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ABSTRACT

The WNT/β-catenin cellular network has been extensively studied in numerous diseases including inflammatory bowel disease (IBD). IBD is a condition that increases the risk of developing colorectal cancer. WIF-1 is an inhibitory protein that acts by blocking the interactions of WNT with its receptor complex, thus leading to downregulation of end products of this pathway. While WIF-1 has been characterized in several cancers, its relationship with IBD has yet to be elucidated. In this study, the expression of WIF-1 in patients with IBD was analyzed in order to provide insights into the pathophysiology and rationale for alternative therapies. Biopsies of both normal and inflamed colonic mucosa from patients with Crohn’s disease or ulcerative colitis were histologically examined for the degree of morphologic changes, immune cell infiltration and presence of WIF-1 through immunohistochemistry. No differences were observed in WIF-1 expression linked to a particular condition, but WIF-1 stain was significantly enhanced in the crypts and lamina propria as inflammation increased in biopsies from patients with both, ulcerative colitis and Crohn’s disease. These findings could give guidance to new therapeutic applications of the WNT/β-catenin system and WIF-1 in IBD.

KEYWORDS: WIF-1, WNT, inflammatory bowel disease, ulcerative colitis, Crohn’s disease

RUNNING TITLE: WIF-1 expression in IBD
INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic, debilitating disease that affects nearly 1.6 million people in the United States (Shivashankar et al., 2017) and the prevalence of IBD has been on the rise for the past several decades (Molodecky et al., 2012). It is subdivided into Crohn’s disease (CD), ulcerative colitis (UC) and indeterminate colitis (does not clinically fit Crohn’s disease or UC). While the exact cause of IBD remains to be formally defined, it is believed that a combination of a breakdown in host immune defenses, favorable genetic predisposition, and gut microbial environmental factors might cause this condition (Cho 2008; Quetglas et al., 2015). The effects of the prolonged inflammation on colonic tissue lead to extensive damage that can promote dysplasia (Wang et al., 2015) and increase the risk of malignancy (Ekbom et al., 1990).

Therapeutic options for patients afflicted with IBD range from aminosalicylates and corticosteroids to immune modulating agents, biologics and monoclonal antibodies targeting specific pathways in the pro-inflammatory cascade. This has allowed for a personalized approach to disease management, but identifying a common initiator of pathogenesis is still being extensively investigated. It has been proposed that finding biomarkers to assess specific aspects of IBD could revolutionize treatment considerations and approach (Quetglas et al., 2015). While progress has been made, it has been limited to monitoring clinical course and identifying relapses (Iskandar and Ciorba, 2012).

The WNT/β-catenin signaling pathway regulates numerous aspects of cell proliferation, differentiation, and movement (Komiya and Habas, 2008). It has also emerged as a widely studied system in multiple autoimmune diseases, and is significantly altered in the pathophysiology of IBD (Shi et al., 2016). This pathway is triggered in early stages of colitis-associated carcinogenesis, eventually leading to high concentrations of β-catenin.
detectable in patient serum with more severe disease (Claessen et al., 2010). Several drugs have been developed to stunt this pathway in order to combat inflammation and several cancers (Urakami et al., 2006; Yao et al., 2011; Duchartre et al., 2015; Vilchez et al., 2016).

WIF-1, a WNT inhibitor protein has recently become an area of focus in carcinogenesis. It binds to WNT proteins directly (Hsieh et al., 1999), affecting binding to the receptor complex and thus disrupting both canonical and noncanonical downstream pathways (Kawano and Kypta, 2003). It has been seen in low levels in patients with cervical cancer showing high expression of β-catenin (Liang et al., 2016). Methylation of WIF-1 led to mutations in APC genes in IBD-associated neoplastic development (Dhir et al., 2008). This methylation is more likely to be detected in non-small cell lung cancer (Guo et al., 2017) and colorectal cancers (Silva et al., 2014; Zhang et al., 2014). Interestingly, WIF-1 has been found to be upregulated in the model of colitis-associated cancer with azoxymethane (AOM) and dextran sulfate sodium (DSS) (Suzuki et al., 2007; Lopez-Dee et al., 2015) using gene microarray technology. High expression of WIF-1 was also observed in a model of benzopyrene and DSS (Koyama et al., 2015). Other inhibitory genes were also found to affect the WNT-pathway in patients with UC (You et al., 2007). All these results indicate that WIF-1 may be useful to evaluate the progression and prognosis of IBD, although its relationship to inflammation remains uncertain.

