Original Article

A galectin-specific signature in the gut delineates Crohn's disease and ulcerative colitis from other human inflammatory intestinal disorders

Rodrigo Papa Gobbi[1] AQ3
Nicolás De Francesco[1]
Constanza Bondar[1]
Cecilia Muglia[1]
Fernando Chirdo[1]
Martín Rumbo[1]
Andrés Rocca[3]
Marta A. Toscano[2]
Alicia Sambuelli[3]
Gabriel A. Rabinovich[2,4†*]
Guillermo H. Docena[1†]

[1]Instituto De Estudios Inmunológicos Y Fisiopatológicos-IIFP, Departamento De Ciencias Biológicas, Facultad De Ciencias Exactas, Universidad Nacional De La Plata, La Plata, Argentina

[2]Laboratorio De Inmunopatología, Instituto De Biología Y Medicina Experimental, Consejo Nacional De Investigaciones Científicas Y Técnicas, Buenos Aires, Argentina

[3]Servicio De Enfermedades Inflamatorias, Hospital De Gastroenterología Bonorino Udaondo, Buenos Aires, Argentina

[4]Facultad De Ciencias Exactas Y Naturales, Universidad De Buenos Aires, Buenos Aires, Argentina

Abstract

Inflammatory bowel diseases (IBD) are chronic and relapsing inflammatory conditions of the gastrointestinal tract including Crohn's disease (CD) and ulcerative colitis (UC). Galectins, defined by shared consensus amino acid sequence and affinity for β-galactosides, are critical modulators of the inflammatory response. However, the relevance of the galectin network in the pathogenesis of human IBD has not yet been explored. Here, we analyzed the expression of relevant members of the galectin family in intestinal biopsies, and identified their contribution as novel mucosal markers in IBD. Colonic biopsies were obtained from 59 IBD patients (22 CD and 37 UC), 9 patients with gut rejection after transplantation, 8 adult celiac patients, and 32 non-IBD donors. Galectin mRNA expression was analyzed by RT-PCR and qPCR using specific primers for individual galectins. A linear discriminant analysis (LDA) was used to analyze galectin expression in individual intestinal samples. Expression of common mucosal-associated galectins (Gal-1, −3, −4, −9) is dysregulated in inflamed tissues of IBD patients compared with noninflamed IBD or control samples. LDA discriminated between different inflammation grades in active IBD and showed that remission IBD samples were clusterized with control samples. Galectin profiling could not distinguish CD and UC. Furthermore, inflamed IBD was discriminated from inflamed tissue of rejected gut in transplanted patients and duodenum of celiac patients, which could not be distinguished from control duodenum samples. The integrative analysis of galectins discriminated IBD from other intestinal inflammatory conditions and could be used as potential mucosal biomarker. © 2015 BioFactors, 00(00):000–000, 2015

Keywords: *inflammatory bowel diseases; galectins; Crohn disease; ulcerative colitis; biomarkers*

Volume 00, Number 00, Month/Month 2015, Pages 00–00

*Address for correspondence: Guillermo H. Docena, PhD; Instituto de Estudios de Inmunológicos y Fisiopatológicos - IIFP, Facultad de Ciencias Exactas, Universidad Nacional de La Plata y Consejo, Nacional de Investigaciones Científicas y Técnicas, Calles 47 y 115, 1900 La Plata, Argentina. Tel.: +54 221 425 0497; Fax: +54 221 422 6947; E-mail: guidoc@biol.unlp.edu.ar.

†GAR and GHD shared senior authorship.

Received 29 October 2015; accepted 10 November 2015

DOI 10.1002/biof.1252

Published online 00 Month 2015 in Wiley Online Library (wileyonlinelibrary.com)

BioFactors

1. Introduction

Galectins are a family of evolutionarily conserved lectins widely distributed in the animal kingdom [1]. These lectins are defined by two common features: a carbohydrate recognition domain composed by a shared consensus amino acid sequence and affinity for β-galactoside containing glycans. To date, 15 members of the galectin family have been identified in different mammalian tissues. According to their architecture, galectins have been classified into prototype (Gal-1, −2, −5, −7, −10, −11, −13, −14, and −15), chimera type (Gal-3), and tandem-repeat type (Gal-4, −6, −8, −9 and −12) [2]. They have been shown to play pivotal roles as regulators of immune cell homeostasis and inflammation, by regulating cell survival and signaling, influencing chemotaxis, and interfering with cytokine secretion [3–5]. Several cell types, including epithelial cells, endothelial cells, and mucosal-associated immune cells are major sources of galectins and key targets of their biological effects in the intestinal ecosystem [6–11].

Inflammatory bowel diseases (IBD) are chronic disorders of the gastrointestinal tract of unknown etiology with a typically relapsing and remitting course. Crohn's disease (CD) and ulcerative colitis (UC) represent the two main forms of IBD [12]. These pathologies typically differ in their histology, type of lesion, location, and prognosis, and both are characterized by heterogeneous symptoms indicative of an underlying inflammatory process [13]. Nevertheless, in both cases, immune, environmental, and genetics factors have been documented as key players that contribute to persistent inflammation and subsequent mucosal lesions leading to a mucosal immune dysfunction [14]. Although the etiology of IBD remains unknown, a growing body of experimental evidence supports a link between chronic inflammatory disorders and a failure of the mucosal immune system to restore homeostasis and restrict the aberrant immune response against the microbiota. In this scenario, the role of galectins in IBD is not fully understood. These endogenous lectins are widely expressed by several intestinal cell subsets [7,15,16] and galectin–glycan interactions have been implicated in the pathogenesis of intestinal inflammation in murine models of colitis [9,17]. In studies using human intestinal samples, Gal-3, −4, and −9 have been implicated in the pathogenesis of IBD by modulating the migratory capacity of fibroblasts and regulating T cell survival [8,18,19]. Given the important roles of glycan-binding proteins in mucosal-associated compartments, delineating their expression profile in physiologic and pathologic settings is relevant for the implementation of potentially novel diagnostic, prognostic, and therapeutic approaches. Here we propose an integrated analysis of the expression profile of multiple galectins involved in intestinal disorders, which discriminates IBD from noninflamed tissues or from other intestinal inflammatory conditions, indicating their potential use as novel mucosal markers for IBD diagnosis and monitoring.

2. Materials and Methods

This study was approved by the Ethics Committee of the Udaondo Hospital for Gastroenterology (Buenos Aires, Argentina). In all cases, tissue samples were obtained with the consent of patients.

TABLE 1 *Clinical features of endoscopic active UC patients (n = 25)*

Characteristics and parameters	n	*Median (range)*
Age		43 (18–75)
Sex		
Male	11	
Female	14	
Intestinal location		
Proctitis	4	
Left-sided colitis	10	
Extensive	11	
Partial Mayo score[a]		
Remission (0–1)	5	
Mild disease (2–4)	11	
Moderate disease (5–6)	3	
Severe disease (7–9)	6	
IBSEN score		
1	8	
2	17	
Histology		
Mild inflammatory infiltrate	12	
Marked inflammatory infiltrate	13	

[a]*Without endoscopy.*
IBSEN: inflammatory bowel disease in SouthEastern Norway.

2.1. Patients and Sample Collection

Colon biopsies were collected from 25 UC patients and 18 CD patients with endoscopic features of active disease (clinical data is shown in Tables 1 and 2) and 22 non-IBD controls (10 women and 12 men, mean age: 47, range: 22–75 years). IBD patients were diagnosed based on clinical, endoscopic, and histopathologic findings according to accepted criteria of Lennard-Jones and Geboes et al. [20,21]. Clinical disease activity was graded according to the partial Mayo Score for UC patients [22] and the Hervey–Bradshaw Index (HBI) for CD patients [21,23]. Total ileocolonoscopy was performed and four biopsies (two for RNA extraction and two for pathology) adjacent to each other were taken from the colonic segment with the most severe activity observed (inflamed IBD samples).

