



Alimentary Tract

Claudin-2: A marker for a better evaluation of histological mucosal healing in inflammatory bowel diseases



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ABSTRACT

Background: Histological mucosal healing has become a paramount target goal to achieve in the treatment of inflammatory bowel diseases. However, there is still a lack of agreement on the best way to reach this goal, since numerous histological scores are available worldwide.

Aims: We investigated whether claudin-2, a member of claudin family involved in the regulation of intestinal tight junctions, might be useful to assess the presence of active disease in patients with inflammatory bowel diseases.

Methods: Biopsies from 123 patients with ulcerative colitis, Crohn's disease, infectious colitides and irritable bowel syndrome patients were tested with immunohistochemistry for claudin-2.

Results: Claudin-2 appeared to be a very sensitive marker of disease activity in inflammatory bowel diseases, but was negative in the other kinds of patients. In addition, immunohistochemistry for claudin-2 showed good reproducibility by different pathologists.

Conclusions: Should these findings be confirmed in more numerous cohorts of patients, and especially in those with minimal or focal residual disease activity, this simple assessment could be useful in the routine daily practice to facilitate the task of pathologists and clinicians in the diagnosis and management of patients with inflammatory bowel diseases.

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1. Introduction

Inflammatory bowel diseases (IBD), which encompass Crohn's disease (CD) and ulcerative Colitis (UC), are chronic relapsing inflammatory disorder of unknown etiology involving the gastrointestinal tract. Histological evaluation of biopsies is central to the diagnosis, and recently has attracted interest as an additional tool for disease management, particularly for UC [1]. Over the years, the therapeutic goal in IBD have evolved from a mere symptomatic relief to mucosal healing (MH) [2,3]. Various definitions of MH are available, and the most recent ones include a combination of endoscopic and histological remission. Histological remission is defined by absence of neutrophil infiltration in the *lamina propria*

together with neutrophil-mediated mucosal injuries in crypt and surface epithelium [4,5].

Epithelial barrier in IBD is impaired and its dysfunction leads to increased permeability, allowing toxic substances to enter from the intestinal lumen into the intraepithelial compartment, thus triggering the development of disease [6,7] or sustaining inflammation. Tight junctions (TJ) regulate the intestinal epithelial barrier and their molecular structure and regulation are involved in the pathogenesis of IBD [8,9]. TJ are composed by transmembrane proteins such as claudins, occludins, and cytosolic proteins, such as zonulin 1 (ZO-1). TJ situated between apical and lateral regions of the polarised epithelial cells regulate the passage of molecules and ions via the paracellular pathway and restrict the lateral movement of molecules in the cells membrane [10].

Claudin-2 is a member of claudin family and forms high conductance, paracellular cation-selective pores, which regulate the paracellular ion selectivity and water permeability [11,12]. In UC, the expression of TJ proteins, such as occludin, claudin-2 and ZO-1,

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plays a crucial role in MH [13]. Claudin-2 is absent or barely detectable in the healthy human colon but is highly expressed in IBD [14] and its expression positively correlates with disease activity [15].

The aim of our study was to document the possible usefulness of immunohistochemical expression measurement of claudin-2 in the assessment of histological healing of IBD in routine clinical practice.

2. Patients

Biopsy samples of patients with an established diagnosis of CD and UC following treatment, cases of diarrhea-predominant irritable bowel syndrome, and non-IBD colitides as controls, evaluated at the Institute of Pathology of ASST Spedali Civili in Brescia Between January 2022 and January 2024, were assessed. Biopsies were obtained according to ECCO's guidelines [16], with at least two specimens collected from the five colorectal segments (ascending, transverse, descending, sigmoid and rectum) and also from the terminal ileum in CD.

3. Methods

The samples were correctly oriented on acetate cellulose filters, fixed in formalin and embedded in paraffin. Sections stained with haematoxylin and eosin (H&E) were evaluated to grade histological activity. The activity was graduated as: mild, when neutrophils were in the *lamina propria* and the crypt epithelium (cryptitis); moderate, when neutrophils were in crypt lumina (crypt abscesses); severe, in the presence of erosions/ulcerations (granulation tissue). The disease was defined quiescent when the biopsies of the different segments involved by the diseases showed signs of chronic disease (crypt distortion, basal plasmacytosis) without presence of neutrophils [17].