In this study, the expression of WIF-1 in colonic biopsies of patients with IBD was investigated. This study aimed to determine whether the level of expression of WIF-1 is correlated with the severity of mucosal inflammation and clinical features. These results may contribute to determining a role of WIF-1 in IBD and provide a rationale for new therapeutic alternatives for this disease.
MATERIALS AND METHODS

Biopsy Collection and Clinical Parameters

The Wilkes University IRB committee reviewed and approved the research protocols prior to the study onset. Patients diagnosed with IBD were scheduled for colonoscopies to identify pre-malignant dysplasia or for assessment of disease activity if the patient was symptomatic. Upon arrival to the clinic (Gastrointestinal Consultants of Northeastern Pennsylvania, Scranton, PA) patients were approached for participation in the study. Written informed consent was read and signed by each patient before obtaining colonic biopsies. Patients without the diagnosis of IBD were not asked to participate. Recorded information about the patients included age, gender, BMI, IBD diagnosis, and family history. Patients were not advised to change their current treatment regimen prior to the study, or prepare for the colonoscopy in any other way other than what their physician instructed them to do. During the procedure, signs of active macroscopic inflammation as loss of vascularity, tissue granularity, friability and mucosal erosion and bleeding were screened (Clark and Turner, 2015). Two biopsies were obtained from normal appearing colonic mucosa and two biopsies, if evident, from inflamed colonic mucosa for the research study purpose. The samples were coded and fixed in Histochoice™mb® (Electron Microscopy Sciences, Hatfield, PA) overnight, processed, and cut in 5 μm sections. Slides were re-coded before staining.

Inflammation Grading

Slides were stained with hematoxylin and eosin (H&E) to analyze and grade inflammation. Representative images of each slide were taken at 400x, randomized and blindly evaluated. Images were graded using a previously published method (Lopez-Dee et al., 2012) with some modifications. The inflammation was graded according to the intensity of leukocytic
infiltration and cryptitis, severity of the mucosal hyperplasia, signs of crypt damage, decrease of goblet cells and presence of granulomas and lymphoid aggregates. (Roger and Feakins, 2013). Biopsies were numerically scored as 0-1.4 (in the range of normal/mild inflammation), 1.5-2.4 (moderate inflammation) and samples showing severe inflammation were scored as 2.5-3.

**Immunohistochemistry (IHC) and Scoring**

Staining for WIF-1 was performed using a mouse polyclonal primary antibody (1:100 dilution) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), followed by an anti-mouse secondary antibody using an ImmPRESS™ HRP Anti-Mouse IgG (peroxidase) Polymer Detection Kit (Vector Laboratories, Burlingame, CA, USA). β-catenin staining was performed using a rabbit polyclonal antibody (ScyTek Laboratories, UT, USA) followed by ImmPRESS™ HRP Anti-Rabbit IgG (peroxidase) Polymer Detection Kit (Vector Laboratories). Both WIF-1 and β-catenin antibodies complexes were marked with 3-3′-diaminobenzidine as chromogen by using the ImmPACT™ DAB Peroxidase Substrate Kit (Vector Laboratories), according to the manufacturer’s recommendations. Pictures of these slides were coded and evaluated by double blind analysis. IMH staining in the crypts and stroma was scored using a semiquantitative method recording the intensity and the distribution of specific staining. From each 100X field, a percentage of positive cells was recorded as follows: negative staining for WIF-1 was scored as 0, weak or light as 1, moderate as 2, and intense as 3. A total score (H-score) was obtained by summing the percentage of stained cells at each intensity multiplied by the intensity of the staining. (Bacus et al., 1988). Evaluations of the β-catenin stained biopsies were performed using the same semiquantitative procedure and recorded separately according to the localization of the staining (membranous, cytoplasmic, and nuclear staining).
**Statistical Analysis**

Statistical analysis was performed with nonparametric ANOVA and Fisher’s test.