The endoscopic disease activity in both types of IBD patients was evaluated according to the Inflammatory Bowel disease in SouthEastern Norway (IBSEN) study published by Frøslie et al. [24]. This index is one of the endoscopic activity scales considered to be used for the European Evidence-Based Consensus for Endoscopy in IBD [25]. Unlike the Mayo subendoscopic scoring, Crohn's Disease Endoscopic Index of Severity (CDEIS), and Simple Endoscopic Score for Crohn's Disease (SES-CD), the IBSEN score has been designed to be utilized for both UC and CD. This allowed us to draw parallels between patients with different diagnosis, but with equivalent degree of severity, condition that we regarded as an advantage for this study. We considered the score of the colonic segment where biopsies were obtained. Briefly, the inflammatory status of the mucosa was scored from 0 to 2 by endoscopic evaluation by the same observer, a gastroenterologist with experience in endoscopic diagnosis of IBD: 0 = normal; 1 light erythema or granularity; 2 = granularity, friability, and bleeding, with or without ulcerations. In this study, we considered as inflammation all findings different to score 0. Therefore, scores of 1 and 2 were considered as "weak inflammation" and "marked inflammation," respectively (the latter includes both moderate and severe changes). Histological degree of inflammation was assessed by an expert pathologist, blindly with respect to the endoscopist grading. The histological severity of mucosal inflammation was determined according to Saverymuttu et al. [26] with minor modifications. Briefly, histologic inflammation was classified as "mild inflammatory infiltrate" and "marked inflammatory infiltrate" (the latter includes moderate and severe inflammation).

Noninflamed samples were taken from noninvolved areas, with no macroscopic inflammation or history of activity in the segment, from 13 UC and 13 CD patients. The study also included 16 IBD patients with quiescent disease, 12 with UC and 4 with CD (mean age: 52.5, range: 18–80 years). Quiescent UC and CD patients were in clinical remission and samples were taken from areas that were previously documented as inflamed but without current signs of endoscopic activity (endoscopic score = 0). Remission was achieved in these patients with the following treatments: mesalazine (2), mesalazine and antibiotics (1), sulfasalazine (3), 6-mercaptopurine (7), 6-mercaptopurine and thalidomide (1), infliximab (1), and infliximab and 6-mercaptopurine (1).

Non-IBD control patients were endoscopically examined because of abdominal pain, constipation, irritable bowel syndrome, screening of colorectal cancer, or history of polyp resection. No signs of macroscopical or histologic features of IBD inflammation were observed in these patients [27].

In addition, samples from patients that underwent gut transplantation, and experienced gut rejection were included (n = 9). Patients aged 10–47 years received isolated intestinal transplantation due to short bowel syndrome secondary to multiple surgical resections as a result of volvulus or traumatic injuries without chronically intestinal pathology. In all cases, no postoperative complications upon the intestinal transplantation were detected and patients were subjected to routine endoscopic follow-up until diagnosis of acute cellular rejection according to international criteria [28]. Patients received corticoids (methylprednisolone or meprednisone) and tacrolimus (4), or corticosteroids (methylprednisolone or meprednisone), tacrolimus and rapamicin or mycophenolate (5) as immunosuppressive treatments.

Duodenal biopsies were obtained from adult patients during routine procedures to diagnose celiac disease (n = 8). Patients aged 20–40 were diagnosed by clinical, serological, and histological criteria. Control nonceliac patients (n = 10) with other gastrointestinal conditions, primarily dyspepsia, with negative serology for celiac disease and normal duodenal histology were also analyzed.

2.2. RNA Isolation and Real-Time RT-PCR

Intestinal tissue samples collected by endoscopy were immediately placed in 0.5 mL RNA later (Ambion, USA) at 4 °C and stored at −80 °C until processing. Total RNA extraction was performed by means of total RNA isolation system (Macherey Nagel, Duren, Germany) following the manufacturer's instructions. Random primers and MMLV-reverse transcriptase (Invitrogen, Carlsbad, CA, USA) were used for reverse transcription of isolated RNA. Real-time quantitative PCR (qPCR) for Gal-1, Gal-3, Gal-4, Gal-9, STAT4, GATA3, and T-bet was performed using SYBR Green PCR Master Mix (BioRad, Hercules, CA, USA) in the iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). The relative expression level of genes was determined using human β-actin as a normalizer. Primers' sequence were as follows: Gal-1 forward: 5′-TGAACCTGGGCAAAGACAGC-3′; reverse: 5′-TTGGCCTGGTCGAAGGTGAT-3′, Gal-3 forward: 5′-GAAGGGAAGAAAGACAGTCG-3′; reverse: 5′-CAAGTGAGCATCA TTCACTG-3′, Gal-4 forward: 5′-CGAGGAGAAGAAGATCA CCC-3′; reverse: 5′-CTCTGGAAGGCCGAGAGG-3′, Gal-9: forward: 5′-CTTTCATCACCACCATTCTG-3′, reverse: 5′-ATGTGGAACCTCT GAGCACTG-3′, STAT4: forward: 5′-GATCTGCAAGACGAATTT GAC-3′; reverse: 5′-TGTCACTCTGATCCATTGTC-3′; GATA3: forward: 5′-GCTTCACAATATTAACAGACCC-3′; reverse: 5′-GTT AAACGAGCTGTTCTTGG-3′; T-bet: forward: AGAATGCCGA-GATTACTCAG: reverse: TACATGGACTCAAAGTTCTCC. β-actin forward: 5′-CCTGGCACCCAGCACAAT-3′; reverse: 5′-GCCGAT CCACACGGAGTACT-3′.

2.3. Statistical Analysis

Comparison between groups was performed using analysis of variance (ANOVA) and Tukey's test. P values lower than 0.05 were considered statistically significant. A normal control range for each galectin was defined as mRNA levels lying between Q1 − 1.5 × IQR and Q3 + 1.5 × IQR (Q1: 1st quartile; Q3: 3rd quartile; IQR: interquartile range, all belonging to the control population).

Linear discriminant analysis (LDA) was used to separate predefined groups using a linear combination of variables (gene expression) [29]. Data were plotted in a multidimensional graph in which similar objects lie close to one another and clusterized. LDA was used to determine whether control and IBD patients can be discriminated, as well as UC from CD

patients. In addition, LDA was assessed to discriminate IBD inflammation, gut transplantation, and celiac patients. Leave-one-out cross-validation was used to confirm graphical segregation. Each sample was randomly excluded from analysis as the remaining database was used to generate an algorithm to predict the excluded sample identity. This procedure was repeated as many times as samples were in the database, always excluding a different sample. Finally, we obtained a percentage of success as cross-validation (CV) % that indicates how many times samples were recategorized within the previously defined group.

A receiver operating characteristic (ROC) curve representing sensitivity (true-positive rate) versus specificity (false-positive rate) was built. The area under the ROC curve (AUC) is a diagnostic measure indicating if a variable (mRNA expression level of galectins or transcription factors, or a linear combination thereof) can be used to distinguish between two groups (control vs IBD). An AUC value of 1 corresponds to a system capable of a perfect discrimination, whereas a value of 0.5 indicates a complete lack of discriminative power.

3. Results

3.1. Galectin Profile is Homogenously Distributed in Different Sections of the Colon

To investigate the profiles of intestinal galectins in IBD and other relevant intestinal pathologies, we first analyzed control biopsies of non-IBD patients corresponding to ascending, transverse, descending, and sigmoid colon, and rectum for Gal-1, Gal-3, Gal-4, and Gal-9 mRNA expression (Fig. 1A). No statistical differences were observed between the different intestinal sections, demonstrating a homogenous and ubiquitous galectin mRNA expression throughout the colon. This finding led us to analyze biopsies of colonic mucosa, irrespective of the colonic segment. Interestingly, we found that Gal-4 was the most highly expressed galectin within the colon, with mRNA levels comparable to those of β-actin, followed by Gal-3, Gal-9, and Gal-1, respectively.

F1

3.2. Galectins are Differentially Expressed in the Inflamed Mucosa of IBD Patients

We next sought to analyze biopsies obtained from colonic inflamed tissues of both UC and CD patients (Fig. 1B). We found a significant deregulation of galectin mRNA expression in severe inflamed tissues compared with biopsies from control patients. Results showed that noninvolved areas from tissues corresponding to IBD patients (noninflamed tissues) showed a similar galectin profile as control samples. Moreover, Gal-1 and Gal-4 were differentially expressed in mild inflamed areas compared with control samples. Regarding areas with more prominent inflammatory infiltrates, all galectins analyzed showed a differential gene expression profile as compared with control tissues. We found that Gal-1 mRNA considerably increased, whereas Gal-3, Gal-4, and Gal-9 mRNA substantially dropped in areas with pronounced inflammation. These results suggest that severe inflammation leads to a significant deregulated expression of the human galectin network. Moreover, galectin expression showed unsatisfactory AUC when mild inflamed and control samples were compared (Fig. 1C). However, we found that all galectins showed a high sensitivity and specificity for delineating marked inflamed areas of IBD patients from control samples ($AUC > 0.8$), which was even more pronounced for Gal-4 ($AUC = 0.99$) than for other galectins analyzed (Fig. 1D).