Claudin-2 immunohistochemistry was performed on 4 μ m sections using the antibody Claudin-2 (Cell Signaling Technology, clone E1H90, dilution 1:100). The primary antibody was detected using a biotin-free polymeric-horseradish peroxidase (HRP)-linker antibody conjugate system (Bond Polymer Refine Detection, Leica BioSystems, Newcastle UK) with heat-induced epitope retrieval, using the Bond III automated immunostainer (Leica BioSystems, Melbourne, Australia).

All immunostained sections were evaluated independently by two pathologists with experience in IBD assessment. Claudin-2 was considered expressed when we observed staining with variable intensity along cytoplasmic cell membranes of glandular epithelium and/or of surface epithelium. The stain was semi-quantified according to the following score: 0-absence of immunostaining in glandular epithelium; 1-immunostaining of <10 % epithelial cells in the glands; 2-immunostaining of at least 50 % epithelial cells in the glands; 3-immunostaining of more than 90 % epithelial cells in the gland.

3.1. Statistical analysis

In both UC and CD patients, the correlation between the histological score and claudin-2 expression was assessed by means of the Kendall's rank correlation coefficient [18]. Inter-observer agreement between the two assessing pathologists was calculated by means of the inter-rater agreement (kappa, K) with 95 % confidence intervals (CI). The strength of agreement was quantified as follows: $K < 0.20$ = poor; $K 0.21-0.40$ = fair; $K 0.41-0.60$ = moderate; $K 0.61-0.80$ = good; $k 0.81-1.00$ = very good [19]. Values of $p < 0.05$ were chosen for rejection of the null hypothesis.

3.2. Ethical considerations

The study protocol was approved by the Ethical Committee of Spedali Civili di Brescia (no. 5923, v.3 of 24/05/2023) and carried out according to the principles of Declaration of Helsinki (1964, updated in 2013). Each patient provided written informed consent for the endoscopic examination and the use of the data for scientific purposes.

4. Results

Overall, biopsy samples from a total of 123 patients, including 81 cases of UC (14 women, 67 men, aged 43 (range 29–51) yrs), 19 cases of CD (10 women, 9 men, aged 45 (range 30–57) yrs), 20 cases of non-IBD colitides (10 women, 10 men, aged 48 (range 32–71) yrs), of which 4 of infective origin (including two cases of Spirochetosis), 10 microscopic lymphocytic/collagenous, 2 ischemic, 2 eosinophilic) and 5 cases of irritable bowel syndrome (4 women 1 man, aged 38 (range 25–47) yrs), were assessed.

The results obtained can be summarized as follows:

- The staining pattern of claudin-2 was located on the lateral cell membranes and tight junctions in the surface epithelium, whereas the nuclei of epithelial and inflammatory cells were negative;
- All cases of non-IBD colitides and irritable bowel syndrome did not express claudin-2 (score 0) (Fig. 1), with the exception of 2 cases of ischemic colitis; a focal positivity was observed only in some cells of the *lamina propria* in a few cases and not in glands;
- In cases of UC the expression of claudin-2 was found only in colonic segments with active disease but not in areas of quiescent colitis (Figs. 2 and 4);
- In CD, claudin-2 expression was limited to the areas with endoscopically visible activity, in both the ileum and in the colon (Fig. 3);

The expression of claudin-2 strongly correlated with the grade of histologic activity (Table 1): in segments with mild activity there was low expression of claudin-2 (score 1) while in the segment with severe activity there was elevated expression of the protein (score 3). Of particular interest was the finding of claudin-2 positivity in 3 cases of CD and 8 cases of UC with minimally active disease, in whom disease activity could have been potentially missed for active treatment during conventional histologic assessment.

The sensitivity of claudin-2 expression to detect active disease was 100 % and the specificity was 91.3 %, with a positive predictive

Table 1
Segment-by-segment correlation between histological score and claudin-2 expression in UC and CD.