Correlations between WIF-1 H-scores and inflammation grades were analyzed using the Fisher transformed Pearson’s correlations. The calculations were performed using the StatView system for Macintosh. (Abacus Concepts, Berkeley, CA). p < 0.05 values were considered significant.
3. RESULTS

Patients Cohort

Clinical histories of patients with IBD were obtained prior to maintenance colonoscopy (Table 1). The patients for this study were stratified based on their IBD diagnosis. Of the 32 patients, 24 patients had a diagnosis of UC, 7 patients had CD, and 1 patient had indeterminate colitis. The population was 62.5% male with a mean age of 54.8 years (range: 26-83 years).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Patients: n = 32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (%)</td>
<td>20 (62.5)</td>
</tr>
<tr>
<td>Patients with ulcerative colitis (%)</td>
<td>24* (75)</td>
</tr>
<tr>
<td>Mean age, years (range)</td>
<td>54.8 (26-83)</td>
</tr>
<tr>
<td>Mean BMI, kg/m² (range)</td>
<td>27.2 (19.6-51.7)</td>
</tr>
<tr>
<td>Positive Family History (%)</td>
<td>4 (12.5)</td>
</tr>
<tr>
<td>Patients on DMARD therapy (%)</td>
<td>16 (50)</td>
</tr>
</tbody>
</table>

*1 patient has indeterminate colitis

Table 1. Demographic data: Various clinical parameters of patients with IBD were taken before maintenance colonoscopy. The list included age, gender, IBD subtype, BMI, family history, and treatment modality.

Inflammation does not correlate with endoscopic changes IBD samples

Biopsies of colonic mucosa (n=56) from patients with IBD were examined to grade the histologic status of inflammation (Figure 1A, 1C and 1E). Analyses were performed separately in normal or grossly inflamed tissue as reported by the endoscopist. In the samples with the diagnosis of UC, differences in the histologic grade of inflammation between appearing normal (n = 21; mean = 1.18 +/- 0.13 SEM) and inflamed mucosa (n = 14; mean = 1.27 +/- 0.21 SEM) were minimal. The differences in the grades of inflammation between normal (n = 5; mean = 0.15 +/- 0.1 SEM) and inflamed (n = 6; mean = 1.08 +/- 0.47 SEM) mucosa in CD patients, however were notable but not statistical differences were observed when they were classified according to the endoscopic findings.
Biopsies were re-classified according to the histological inflammation by using a numerical range of 0-1.4 (when the biopsy was normal or showing mild inflammation) (n=16), graded as 1.5-2.4 (when the biopsy showed moderate inflammation) (n=27) and samples showing severe inflammation were assigned with scores of 2.5-3 (n=13). When the histologic grade of inflammation was correlated with the type of disease, no statistical significance was observed regarding the grade of inflammation between biopsies obtained from patients with UC and patients with CD. However, patients with UC showed a trend for highest grades of inflammation (p=0.5584) (Figure 2A)

**WIF-1 expression correlates with the grade of inflammation in both UC and CD**

The mucosal biopsies were examined for expression of WIF-1 through IHC (Figure 1B, 1D and 1F). Biopsies with mild inflammation showed a light to moderate cytoplasmic staining in the luminal epithelium and some cells of the colonic crypts. Most of the leukocytes were negative for WIF-1. When severe inflammation was present sometimes causing complete disruption of the glands in the mucosa, leukocytes and remnant epithelial cells showed a very intense expression for WIF-1. Endothelial cells displayed intense WIF-1 expression in the submucosa as well.

WIF-1 staining in the crypts was not related with the type of condition, UC or CD (p=0.5584) (Figure 2B). Same results were observed when WIF-1 was evaluated in the stroma (p=0.7257) (Figure 2C).

When WIF-1 expression in all the biopsies was compared with the degree of mucosa integrity and severity of the inflammation a significant correlation was observed in the crypts (correlation: 0.367; p=0.0055) (Figure 3A). WIF-1 expression in the lamina propria showed a stronger correlation in all the samples as well (correlation: 0.238; p = 0.0797) (Figure 3B).
Biopsies showing regenerative changes such as chronic inflammation with irregular shaped glands and glandular budding displayed focal and intense nuclear expression of WIF-1 (Figure 4A-4E). This feature was observed in just 25% of the biopsies and was not related with inflammation grade, WIF-1 Hscores, presence of symptoms or family history (data not shown). However, biopsies of patients treated with immunomodulators plus corticosteroids (CI) showed highest percentages of nuclear staining (p=0.0828), (Figure 4G)