However, analysis of individual galectins in control samples did not enable us to define a cutoff value of gene expression to distinguish control vs IBD samples (Fig. 1B). Our results showed that although individual analysis of gene expression corresponding to these galectins may provide an approximation of the mucosal status, it can discriminate noninflamed from inflamed tissues in a restricted percentage of cases (Gal-1 = 40%; Gal-3 = 0%; Gal-4 = 42%; and Gal-9 = 58%). These results prompted us to run a multiparametric analysis of galectin gene expression including all galectins in the same matrix.

3.3. Galectin mRNA Expression Distinguishes Inflamed IBD Mucosa from Control Tissue and Reflects Disease Activity

Seeking for an integrated analysis of the galectin signature in IBD, we clusterized gene expression of galectins under different conditions using the LDA. The leave-one-out cross-validation analysis allowed us to evaluate the performance of the estimates. The LDA showed no significant differences between control and noninflamed IBD samples, whereas inflamed IBD samples could be discriminated (Fig. 2A). Remarkably, also remission IBD samples were similarly clusterized as control samples, suggesting that once inflammation is resolved by treatment, the pattern of galectin expression recapitulates that found in healthy mucosa. The cross-validation (CV) analysis rendered the following CV percentages: inflamed IBD, 81.40% (n = 43); noninflamed IBD, 23.08% (n = 26); remission IBD, 25.00% (n = 16), and control, 31.82% (n = 22). The leave-one-out cross-validation analysis classified 35/43 IBD samples as belonging to the inflamed cluster.

F2

When we investigated the galectin gene expression profile in samples from tissues with different degree of endoscopic inflammation, we found that the LDA reflected disease activity (Fig. 2B). Samples from areas with mild inflammation were discriminated from those with marked inflammation and control samples. The leave-one-out cross-validation analysis prompted us to correlate the progression of the disease with the integrated galectin expression. We found CV values for mild inflamed, active inflamed, or control samples tissues of 66.67% (n = 18), 72.00% (n = 25), and 82.82% (n = 22), respectively.

3.4. Galectin mRNA Expression Profile does not Discriminate between CD and UC

Although the LDA showed a good performance to delineate IBD from control samples, with an AUC = 0.97 (Fig. 3A), CV_{IBD} = 92% and $CV_{CONTROL}$ = 82%, it could not discriminate CD from UC samples (Fig. 3B). The leave-one-out cross-validation analysis rendered the following percentages: CV_{CD} = 44.44%

F3

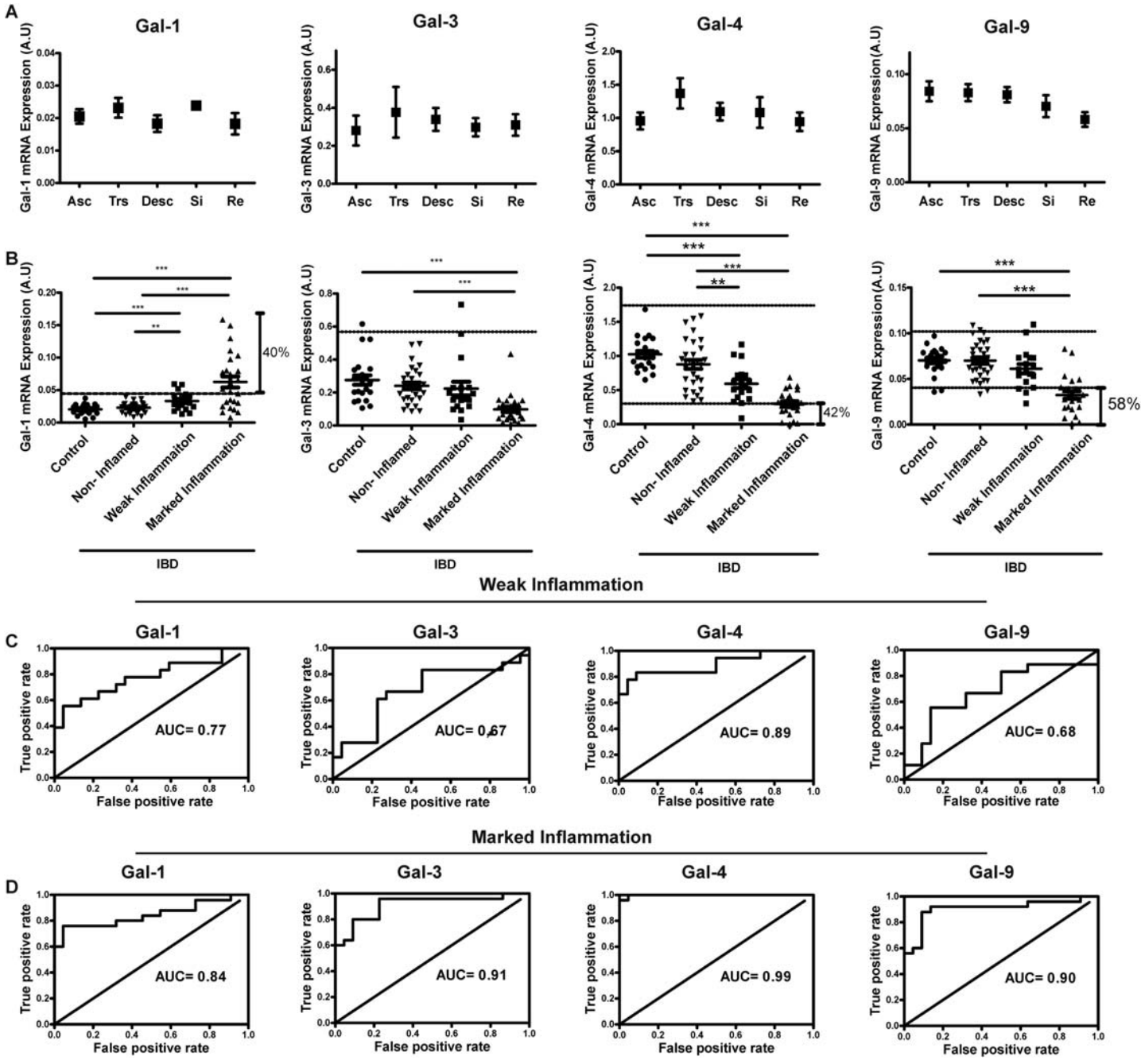

FIG 1

*Galectin gene expression profile in human colon samples. (A) Galectin mRNA expression by qPCR in the different sections of the healthy colon: Ascending (Asc), transverse (Trs), descending (Desc), and sigmoid colon (Re). (B) mRNA levels of individual galectins in control patients and inflamed tissues of patients with IBD. Receiver operating characteristic (ROC) curve for the individual galectin gene expression and corresponding area under the curve (AUC) statistics for evaluation of samples with (C) mild inflammation or (D) active inflammation. Galectin mRNA levels were normalized relative to human β-actin. Samples were analyzed in duplicate and data are expressed as mean values ± standard error of the mean (SEM). Line indicates the normal control range (***P < 0.001, **P < 0.01, *P < 0.05).*

(n = 18), CV_{UC} = 44.00% (n = 25), and $CV_{CONTROL}$ = 81.82% (n = 22). In order to improve disease discrimination analysis, we next incorporated CD- or UC-associated immune markers (*stat-4*, *gata-3*, and *t-bet*). The LDA of the immune markers (Fig. 3C) did not distinguish IBD from control samples (AUC = 0.72, CV_{IBD} = 58%, and $CV_{CONTROL}$ = 77%). Nevertheless, the addition of immune markers to galectin expression analysis (Fig. 3D) discriminated control from IBD samples, although CD and UC were still indiscernible (CV_{CD} = 31.25%; CV_{UC} = 40.91%, and $CV_{CONTROL}$ = 86.36%). Overall, these findings indicate that