UC segments	Kendall's tau	P
Ascending	0.94	<0.0001
Transverse	0.88	<0.0001
Descending	0.94	<0.0001
Sigmoid	0.89	<0.0001
Rectum	0.83	<0.0001
CD segments		
Ileum	0.99	<0.0001
Ascending	0.97	$P = 0.0002$
Transverse	N/A	N/A
Descending	N/A	N/A
Sigmoid	N/A	N/A
Rectum	N/A	N/A

N/A= not available for statistical analysis; in these segments, there were only a few patients with active disease, all of them displaying concordance between the two variables in the few areas of activity.

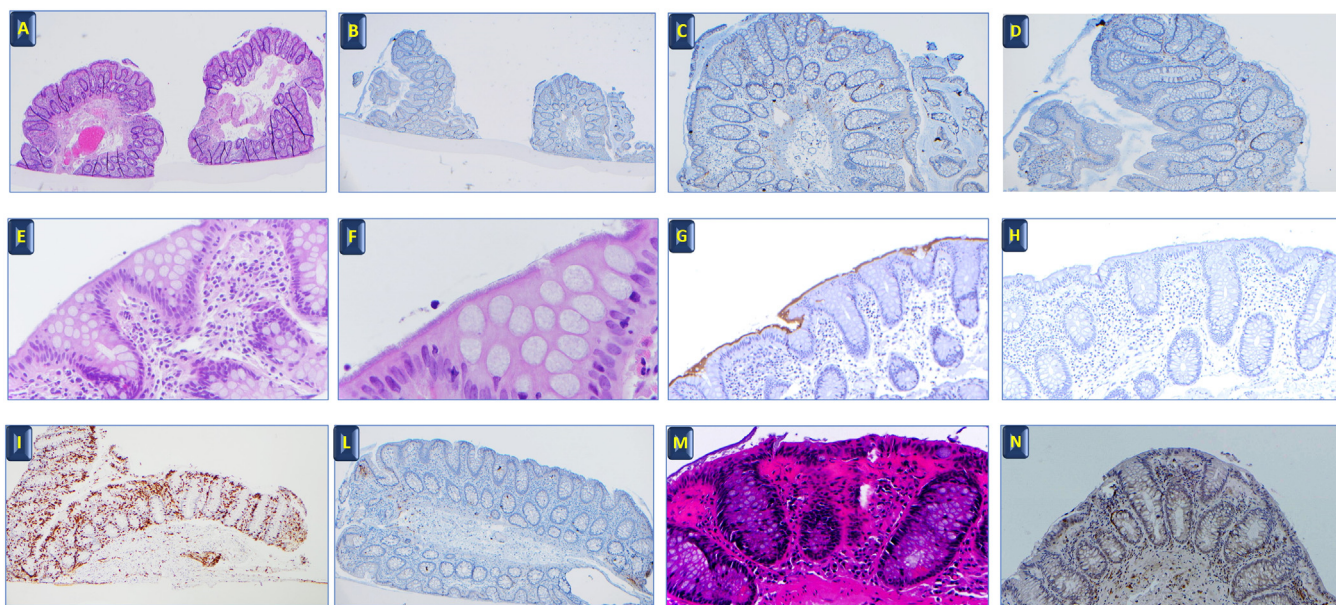


Fig. 1. A-B-C-D: Normal colonic mucosa, non reactive for claudin-2; E-F: *Spirochaetae* adherent to the mucosal surface, H&E, x20, x40, highlighted in G by anti-*Treponema pallidum* antibodies, x40; H: Complete absence of expression of Claudin-2 in a IBS patient, x20; I: lymphocytic colitis (CD3 immunohistochemistry highlights the pathologically increased intraepithelial T lymphocytes), x20; L: absence of expression of claudin-2 in the same patient, x20; M: collagenous colitis (trichrome stain), x20, showing in N: no claudin-2 expression in glandular and surface epithelium, 20x.