No relationship was observed between WIF-1 expression and the presence or absence of symptoms or family medical history (data not shown). No statistical differences were observed among the types of treatment. However, patients receiving biologic agents (B) showed a trend for lowest WIF-1 Hscores in the stroma comparing with the ones in biopsies from patients receiving immunosuppressing and corticosteroids (p=0.1200) or aminosalicylates (p=0.1977) (Figure 5)

**β-catenin expression is increased in IBD colonic biopsies**

β-catenin overexpression was localized in the cytoplasmic. No nuclear staining was detected in any of the biopsies even in the ones displaying the highest grades of inflammation. These results concur with previous reports showing that nuclear staining is rarely observed in UC biopsies and colitis models (Randall-Demllo S, et al 2016). UC biopsies showed significant higher H-scores of cytoplasmic β-catenin when compared to CD biopsies (p=0.0282) (Figure 2D). These results underscore the relevance of β-catenin in the pathophysiology of UC (Soleti et al., 2013). Interestingly, H-scores of membranous β-catenin were correlated with inflammation grade in CD patients (correlation=0.852, p value=0.0047) (data not shown). No correlation between WIF-1 (stromal or epithelial) H-scores and β-catenin were detected.
DISCUSSION

The WNT/β-catenin pathway has been extensively studied in normal cellular physiology, inflammation and neoplastic conditions. The inhibitory factor WIF-1 has been examined for its role in cancer, but investigations into its involvement with pre-malignant conditions, such as IBD, were limited. This study aims to correlate the degree of inflammation with the expression of WIF-1 in the colonic mucosa of patients with IBD. We have found that in this small group of patients, WIF-1 expression was significantly greater in inflamed mucosa in both the glands and leukocytes of the lamina propria compared to unaffected colonic specimens. These results confirm observations by Koyama and colleagues (2015), where they noted significant upregulation in WIF-1 in the BP/DSS mouse model of colitis and rapid tumorigenesis.

During our analysis, we noted that the histological differences between normal and inflamed colon mucosa were clearly seen with CD patients, but was very limited in patients suffering from UC. This discrepancy could be explained by the occurrence of what is called patchiness, which could be present to varying degrees on endoscopic and gross inspection, as well as histologic examinations (Bernstein et al., 1995; Kleer and Appelman, 1998; Kim et al., 1999; Joo and Odze, 2010). While a patchy involvement of the colon suggests CD, contiguous areas of the colon are affected in UC. Furthermore, a grossly inflamed mucosa in a patient with an active disease could be quite noticeable. Friable mucosa, hemorrhage, erosions and ulcers are clear indication of active disease. However, during the healing stage, the mucosa may appear grossly normal, but mild to moderate inflammation may still persist histologically. These are subtle microscopic changes that might be detected during the quiescent or resolving stage of the disease, further complicating the accurate diagnosis of CD or UC (Odze, 2003). Anomalies in the expression of members of the Wnt pathway in colonic
epithelial cells have been observed in biopsies of patients with IBD and animal models of colitis (Kini et al., 2015). Additionally, there are multiple conditions that can mimic IBD (Yantiss and Odze, 2007). In spite of this, instances where healing is noted over time (Odze, 2003; Yantiss and Odze, 2007) can provide a means of assessing the clinical course and the therapeutic response (Mazzuoli et al., 2013; Peyrin-Biroulet et al., 2014). In this study, we related the inflammatory findings in the colonic mucosa with the expression of WIF-1. Even though the differences between endoscopically normal and inflamed colon in UC were minimal, we do not feel it affected our correlations with degree of inflammation and WIF-1 expression. WIF-1 protein is seems to be expressed more as the grade of inflammation increases in these inflamed biopsies of patients regardless the condition. Therefore, WIF-1 might be a potential marker for detecting active disease and tracking remission in IBD. No statistical relationship was observed between WIF-1 Hscores and presence of symptoms or family medical history. However, a trend for highest WIF-1 expression in the stroma was observed in patients receiving corticosteroids with immunomodulators. Stromal WIF-1 may be linked to most severe cases of IBD, the ones unresponsive to most treatments.