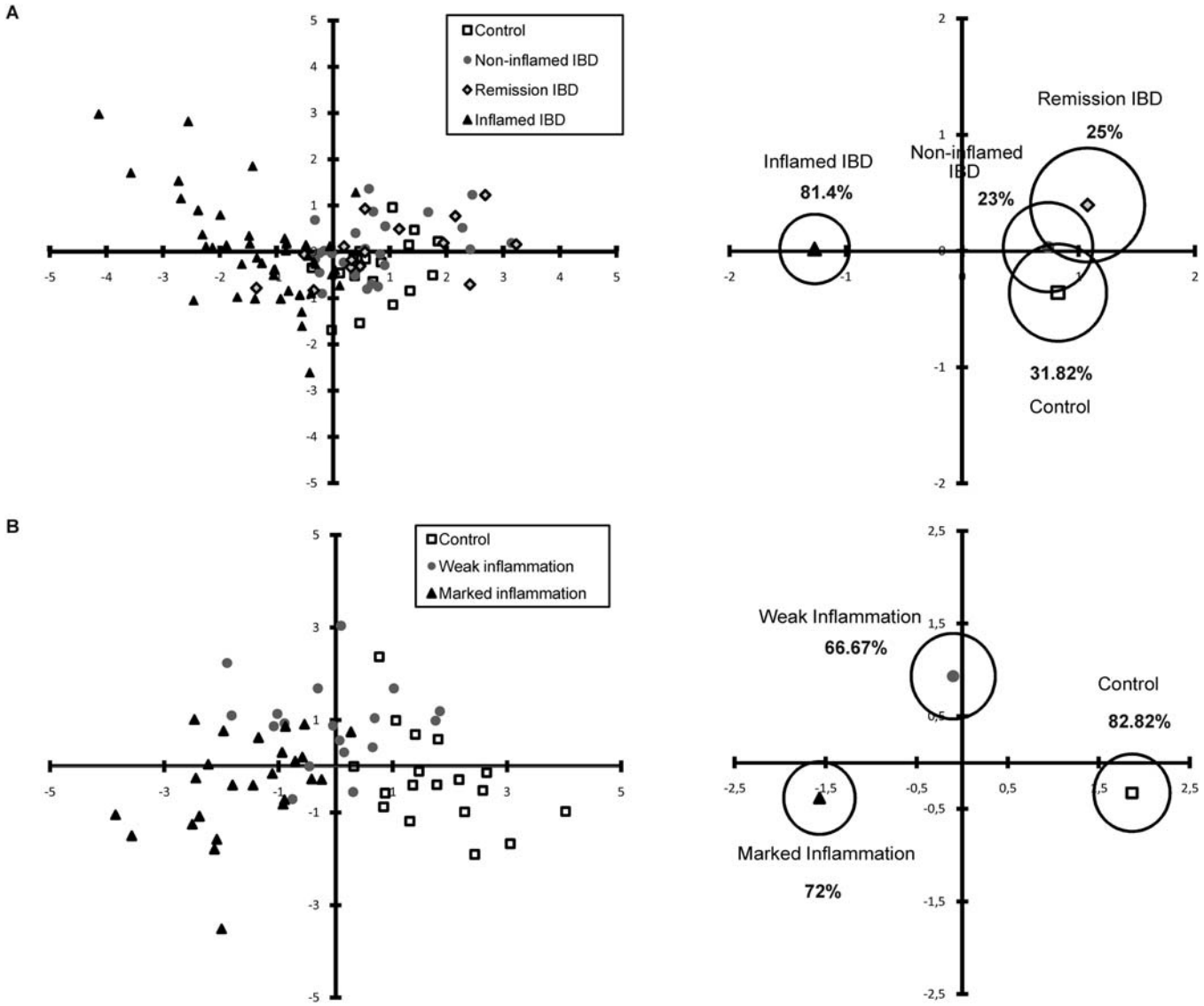

FIG 2 *Linear discriminant analysis of galectin gene expression profile in control and IBD colonic samples. (A) Multivariate analysis of Gal-1, −3, −4, and −9 mRNA expression in inflamed active IBD, noninflamed IBD, remission IBD, and control samples. (B) Analysis of galectin gene expression in biopsies from patients with different disease activity, and control samples. Right panels show the centroids corresponding to the analysis of mRNA expression of the four galectins. Samples were analyzed in duplicate.*

analysis of mucosal galectin gene expression mostly contributed to discriminate between IBD and control samples. Additionally, our results suggest that these endogenous lectins are equally deregulated in CD and UC inflamed tissues.

3.5. Galectins can Discriminate Inflamed IBD from Other Types of Intestinal Inflammatory Responses

To understand whether the aberrant pattern of galectin gene expression observed in IBD patients is specific of the inflammatory processes occurring in UC and CD, we performed the same analysis in biopsies from patients with other intestinal inflammatory conditions. We analyzed intestinal biopsies from small bowel of patients with active celiac disease, patients that received gut transplantation and underwent rejection, and healthy control patients. When performing the LDA, we found a clear discrimination of the inflammatory response between celiac disease and gut transplant rejection (Fig. 4A). However, F4 the centroid corresponding to celiac samples was close to control duodenum samples. In fact, cross-validation analysis showed that half of the samples from celiac patients were redefined as control samples, while 78% of samples from rejected gut were gathered as themselves, and distant from normal and celiac centroids (CV_{CELIAC} = 50.00%; $CV_{REJECTION}$ = 77.78%; $CV_{CONTROL}$ = 70.00%). These data suggest that the LDA could not distinguish inflammation in celiac patients from control duodenum. On the contrary, inflammation in gut rejection samples

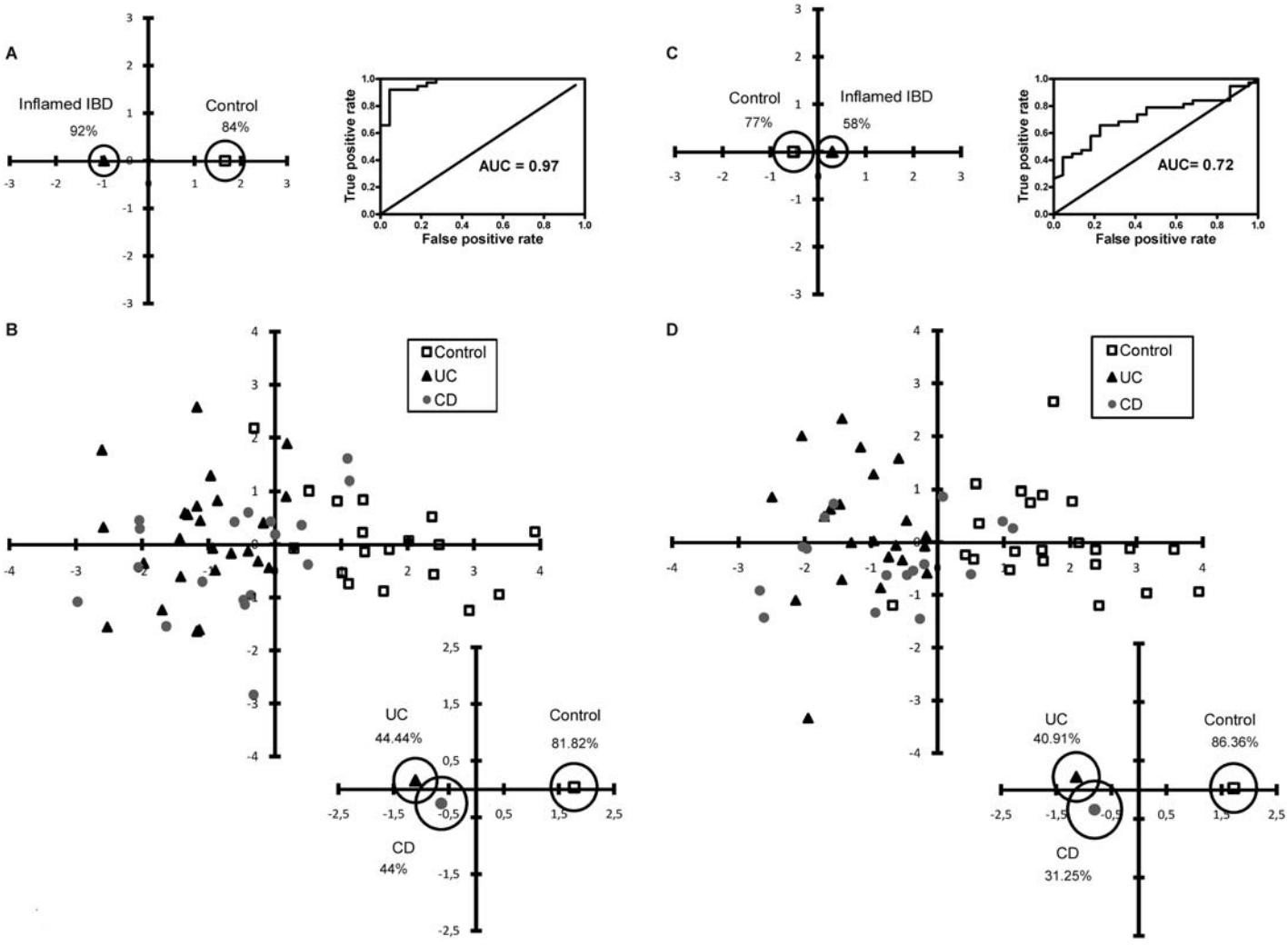

FIG 3

Linear discriminant analysis of galectin gene expression profile and T cell-associated transcription factors in control and IBD colonic samples. (A) Multivariate analysis of galectin mRNA expression in active IBD and control samples. ROC and AUC statistics for discrimination of IBD and control samples were calculated. (B) Multivariate analysis for discrimination of active CD from UC. Centroids for inflamed CD and UC vs control patients were plotted using Gal-1, Gal-3, Gal-4, and Gal-9 mRNA expression. (C) Discriminant analysis of transcription factor (GATA-3, STAT-4, and T-bet) mRNA expressions in active IBD and control samples. ROC and AUC statistics were calculated. (D) Multivariate analysis and clustering of inflamed CD and UC vs control samples with centroids were plotted using Gal-1, Gal-3, Gal-4,Gal-9, GATA-3, STAT-4, and T-bet mRNA expression. Samples were analyzed in duplicate.

