follow-up of ulcerative colitis in Taiwan," *Journal of the Chinese Medical Association*, vol. 75, pp. 151–155, 2012.

[21] F. Portela, F. Magro, P. Lago et al., "Ulcerative colitis in a southern european country: a national perspective," *Inflammatory Bowel Diseases*, vol. 16, no. 5, pp. 822–829, 2010.

[22] M. Henriksen, J. Jahnsen, I. Lygren, M. H. Vatn, and B. Moum, "Are there any differences in phenotype or disease course between familial and sporadic cases of inflammatory bowel disease? Results of a population-based follow-up study," *The American Journal of Gastroenterology*, vol. 102, no. 9, pp. 1955–1963, 2007.

[23] G. Riegler, M. T. Tartaglione, R. Carratú et al., "Age-related clinical severity at diagnosis in 1705 patients with ulcerative colitis: a study by GISC (Italian Colon-Rectum Study Group)," *Digestive Diseases and Sciences*, vol. 45, no. 3, pp. 462–465, 2000.

[24] I. C. Solberg, I. Lygren, J. Jahnsen et al., "Clinical course during the first 10 years of ulcerative colitis: results from a population-based inception cohort (IBSEN Study)," *Scandinavian Journal of Gastroenterology*, vol. 44, no. 4, pp. 431–440, 2009.

[25] L. S. Kiss and P. L. Lakatos, "Natural history of ulcerative colitis: current knowledge," *Current Drug Targets*, vol. 12, no. 10, pp. 1390–1395, 2011.

[26] A. Lau, N. Chande, T. Ponich, and J. C. Gregor, "Predictive factors associated with immunosuppressive agent use in ulcerative colitis: a case-control study," *Alimentary Pharmacology and Therapeutics*, vol. 28, no. 5, pp. 606–613, 2008.

[27] B. M. Calkins, "A meta-analysis of the role of smoking in inflammatory bowel disease," *Digestive Diseases and Sciences*, vol. 34, no. 12, pp. 1841–1854, 1989.

[28] H. S. Odes, A. Fich, S. Reif et al., "Effects of current cigarette smoking on clinical course of Crohn's disease and ulcerative colitis," *Digestive Diseases and Sciences*, vol. 46, no. 8, pp. 1717–1721, 2001.

[29] I. S. Soon, N. A. Molodecky, D. M. Rabi, W. A. Ghali, H. W. Barkema, and G. G. Kaplan, "The relationship between urban environment and the inflammatory bowel diseases: a systematic review and meta-analysis," *BMC Gastroenterology*, vol. 12, article 51, 2012.

[30] Instituto Brasileiro de Geografia e Estatística, *Distribuição Percentual da População nos Censos Demográficos, Segundo as Grandes Regiões, as Unidades da Federação e a Situação do Domicílio—1960/2010*, Instituto Brasileiro de Geografia e Estatística, 2011, http://www.censo2010.ibge.gov.br/sinopse/index.php?dados=9&uf=00.

[31] A. N. Ananthakrishnan, E. L. McGinley, D. G. Binion, and K. Saeian, "Ambient air pollution correlates with hospitalizations for inflammatory bowel disease: an ecologic analysis," *Inflammatory Bowel Diseases*, vol. 17, no. 5, pp. 1138–1145, 2011.

[32] L. Lakatos, T. Pandur, G. David et al., "Association of extraintestinal manifestations of inflammatory bowel disease in a province of western Hungary with disease phenotype: results of a 25-year follow-up study," *World Journal of Gastroenterology*, vol. 9, no. 10, pp. 2300–2307, 2003.

[33] C. Gower-Rousseau, L. Dauchet, G. Vernier-Massouille et al., "The natural history of pediatric ulcerative colitis: a population-based cohort study," *American Journal of Gastroenterology*, vol. 104, no. 8, pp. 2080–2088, 2009.

[34] O. Hoie, F. L. Wolters, L. Riis et al., "Low colectomy rates in ulcerative colitis in an unselected European cohort followed for 10 years," *Gastroenterology*, vol. 132, no. 2, pp. 507–515, 2007.

[35] L. E. Targownik, H. Singh, Z. Nugent, and C. N. Bernstein, "The epidemiology of colectomy in ulcerative colitis: results from a population-based cohort," *The American Journal of Gastroenterology*, vol. 107, no. 8, pp. 1228–1235, 2012.

[36] G. G. Kaplan, C. H. Seow, S. Ghosh et al., "Decreasing colectomy rates for ulcerative colitis: a population-based time trend study," *The American Journal of Gastroenterology*, vol. 107, no. 12, pp. 1879–1887, 2012.

[37] S. H. Park, Y. M. Kim, S. Yang et al., "Clinical features and natural history of ulcerative colitis in Korea," *Inflammatory Bowel Diseases*, vol. 13, no. 3, pp. 278–283, 2007.