Focal nuclear WIF-1 stain was observed in chronically inflamed tissues where glands displayed regenerative changes such as irregularity in shape, budding and branching. These reactive changes are usually manifested with enlargement of the nuclei and increasing mitotic activity. As this nuclear staining is not related with the intensity of WIF-1, we don’t believe it is a technical issue caused by increased time exposure of the chromogen. A more diffuse and intense staining would be expected if that would be the case. We found this nuclear pattern in only 25% of the biopsies and at a range from 1% to 23% cryptic cells per sample. Mitotic figures were detected in those focal areas with nuclear staining, suggesting that WIF-1 might be involved in mitosis and proliferation. Nuclear WIF-1 expression was detected in cells undergoing atypical mitosis in cervical cancers as well (Liang et al., 2016). A translocation of
WIF-1 is probably as elements of the nuclear T-cell factor/lymphoid enhancer factor (TCF/LEF) are present in the WIF-1 promoter (Tang et al., 2017). In addition, WIF-1 negative regulation is not mediated by a single mechanism and the functions of each of its domains are not yet clear (Kerekes K et al. 2015).

It has been proposed that complex mechanisms outside of the WNT pathways may influence the regulation of WIF-1 and its epigenetic silencing by methylation. Elevated methylation of WIF-1 has been observed in colorectal adenomas and carcinomas compared to normal mucosa (Voorham et al., 2013). In a study with 247 patients, it was found that methylated WIF-1 detected more advanced stages of neoplasia with a higher sensitivity and specificity compared with fecal occult blood testing (Amiot et al., 2014). A miRNA identified in colorectal cancer metastases (miR-181a) binds to and inactivates WIF-1, leading to increased tumor burden and poorer prognosis independent of other clinical parameters (Ji et al., 2014). WNT signaling has been shown to be involved with regulating cyclooxygenase (Araki et al., 2003), which may provide an explanation to the improved prognostic outlook of those diagnosed with colorectal cancer taking aspirin as part of their daily medication regimen (Bains et al., 2016). There is some evidence that TGF-β regulatory mechanisms may have a role in WNT transcriptional activities by linkage with Hippo cell growth pathways (Attisano and Wrana, 2013). Notch ligands have been shown to interact with both TGF-β and WNT signaling in HCT-116 colon cancer cells (Bordonaro et al., 2011) and these anomalies are present even before inflammation become evident (kini et al., 2015).

Given that IBD is considered a pre-malignant condition, our findings may be considered contradictory to what has been observed in early events of colorectal carcinogenesis, in that WIF-1 was methylated during progression of the process (Dhir et al., 2008; Silva et al., 2014; Zhang et al., 2014). However, evidence of increased free β-catenin stabilization as a possible preceding event to tumorigenesis (Bienz and Clevers, 2000) may
provide insight into this paradigm. In addition to the proposed crosstalk previously mentioned between inflammatory pathways, tumor suppressors and oncogenes that are end products or regulators of the WNT system (Bienz and Clevers, 2000; Polakis, 2012) could contribute to the debate. Analysis of biopsies of colorectal cancers showed that WIF-1 was increasingly expressed through higher TNM staging (Huang et al., 2014). It is evident, that more studies will be needed to further elucidate the role of WIF-1 in inflammation and carcinogenesis.

H-scores of cytoplasmic β-catenin were significantly higher in UC compared with CD. These findings are not surprising as early disruption of the WNT pathway is well known in the pathophysiology of IBD. These β-catenin H-scores were consistently high in UC regardless the inflammation grade and WIF-1 scores suggesting an independent role of WIF-1 in IBD. Only membranous and/or cytoplasmic expression was detected in our samples. Translocation of β-catenin to the nucleus might be observed later in the carcinogenesis process. In addition, it has been reported that expression of β-catenin could be modified by the treatment with aminosalicylates (Munding et al., 2012). Most of the biopsies evaluated for this study were obtained during routine colonoscopy. These patients were treated and closely monitored by the gastroenterologist, thus a more aberrant expression of β-catenin may not be yet detected.