showed a galectin expression pattern discernible from other samples (Fig. 4A). When gene expression of individual galectins was compared between control duodenum and inflammation associated with small bowel rejection, Gal-1 showed the main difference with a fourfold increased expression in gut rejection samples. Significant differences were also observed in Gal-3 expression levels (Fig. 4B). However, no differences in individual galectin gene expression profiles were observed between control and celiac patients (data not shown).

Finally, we analyzed whether variations in the galectin expression pattern occurred between distinct inflammatory disorders. Because celiac disease and gut rejection affect mainly the small bowel, whereas IBD (CD and UC) affect the colon, we next compared the galectin expression signature in small bowel and colon from healthy control individuals. Of note, we found differential expression of individual galectins in both tissues examined (Fig. 5A). The four galectins analyzed were highly expressed in the normal colon as compared with healthy duodenum, being Gal-4 the most highly expressed galectin. In addition, Gal-9 displayed the greatest changes in expression (3.5-fold in colon). The integrated analysis of galectins rendered different clusters of gene expression profile corresponding to healthy small bowel and colon (Fig. 5B), and leave-one-out cross-validation analysis confirmed the graphical segregation: $CV_{SMALL\ BOWEL} = 90\%$; $CV_{COLON} = 90.91\%$. F5

Finally, even though the galectin expression profile of the duodenum differed from that of the colon, the integrated analysis showed distant centroids when samples of active IBD

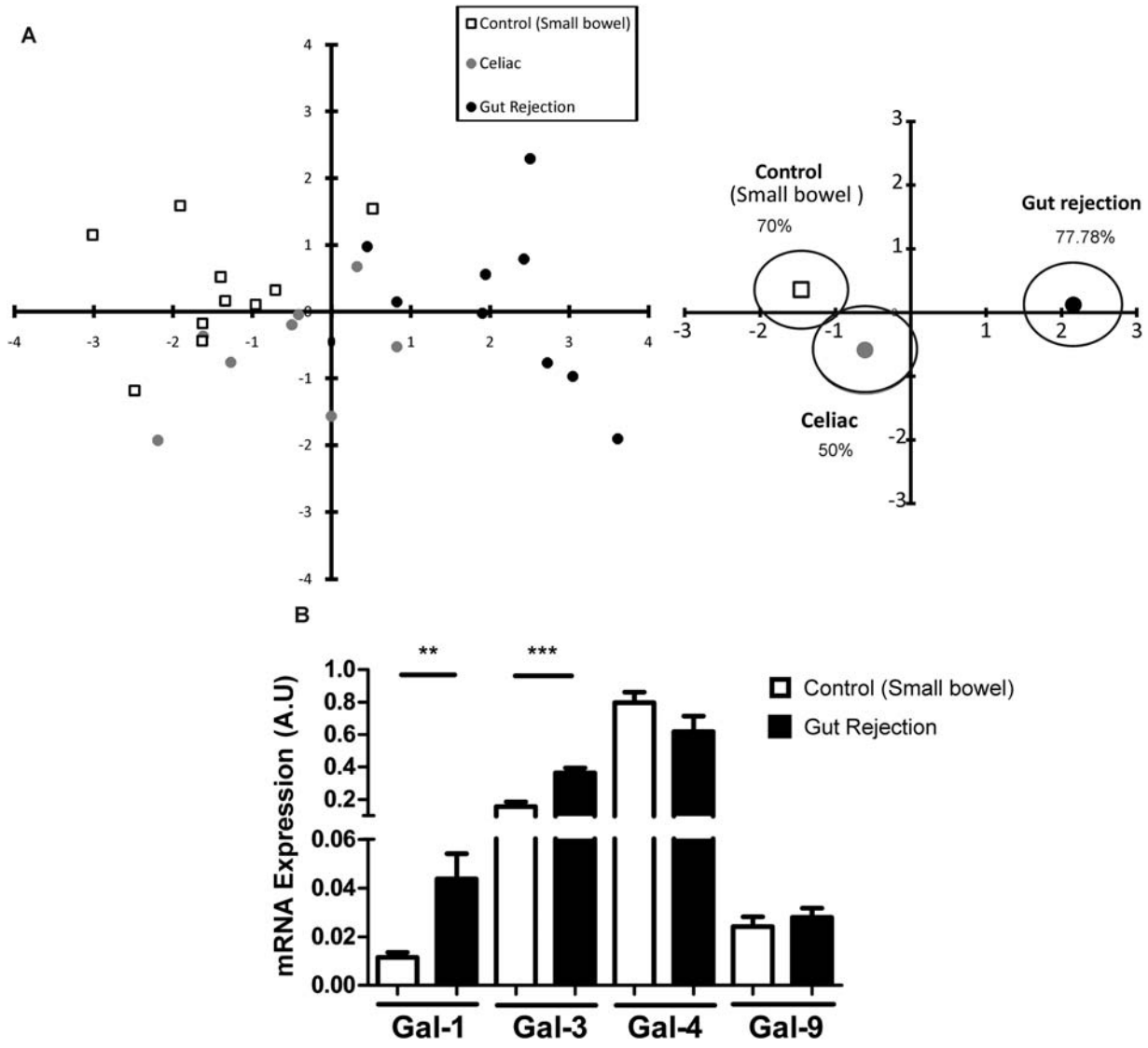

FIG 4 *Individual and linear discriminant analysis of galectin gene expression in inflamed tissues of celiac patients, patients with gut rejection and control patients. (A) LDA and centroids plotted using galectin gene expression profiles of samples from control duodenum, celiac, and gut rejection in transplanted patients. (B) mRNA expression corresponding to galectins in normal duodenum and gut rejection. Samples were analyzed in duplicate and data are expressed as mean values ± standard error of the mean (SEM) (***P < 0.001, **P < 0.01).*

were compared with controls and gut rejection specimens ($CV_{CONTROL\ COLON}$ = 86.66%, $CV_{CONTROL\ SMALL\ BOWEL}$ = 80%, CV_{IBD} = 73.68%; $CV_{GUT\ REJECTION}$ = 66.67%) (Fig. 5B). Overall, these findings indicate substantial differences in galectin expression profiles in IBD (CD and UC) patients as compared to healthy individuals and patients suffering celiac disease or gut rejection.

4. Discussion

In the past few years, a number of experimental mouse models of IBD revealed the critical role of galectins in the resolution of gut inflammation and attenuation of colitis [7,9,18]. In this study, we identified a mucosal galectin signature, which could be potentially used as a mucosal marker for different intestinal inflammatory conditions, including IBD. We analyzed Gal-1, Gal-3, Gal-4, and Gal-9 mRNA by qPCR and found that galectin gene expression can discriminate intestinal inflammation in IBD from celiac disease patients and patients undergoing rejection following gut transplantation. Furthermore, active IBD specimens, regardless of whether they were EC or UC, could be discriminated from noninflamed, quiescent IBD mucosa or from non-IBD control tissues. Noticeably, disease activity was reflected in the performed linear discriminant analysis. In addition, analysis of biological specimens from 22 control and 59 IBD patients demonstrated that galectins are homogeneously expressed throughout the different sections of healthy colon, with a deregulated expression in inflamed tissues.