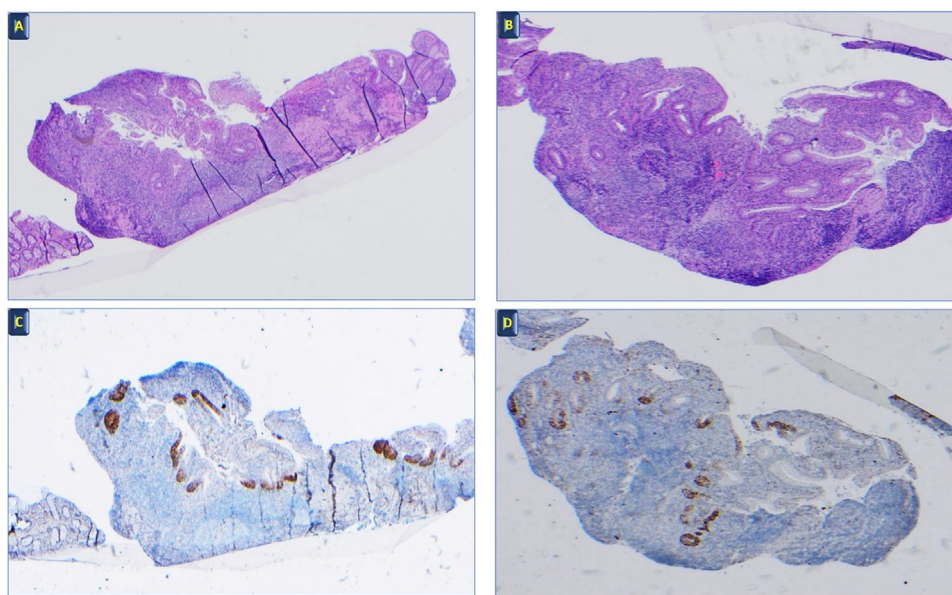


Fig. 2. A-B: UC with moderate/ severe activity. H&E, x20; C-D: in the same patient, the glandular epithelium expresses claudin-2, x20.

value of 98.04 % and a negative predictive value of 100 %. Concerning the inter-rater agreement, this is reported in Table 2. Overall, there was a good to very good concordance between observers in all the examined segments, in both UC and CD.

5. Discussion

Claudin-2 expression, in physiologic conditions, is absent in crypts elements due to the intact presence of the TJ. Its expression, however, changes under different pathological conditions such as cancer, infectious diseases, and inflammation [20]. In fact, an increased expression of claudin-2 is found in UC [14,21,22], CD [23], HIV enteropathy [24], and celiac disease [25]. In active UC, where there are structural changes of the TJ, claudin-2 is up-regulated [26] and its expression extends beyond the crypt base where pro-

Table 2
Segment-by-segment K coefficient for claudin-2 expression in UC and CD.

UC	K	95 % CI
Ascending	0.837	0.738–0.936
Transverse	0.799	0.678–0.919
Descending	0.895	0.810–0.971
Sigmoid	0.913	0.853–0.973
Rectum	0.921	0.861–0.981
CD		
Ileum	1.00	1.00–1.00
Ascending	0.935	0.843–1.00
Transverse	1.00	1.00–1.00
Descending	1.00	1.00–1.00
Sigmoid	1.00	1.00–1.00
Rectum	1.00	1.00–1.00