In conclusion, this study showed elevated WIF-1 expression as tissue damage and inflammation progressed in IBD. Most of the current therapies for IBD are based on suppressing and/or regulating the immune response, as well as managing symptoms and patient discomfort. This study may hopefully contribute to shed some light into the role of WIF-1 as a potential marker and therapeutic aid for IBD.
ACKNOWLEDGEMENTS

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**LEGENDS**

**Figure 1.** Inflammation grading, WIF-1 and β-catenin expression in colonic biopsies of patients with the diagnosis of IBD: Slides of colonic specimens were stained with H&E and examined for leukocytic infiltration and glandular erosion. Grading was performed via a scale based on tissue morphology and leukocytic infiltration: 0, histologically normal tissue; 1, leukocyte infiltration without breakdown of the lamina propria; 2, mild mucosal hyperplasia and signs of crypt damage; 3, extensive infiltration of leukocytes leading to destruction of goblet cells and mucosa architecture. The examples shown are grades 0.5 (A), grade 2 (B) and grade 3 (C). (H&E stain, 100x magnification). Expression of WIF-1 in biopsies of patients with IBD: IMH staining in the crypts and stroma was scored using a semiquantitative method recording the intensity and the distribution of specific staining. A total score (H-score) was obtained by summing the percentage of stained cells at each intensity multiplied by the intensity of the staining. Mild staining was recorded with low H-scores (D) a moderate cytoplasmic staining in the luminal epithelium and stroma, (B, brown color). As inflammation progressed, more intense cytoplasmic staining in more cryptal, pericryptal and stromal cells was observed (E). When severe inflammation with complete abrasion of the glands was displayed, WIF-1 staining was very intense in highest number of cells (highest H-scores recorded) (F). Expression of β-catenin in biopsies of patients with IBD; H-scores for β-catenin staining were recorded as well as the presence of membranous, cytoplasmic and nuclear staining. Biopsies showed predominantly an intense cytoplasmic staining. No nuclear staining was observed in any of the samples (G, H and I). (100x and 400x original magnification)

**Figure 2.** Analysis of the Inflammation grade by the type of disease. When the histologic grade of inflammation was related to the type of disease, no statistical significance was observed between biopsies obtained from patients with UC and patients with CD. However, patients with UC showed a trend for highest grades of inflammation (p=0.3324) (A). WIF-1 staining in the crypts was not particularly linked to the type of condition (UC or CD) (p=0.5584) (B). Same results were observed when WIF-1 expression was evaluated in the stroma (p=0.7257) (C). Cytoplasmic expression of β-catenin showed a significant increase in UC biopsies when compared to CD biopsies (p=0.0282) (D)
**Figure 3.** Correlation between Inflammation Grade and WIF-1 Expression in IBD. Correlation between inflammation grade in UC and CD biopsies and WIF-1 Hscores were evaluated separately in epithelial cells (A) and lamina propria (stroma) (B). When WIF-1 expression in all the biopsies was compared with the severity of the inflammation a significant correlation was observed in the crypts (Correlation 0.367, Z-value=2.774, p=0.0055) (A). WIF-1 expression in the lamina propria showed a lesser correlation (Correlation 0.238, Z-value=1.753, p-value= 0.0797) (B).

**Figure 4.** Nuclear staining of WIF-1 in IBD: Biopsies showing regenerative changes such as chronic inflammation with irregular shaped glands and glandular budding displayed focal and intense nuclear expression of WIF-1 (C and F, 100X original magnification); A and D show details of cryptal nuclear staining; B and E showed negative controls (400x original magnification). This feature was observed in 25% of the biopsies and it was not related with inflammation grade, WIF-1 Hscores, presence of symptoms or family history. However, biopsies of patients treated with immunomodulators plus corticosteroids (CI) showed highest percentages of nuclear staining (p=0.0828) (B). (A: aminosalicylates, B: biologic therapies, AC: aminosalicylates plus CI: immunomodulators plus corticosteroids, I: immunomodulators only, N: no treatment)

**Figure 5.** WIF-1 Hscores and type of treatment in patients with IBD: Distribution of WIF-1 scores according to the type of treatment (B). Patients under treatment with biologic agents showed a trend for lowest WIF-1 Hscores in the stroma comparing with the ones in biopsies from patients receiving immunosuppressing and corticosteroids (p=0.1200) or aminosalicylates (p=0.1977). A: aminosalicylates, B: biologic therapies, AC: aminosalicylates plus CI: immunomodulators plus corticosteroids, I: immunomodulators only, N: no treatment.