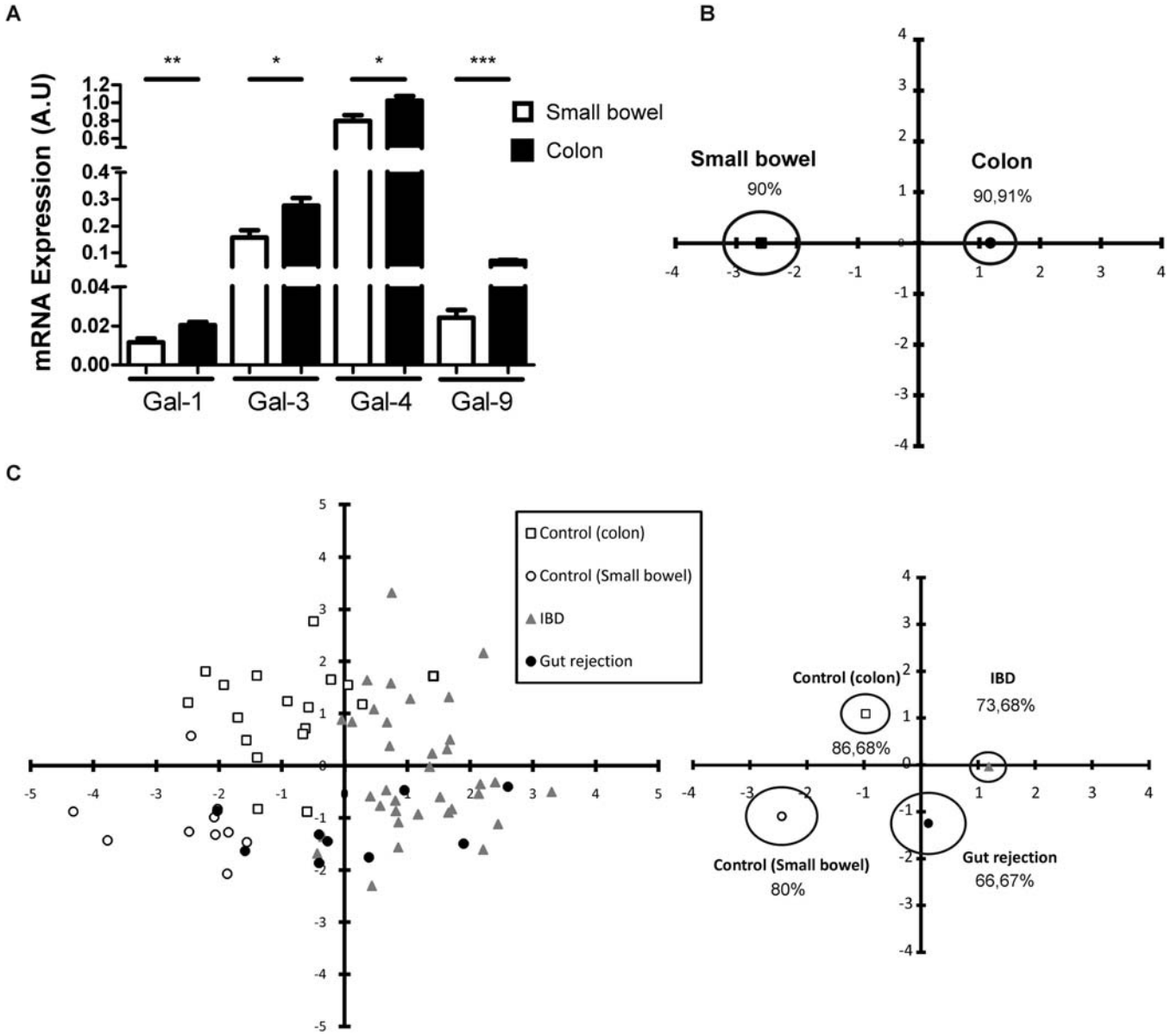

FIG 5 *Linear discriminant analysis of galectin gene expression profiles of control duodenum, control colon, inflamed tissues of active IBD, and gut rejection. (A) mRNA expression corresponding to galectins in normal duodenum and colon. (B) Multivariate analysis of galectin mRNA expression in samples of control duodenum and colon. Centroids were plotted based on Gal-1, Gal-3, Gal-4, and Gal-9 mRNA expression. (C) Multivariate analysis for discrimination of samples from active IBD, control colonic tissue, and samples from rejected gut. Centroids were plotted according to the mRNA expression corresponding to the four galectins examined. Samples were analyzed in duplicate and data are expressed as mean values ± standard error of the mean (***P < 0.001, **P < 0.01, *P < 0.05).*

Galectins analyzed in this study were selected based on their critical involvement in gut mucosa homeostasis [19,30–32]. It has been reported that Gal-1 exerts a protective and immunomodulatory activity during TNBS-induced colitis [30], which might be mediated through the induction of IL-10 secretion and modulation of T cell survival [4,33]. On the other hand, Muller et al. showed that Gal-3 promotes death of activated T cells [7], while Lippert et al. showed the ability of this chimera-type galectin to control migration of lamina propria fibroblasts, thus promoting stricture and fistula formation in Gal-3-depressed mucosa [15,18]. Moreover, Nishida et al. demonstrated that *i.v.* administration of high doses of Gal-4 leads to delayed recovery of DSS-induced colitis [34]. Further, Hokama et al. demonstrated increased synthesis of Gal-4 by intestinal epithelial cells and proinflammatory activity. This lectin interacts with colonic lamina propria $CD4^+$ T cells, forming surface lattices and stimulating IL-6 production and PKC θ activation, with the subsequent delay recovery from acute intestinal injury [34,35]. On the other hand,

Paclik et al. documented that low continuous doses of Gal-4 reduced the disease activity index in a colitis model by induction of mucosal T cell apoptosis as well as suppression of proinflammatory cytokines [8]. These apparent discrepancies could be reconciled, at least in part by the different concentrations of this tandem-repeat galectin as well as the roles of endogenous versus exogenous Gal-4 and the impact of local versus systemic delivery of this lectin in experimental IBD. Finally, Gal-9 has been shown to promote apoptosis of Th1 cells through interaction with T-cell immunoglobulin and mucin domain-3 (TIM-3) [36]. It has been reported that expression of TIM-3 and Gal-9 is decreased in CD and UC patients inducing an imbalance of Th17 and T regulatory (Treg) cells [36,37]. In summary, experimental IBD models show that Gal-1, −3, −4, and −9 play key roles in the intestinal homeostasis and may contribute to the pathogenesis of IBD through different, although partially overlapping mechanisms.

Here we found that all members of the galectin family analyzed were homogeneously expressed through different sections of the large bowel. Although individual galectin gene expression profiles were differentially induced in active IBD, we could not identify a potential biological indicator of disease activity with enough predictive value to be clinically used as a mucosal biomarker. Nevertheless, the multivariate and integrated analysis of gene expression of these four galectins predicted in 82% of the active IBD samples (mild and actively inflamed) that they were indeed obtained from IBD patients. Of note, this analysis reflected the disease activity in inflamed tissues, while healing in UC and CD patients could not be distinguished from control or non-inflamed mucosa. However, active CD and UC could not be discriminated by our multivariate and integrated analysis, thus emphasizing the fact that galectins are similarly deregulated in both inflammatory conditions. On the contrary, when galectins were analyzed in inflamed tissues of rejected grafts from patients that underwent gut transplantation, we found that the LDA distinguished active IBD from rejected inflamed mucosa and control mucosa. Interestingly, when other inflammatory conditions such as celiac disease were analyzed, a completely different scenario was revealed. Duodenal samples from control adults and patients with active celiac disease could not be discriminated; however, they were clearly different from duodenal samples from patients undergoing gut rejection. Thus, integrated analysis of galectin gene expression reflects the differential regulation of these endogenous glycan-binding proteins in each inflammatory condition. In this regard, one might hypothesize that variations in galectin expression could reflect differences in the underlying mechanisms that govern intestinal inflammatory disorders and might correlate with the natural history of the disease in each individual patient.