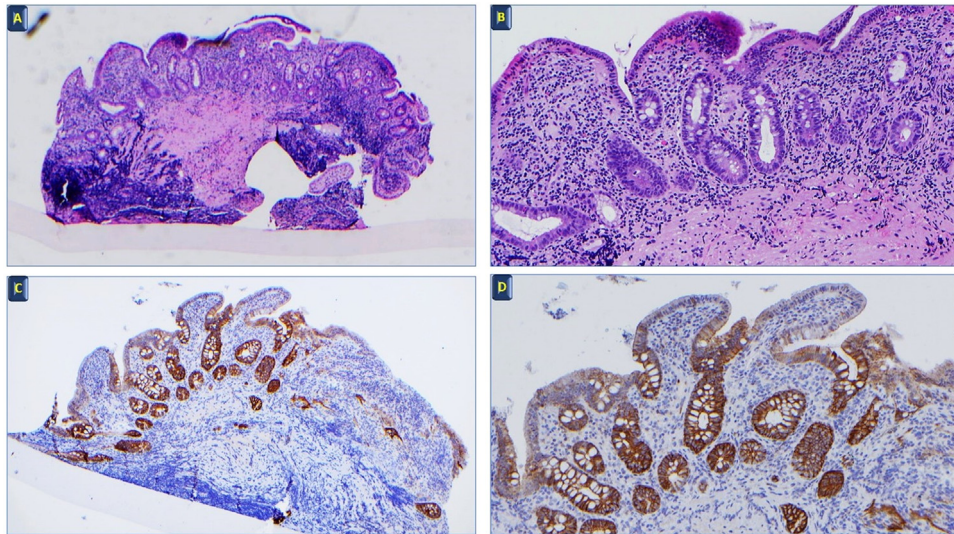


Fig. 3. A-B: active CD. H&E, x10, x20: C-D: in the same patient, the glandular epithelium expresses claudin-2, x10, x20.

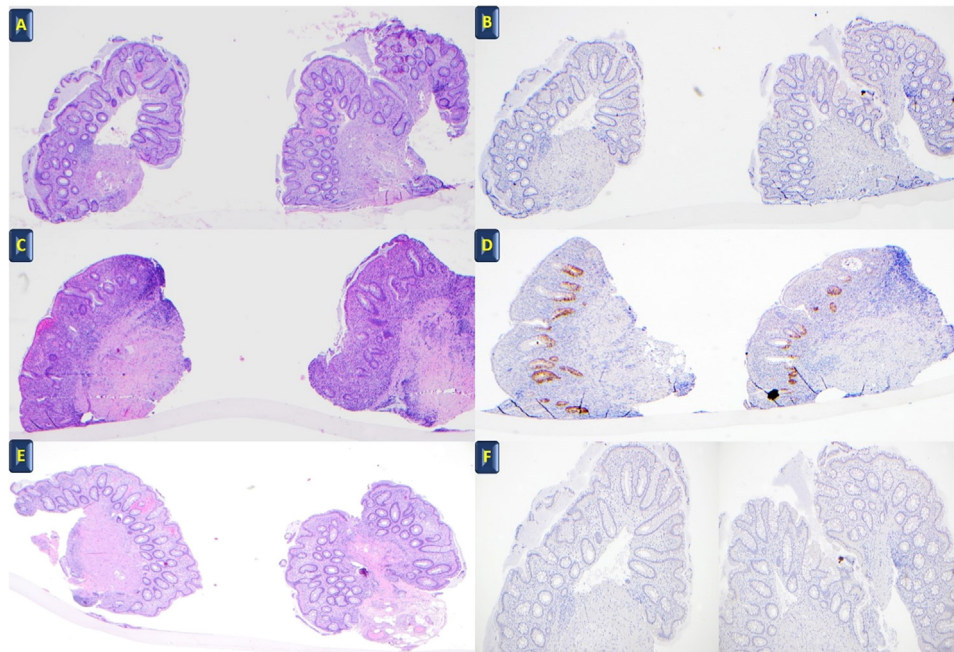


Fig. 4. Representative case of UC in remission in the proximal sigmoid colon and rectum, where claudin-2 was negative (A-B-E-F), and active in the distal sigmoid colon that showed positivity for claudin-2 (C-D).

liferative cells reside. Some studies also suggested the presence of elevated claudin-2 in quiescent disease [26,27].

An altered claudin-2 expression along TJ may cause decreased active absorption and increased mucosal leakiness. The inflammatory cytokines, such as interleukin (IL)-13 and tumor necrosis factor- α (TNF- α), play a critical role in barrier dysfunction [14] by modifying the expression and distribution of TJ proteins [28]. IL-13, an anti-inflammatory Th2 cytokine, is significantly elevated in UC patients compared to control patients or patients with CD [29,30] and causes an upregulation of the claudin-2 gene, thus producing an increase in claudin-2 expression [14]. The proinflammatory cytokine TNF- α is elevated in CD [31] and causes barrier dysfunction by increasing myosin light chain kinase (MLCK) phosphorylation [32]. Of interest, MLCK activation induces IL-13 expression and claudin-2 synthesis [33].

The main treatment target in UC and CD is MH. At present, it is recommended that histological evaluation should be included in the definition of MH [3,34], but histological remission and MH have not been completely defined so far. Several histological scoring systems, to differentiate active vs quiescent disease and to evaluate the therapeutic efficacy, have been proposed to date [35].

In this study we attempted to give a concrete answer, applicable to the daily routine, to the puzzling question of evaluating MH in IBD. In fact, despite the many indices proposed, the histological definition of MH still remains controversial. The situation is further complicated by the fact that nearly all scores include morphological elements that require a subjective evaluation and therefore are a potential source of disagreement [36–38]. Borrowing from the experience with histological assessment in celiac disease, one could thus repeat that “The greater the number of diagnostic cat-

egories of a method the lower the interobserver and intraobserver agreement, with a consequent reduction in its diagnostic reproducibility” [39]. This concept applies perfectly to the controversy over MH evaluation in IBD. For this reason, attempts to simplify histologic scoring have been made with the Simplified Histologic Mucosal Healing Score (SHMHS) [17] and the Picasso score [40], where only neutrophil granulocytes are considered as a sign of activity [41]. However, even these attempts fail in defining a minimum number of biopsies needed. Moreover, the evaluation of basal plasmacytosis, and particularly the threshold value that marks the boundary of a possible recurrence of the disease [42], is dismissed with generic terms as “focal” and “diffuse” [43,44], and the role of eosinophils [45,46] is also neglected, adding further interpretative complications.

In our study claudin-2 staining proved to be highly sensitive to detect the presence of active IBD. The biological explanation of this accuracy likely resides in the alteration of the TJ in the glandular structures present even in the phases of minimal activity. Although there is no doubt that the diagnosis mainly relies on H&E staining, we feel that claudin-2 could be a useful additional biomarker of MH, possibly useful for a better clarification of the location and degree of activity of the disease, and especially to identify areas with minimal disease activity, areas that sometimes can be missed. Of course, this would require a specifically addressed trial.

Immunohistochemistry is a simple technique, available in all pathology laboratories, requiring no additional training, and with minimal costs. In this regard, the use of cellulose acetate filters, which helps in the orientation of the biopsies and allows to fit several biopsies in one or two slides, considerably reduces the economic costs of immunohistochemistry and its subsequent evaluation. It is also worth noting that claudin-2 expression was precise in identifying areas of CD activity.

To date, no histological index has been yet validated for CD and all the available score are developed based on UC and loosely retrofitted to CD. Therefore, claudin-2 could help to quantify the superficial extension of histologic disease activity, potentially supporting the research on the role of microscopic disease activity in CD assessment.

In our present experience, the only other positive cases to claudin-2 were biopsies of acute severe ischemic colitis, possibly due to the critical alteration of crypts that occurs in ischemic damage. However, the differential diagnosis of active IBD and ischemic colitis is usually straightforward, since several other clinical factors point to one or the other pathological condition, and we believe that this should not represent a concern in daily practice.

In conclusion, we feel that claudin-2 immunohistochemistry, because of its simplicity of application and evaluation, could represent a biomarker of mucosal activity/healing and a practical tool to simplify the evaluation of microscopic disease severity in the daily clinical practice. Should these findings be replicated in larger series, especially concerning minimally active disease, we hope that claudin-2 could be a further histological “weapon” to better direct the therapy, with a view to continuous fruitful cooperation between pathologists and gastroenterologists...a further stride in STRIDE [47].

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Author contributions

Vincenzo Villanacci, Gabrio Bassotti: Conceptualization (equal)- data curation (equal)- formal analysis (equal)- investigation (equal)- methodology (equal)- writing original draft (lead)-review and editing; Rachele Del Sordo, Francesco Lanzarotto, Chiara Ricci,

Angelo Sidoni, methodology (equal)- writing original draft; Stefania Manenti, Sara Mino, Mattia Bugatti review and editing.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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