Although this integrated algorithm could efficiently manage to discriminate patients with IBD diagnosis, CD could not be distinguished from UC even when immune markers were differentially expressed (at the mRNA level) in both inflammatory disorders (e.g., *gata-3*, a Th2 transcription factor associated with UC, and *stat-4* and *t-bet,* Th1 transcription factors associated with CD) were included in the multivariate analysis [38,39]. In f act, using the ROC curve, we observed a better performance of the galectin gene expression profile (AUC = 0.97), when compared to analysis of the expression of transcription factors (AUC = 0.72) for discriminating IBD and control samples. Furthermore, LDA showed close centroids corresponding to control samples, nonactive and quiescent IBD distinguishable from active IBD. These findings suggest that inflammation leads to

TABLE 2 ***Clinical features of endoscopic active CD patients (n = 18)***

Characteristics and parameters	n	*Median (range)*
Age		43 (20–73)
Sex		
Male	6	
Female	14	
Age of diagnostic		
<30	6	
30–40	6	
>40	8	
Intestinal Location		
Colon only	18	
Small bowel and colon disease behavior	2	
Non stricturing or fistulizing	11/5p	
Stricturing	2/1p	
Fistulizing	0/1p	
HBI		
Remission (<4)	5	
Mild disease (5–7)	11	
Moderate to severe disease (8–12)	4	
IBSEN score		
1	6	
2	14	
Histology		
Mild inflammatory infiltrate	11	
Marked inflammatory infiltrate	9	

p: with perianal compromise.
HBI: Harvey–Bradshaw index.
IBSEN: inflammatory bowel disease in SouthEastern Norway.

deregulation in galectin gene expression profiles, likely aimed at limiting the inflammatory process and restoring mucosal homeostasis. In this regard, by studying intestinal necrosis in neonates diagnosed with necrotizing enterocolitis, Sylvester et al. found that the multivariate and integrated analysis of clinical parameters in combination with urine peptide biomarkers, improved the prognostic accuracy of disease progression in comparison with clinical or analytical parameters individually analyzed [29].

In this work, we provided evidence that the galectin gene expression profile is significantly deregulated in active inflamed mucosa from IBD (both CD and UC) patients and reflects disease activity. Since Gal-1 is a well-known anti-inflammatory factor, and it is upregulated in the active inflamed mucosa, we suggest that it may play a resolving role in IBD by controlling the survival of activated proinflammatory Th1 and Th17 cells and triggering regulatory circuits mediated by tolerogenic dendritic cells, FoxP3$^+$ Treg cells and M2-type macrophages [3,5,40]. Similar speculations could be made with regards to Gal-3 and Gal-4, which have been shown to modulate T cell activation, proinflammatory cytokine synthesis and T cell apoptosis [7,8]. Further studies are needed to address the specific role of endogenous galectins in the pathogenesis of CD and UC.

Although serum and fecal markers are increasingly used [41–43], colonoscopy and mucosal sampling are routine procedures for diagnosis and monitoring the disease in IBD patients and remains the only reliable procedure to assess mucosal inflammation. In fact, frequent lack of correlation is observed between clinical, endoscopic, and histological findings [44]. As shown in Tables 1 and 2, according to the partial Mayo score without endoscopy and the HBI, 20% (5/25) of UC patients and 25% (4/18) of CD patients showed endoscopic signs of inflammation but no clinical manifestations.

Different criteria about disease remission impact in the management of the patient and results of clinical trials. Currently, there is no international consensus that defines endoscopic mucosal healing [45], and histological assessment is still controversial and need further examination [27]. Therefore, both mucosa with no abnormalities and mucosa with mild inflammation might be considered as "healing." Given that changes in mRNA expression are fast events, galectin gene expression in intestinal samples may constitute potential mucosal biomarkers to rapidly assess the evolution and severity of inflammation during active disease, to predict possible relapses, or to monitor the inflammation status of the mucosa once pharmacological treatment has been initiated.

In conclusion, in this study, we identified the relevance of mucosal galectins as potential markers of the severity of mucosal inflammation in IBD patients. Quantification of Gal-1, Gal-3, Gal-4, and Gal-9 mRNA in intestinal biopsies and the ensemble multivariate analysis provide an improved tool to distinguish active IBD from control patients, quiescent IBD, and other intestinal inflammatory disorders and to assess disease activity in IBD patients. Further studies will be necessary to determine whether galectin gene expression may reflects changes occurring during the natural course of IBD or following different treatments in clinical trials.

Funding

This work was supported by grants from the National Agency for Research (PICT 2012 −1772), University of La Plata (X455), and The Broad Medical Research Program at CCFA (IBD-0378, 2013).

Acknowledgements

All authors have made substantial contributions to all the following: the conception, design of the study and analysis and interpretation of the data (RPG, NDF), biopsies sampling and data acquisition (RPG, CB, AR), drafting the article (RPG, NDF), revising it critically for important intellectual content (CM, FC, MR, MAT, GAR, AS), and final approval of the version to be submitted (GAR, AS, GD).

Conflicts of Interest and Source of Funding

Declaration of personal interests: Authors have no conflicts of interest to declare that are relevant to this manuscript.

References

[1] Cooper, D. N. W. (2002) Galectinomics: finding themes in complexity. Biochim. Biophys. Acta 1572, 209–231. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12223271 [Accessed July 7, 2015].

[2] Liu, F. -T. and Rabinovich, G. (2005) Galectins as modulators of tumour progression. Nat. Rev. Cancer 5, 29–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15630413 [Accessed June 16, 2014].

[3] Ilarregui, J. M., Croci, D. O., Bianco, G., et al. (2009) Tolerogenic signals delivered by dendritic cells to T cells through a galectin-1-driven immunoregulatory circuit involving interleukin 27 and interleukin 10. Nat. Immunol. 10, 981–991. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19668220 [Accessed June 10, 2014].

[4] Toscano, M. A., Ilarregui, J. M., Bianco, G. A., et al. (2014) Dissecting the pathophysiologic role of endogenous lectins: glycan-binding proteins with cytokine-like activity? Cytokine Growth Factor Rev. 18, 57–71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17321195 [Accessed June 23,

[5] Toscano, M. A., Bianco, G. A., Ilarregui, J. M., et al. (2007) Differential glycosylation of TH1, TH2 and TH-17 effector cells selectively regulates susceptibility to cell death. Nat. Immunol. 8, 825–834. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17589510 [Accessed June 10, 2014].

[6] Rubinstein, N., Ilarregui, J. M., Toscano, M. A., et al. (2004) The role of galectins in the initiation, amplification and resolution of the inflammatory response. Tissue Antigens 64, 1–12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15191517 [Accessed June 27, 2014].

[7] Müller, S., Schaffer, T., Flogerzi, B., et al. (2006) Galectin-3 modulates T cell activity and is reduced in the inflamed intestinal epithelium in IBD. Inflamm. Bowel Dis. 12, 588–597. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16804396 [Accessed June 23, 2014].

[8] Paclik, D., Danese, S., Berndt, U., et al. (2008) Galectin-4 controls intestinal inflammation by selective regulation of peripheral and mucosal T cell apoptosis and cell cycle. PLoS One 3, e2629 Available at: http://www.pubmedcentral.nih.gov/articlerender.

fcgi?artid=2440804&tool=pmcentrez&rendertype=abstract [Accessed June 16, 2014].

[9] Paclik, D., Berndt, U., Guzy, C., et al. (2008) Galectin-2 induces apoptosis of lamina propria T lymphocytes and ameliorates acute and chronic experimental colitis in mice. J. Mol. Med. (Berl). 86, 1395–1406. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18064431 [Accessed June 16, 2014].

[10] Nio-Kobayashi, J., Takahashi-Iwanaga, H., and Iwanaga, T. (2009) Immunohistochemical localization of six galectin subtypes in the mouse digestive tract. J. Histochem. Cytochem. 57, 41–50. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2605710&tool=pmcentrez&rendertype=abstract [Accessed December 12, 2014].

[11] Kaltner, H., Seyrek, K., Heck, A., et al. (2002) Galectin-1 and galectin-3 in fetal development of bovine respiratory and digestive tracts. Comparison of cell type-specific expression profiles and subcellular localization. Cell Tissue Res. 307, 35–46. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11810312 [Accessed December 12, 2014].

[12] Xavier, R. J. and Podolsky, D. K. (2007) Unravelling the pathogenesis of inflammatory bowel disease. Nature 448, 427–434. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17653185 [Accessed May 24, 2014].

[13] Baumgart, D. C. and Sandborn, W. J. (2007) Inflammatory bowel disease: clinical aspects and established and evolving therapies. Lancet 369, 1641–1657. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17499606 [Accessed January 18, 2015].

[14] Baumgart, D. C. and Carding, S. R. (2007) Inflammatory bowel disease: cause and immunobiology. Lancet 369, 1627–1640.

[15] Lippert, E., Falk, W., Bataille, F., et al. (2007) Soluble galectin-3 is a strong, colonic epithelial-cell-derived, lamina propria fibroblast-stimulating factor. Gut 56, 43–51. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1856646&tool=pmcentrez&rendertype=abstract [Accessed June 16, 2014].

[16] Gitt, M. A., Colnot, C., Poirier, F., et al. (1998) Galectin-4 and galectin-6 are two closely related lectins expressed in mouse gastrointestinal tract. J. Biol. Chem. 273, 2954–2960. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9446608 [Accessed June 23, 2014].

[17] Srikrishna, G., Turovskaya, O., Shaikh, R., et al. (2005) Carboxylated glycans mediate colitis through activation of NF-kappa B. J. Immunol. 175, 5412–5422. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16210648 [Accessed June 23, 2014].

[18] Lippert, E., Gunckel, M., Brenmoehl, J., et al. (2008) Regulation of galectin-3 function in mucosal fibroblasts: potential role in mucosal inflammation. Clin. Exp. Immunol. 152, 285–297. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2384104&tool=pmcentrez&rendertype=abstract [Accessed June 16, 2014].

[19] Morimoto, K., Hosomi, S., Yamagami, H., et al. (2011) Dysregulated upregulation of T-cell immunoglobulin and mucin domain-3 on mucosal T helper 1 cells in patients with Crohn's disease. Scand. J. Gastroenterol. 46, 701–709. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21463244 [Accessed June 23, 2014].

[20] Lennard-Jones, J. E. (1989) Classification of inflammatory bowel disease. Scand. J. Gastroenterol. Suppl. 170, 2–6. discussion 16–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2617184 [Accessed January 26, 2015].

[21] Lewis, J. D., Chuai, S., Nessel, L., et al. (2008) Use of the noninvasive components of the Mayo score to assess clinical response in ulcerative colitis. Inflamm. Bowel Dis. 14, 1660–1666. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2597552&tool=pmcentrez&rendertype=abstract [Accessed September 9, 2015].

[22] Harvey, R. F. and Bradshaw, J. M. (1980) A simple index of Crohn's-disease activity. Lancet (London, England) 1, 514 Available at: http://www.ncbi.nlm.nih.gov/pubmed/6102236 [Accessed October 7, 2015].

[23] Panaccione, R., Loftus, E. V., Binion, D., et al. (2011) Efficacy and safety of adalimumab in Canadian patients with moderate to severe Crohn's disease: results of the Adalimumab in Canadian SubjeCts with ModErate to Severe Crohn's DiseaSe (ACCESS) trial. Can. J. Gastroenterol. 25, 419–425. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3186726&tool=pmcentrez&rendertype=abstract [Accessed October 7, 2015].

[24] Frøslie, K. F., Jahnsen, J., Moum, B., et al. (2007) Mucosal healing in inflammatory bowel disease: results from a Norwegian population-based cohort. Gastroenterology 133, 412–422. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17681162 [Accessed November 14, 2014].

[25] Annese, V., Daperno, M., Rutter, M. D., et al. (2013) European evidence based consensus for endoscopy in inflammatory bowel disease. J. Crohns Colitis. 7, 982–1018. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24184171 [Accessed November 6, 2014].

[26] Saverymuttu, S. H., Camilleri, M., Rees, H., et al. (1986) Indium 111-granulocyte scanning in the assessment of disease extent and disease activity in inflammatory bowel disease. A comparison with colonoscopy, histology, and fecal indium 111-granulocyte excretion. Gastroenterology 90, 1121–1128. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3956932.

[27] Villanacci, V., Antonelli, E., Geboes, K., et al. (2013) Histological healing in inflammatory bowel disease: a still unfulfilled promise. World J. Gastroenterol. 19, 968–978. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3582008&tool=pmcentrez&rendertype=abstract [Accessed November 14, 2014].

[28] Nishida, S., Hadjis, N. S., Levi, D. M., et al. (2004) Intestinal and multivisceral transplantation after abdominal trauma. J. Trauma 56, 323–327. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14960974 [Accessed February 3, 2015].

[29] Sylvester, K. G., Ling, X. B., Liu, G. Y., et al. (2013) A novel urine peptide biomarker-based algorithm for the prognosis of necrotising enterocolitis in human infants. Gut 1–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24048736 [Accessed June 23, 2014].

[30] Santucci, L., Fiorucci, S., Rubinstein, N., et al. (2003) Galectin-1 suppresses experimental colitis in mice. Gastroenterology 124, 1381–1394. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0016508503002671 [Accessed June 16, 2014].

[31] Shan, M., Gentile, M., Yeiser, J. R., et al. (2013) Mucus enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals. Science 342, 447–453. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4005805&tool=pmcentrez&rendertype=abstract [Accessed June 22, 2015].

[32] Nishida, A., Lau, C. W., and Mizoguchi, A. (2015) Examination of the role of galectins in intestinal inflammation. Methods Mol. Biol. 1207, 231–248. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25253144 [Accessed July 7, 2015].

[33] Leij, J., van der Berg, A., van den Harms, G., et al. (2007) Strongly enhanced IL-10 production using stable galectin-1 homodimers. Mol. Immunol. 44, 506–513. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16581128 [Accessed June 23, 2014].

[34] Nishida, A., Nagahama, K., Imaeda, H., et al. (2012) Inducible colitis-associated glycome capable of stimulating the proliferation of memory CD4+ T cells. J. Exp. Med. 209, 2383–2394. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3526363&tool=pmcentrez&rendertype=abstract [Accessed June 22, 2014].

[35] Hokama, A., Mizoguchi, E., Sugimoto, K., et al. (2004) Induced reactivity of intestinal CD4(+) T cells with an epithelial cell lectin, galectin-4, contributes to exacerbation of intestinal inflammation. Immunity 20, 681–693. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15189734 [Accessed June 23, 2014].

[36] Zhu, C., Anderson, A. C., Schubart, A., et al. (2005) The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. Nat. Immunol. 6, 1245–1252. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16286920 [Accessed June 4, 2014].

[37] Shi, F., Guo, X., Jiang, X., et al. (2012) Dysregulated Tim-3 expression and its correlation with imbalanced CD4 helper T cell function in ulcerative colitis. Clin. Immunol. 145, 230–240. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23117395 [Accessed November 14, 2014].

[38] Christophi, G. P., Rong, R., Holtzapple, P. G., et al. (2012) Immune markers and differential signaling networks in ulcerative colitis and Crohn's disease. Inflamm. Bowel Dis. 18, 2342–2356. Available at: http://www.pubmedcentral.

nih.gov/articlerender.fcgi?artid=3407828&tool=pmcentrez&rendertype=abstract [Accessed June 16, 2014].

[39] Neurath, M. F. (2002) The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. J. Exp. Med. 195, 1129–1143. Available at: http://www.jem.org/cgi/doi/10.1084/jem.20011956 [Accessed June 16, 2014].

[40] Starossom, S. C., Mascanfroni, I. D., Imitola, J., et al. (2012) Galectin-1 deactivates classically activated microglia and protects from inflammation-induced neurodegeneration. Immunity 37, 249–263.

[41] Molander, P., Färkkilä, M., Ristimäki, A., et al. (2015) Does fecal calprotectin predict short-term relapse after stopping TNFα-blocking agents in inflammatory bowel disease patients in deep remission? J. Crohns Colitis. 9, 33–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25052347 [Accessed September 2, 2015].

[42] García-Sánchez, V., Iglesias-Flores, E., González, R., et al. (2010) Does fecal calprotectin predict relapse in patients with Crohn's disease and ulcerative colitis? J. Crohns Colitis. 4, 144–152. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21122498 [Accessed September 14, 2015].

[43] Høie, O., Aamodt, G., Vermeire, S., et al. (2008) Serological markers are associated with disease course in ulcerative colitis. A study in an unselected population-based cohort followed for 10 years. J. Crohns Colitis. 2, 114–122. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21172201 [Accessed September 16, 2015].

[44] Bouguen, G., Levesque, B. G., Feagan, B. G., et al. (2015) Treat to target: a proposed new paradigm for the management of Crohn's disease. Clin. Gastroenterol. Hepatol. 13, 1042–1050.e2. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1542356513013013.

[45] Travis, S. P. L., Higgins, P. D. R., Orchard, T., et al. (2011) Review article: defining remission in ulcerative colitis. Aliment. Pharmacol. Ther. 34, 113–124. Available at: http://doi.wiley.com/10.1111/j.1365-2036.2011.04701.x.

AQ1: Please check if the inserted short title is appropriate and correct wherever required.

AQ2: Please provide five authors' names before placing the et al. in references.

AQ3: Please confirm that given names (red) and surnames/family names (green) have been identified correctly.