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Original Article

Detection of Calprotectin S100A8/A9 in Inflammatory Bowel Disease by Immunochemiluminescence and Immunohistochemistry

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Abstract: Calprotectin (S100A8/A9) constitutes approximately 60% of the cytosolic protein content of neutrophils and serves as a marker of leukocyte activation and migration, providing valuable insight into the intensity and pattern of inflammation. This study aimed to evaluate the tissue expression and quantification of calprotectin in anorectal samples from patients with inflammatory bowel disease (IBD) using immunochemiluminescence and immunohistochemistry. Anti-calprotectin antibodies were conjugated with acridine ester for chemiluminescent detection and applied to tissue extracts. In parallel, indirect immunohistochemistry was performed on paraffin-embedded anorectal sections from patients with Crohn's disease and ulcerative colitis, and from non-IBD controls. Quantitative and semiquantitative analyses were compared between groups. Calprotectin levels were significantly elevated in IBD tissues compared with controls ($p < 0.05$) in both detection methods. The chemiluminescent assay demonstrated higher analytical sensitivity, enabling quantification even in samples with mild histological inflammation. Increased tissue calprotectin reflects enhanced neutrophil infiltration and activation within the intestinal mucosa, corroborating its role as a local biomarker of inflammatory activity in IBD.

Keywords: Inflammatory bowel disease; Calprotectin; Chemiluminescent Immunoassay; Crohn Disease; Ulcerative Colitis.

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1. Introduction

Inflammatory bowel diseases (IBD), which include Crohn's disease (CD) and ulcerative colitis (UC), are chronic, multifactorial disorders characterized by recurrent episodes of inflammation affecting the gastrointestinal tract [1]. Although the incidence, anatomical distribution, clinical presentation, progression, and therapeutic response may differ, both CD and UC share similar etiopathogenic mechanisms [2, 3]. Experimental and clinical studies suggest that these disorders result from the combined influence of environmental, immunological, microbiological, and genetic factors that compromise the integrity of the intestinal mucosal barrier [4]. Trans-epithelial migration of enteric bacteria activates the innate immune system, triggering the inflammatory cascade [5, 6].

Because there are no pathognomonic features that clearly distinguish CD from UC, other forms of enterocolitis, or irritable bowel syndrome, diagnosis is based on an integrated evaluation of clinical data, disease history, lesion distribution, and complementary imaging, laboratory, endoscopic, and histopathological findings [7,8]. The inflamed intestinal mucosa contains abundant neutrophils and proteins derived from these cells, including alpha-1-antitrypsin, elastase, calprotectin, and lactoferrin. Calprotectin, a calcium- and zinc-binding protein of the S100 family, accounts for approximately 60% of the cytosolic protein content of neutrophils and plays both immunoregulatory and antimicrobial roles [9]. Elevated calprotectin levels in IBD result from increased neutrophil infiltration into the intestinal mucosa and transmigration into the intestinal lumen. Other neutrophil-derived proteins such as elastase, myeloperoxidase, and lysozyme have also been investigated as potential markers of gastrointestinal inflammation. However, calprotectin offers an advantage over these molecules due to its abundance in the neutrophil cytosol [9].

Calprotectin concentrations in active IBD have been reported to range from 200 mg/kg to 20,000 mg/kg, supporting the hypothesis that its elevation reflects neutrophil migration from inflamed mucosa into the intestinal lumen [10, 11]. Immunochemiluminescence assays are based on the detection of light emissions generated by the reaction between an antigen and an antibody labeled with a chemiluminescent compound such as acridine ester. This method provides greater analytical sensitivity than spectrophotometric or colorimetric assays, allowing detection in the femtomole to attomole range (10^{-15} to 10^{-18} mol) with high stability [12, 13].

Previous studies from our laboratory have demonstrated that biomolecules labeled with acridine esters can specifically and quantitatively detect different antigens with superior sensitivity compared with traditional histochemical methods [14–16]. While fecal and serum calprotectin levels are widely used to monitor intestinal inflammation, these measurements primarily reflect luminal or systemic inflammatory activity rather than the local mucosal response. Direct assessment of calprotectin expression in intestinal tissue may provide more precise information regarding the spatial distribution and intensity of inflammation at the cellular level. Moreover, tissue-based detection enables the evaluation of histopathological patterns in parallel with biomarker quantification, offering potential advantages for research and diagnostic standardization [19-20]. Considering the limited number of studies exploring tissue calprotectin using highly sensitive analytical methods, our study aimed to investigate the expression and quantification of calprotectin (S100A8/A9) in anorectal tissues of patients with Crohn's disease and ulcerative colitis by combining immunochemiluminescence and immunohistochemistry.

2. Methods

2.1 Cases Selection

Anorectal tissue samples were obtained by biopsy (Professor Medina Biopsy Forceps, 3 mm) from 39 patients with a confirmed diagnosis of Crohn's disease (CD; n = 9) or ulcerative colitis (UC; n = 30), all under regular follow-up at the Proctology Outpatient Clinic of Hospital Barão de Lucena, Pernambuco, Brazil. As controls (n = 6), anorectal tissue samples from cadaveric donors with post-mortem intervals of less than 24 hours were used. To minimize protein degradation in control samples, tissues were collected during routine autopsies, immediately fixed in 10% buffered formalin, and processed under the same laboratory conditions and timelines as patient samples.

Inclusion criteria comprised confirmed diagnosis of CD or UC established by combined clinical, radiological, endoscopic, and histopathological criteria. Exclusion criteria included indeterminate colitis or recent surgical intervention for gastrointestinal disorders. Sociodemographic and clinical variables (age, sex, lesion location, history of cancer, and associated diseases) were collected through structured questionnaires.

2.2 Anti-Calprotectin Conjugation with Acridinium Ester

Conjugation of acridinium ester (AE) to monoclonal anti-calprotectin antibodies (S100A8/A9, clone MRP8/14, BMA Biomedicals, Switzerland) was performed using the Acridinium Ester Labeling Kit (Enzo Life Sciences, Catalog No. ADI-907-001). A mixture of 100 μL of antibody and 1 μL of AE solution was incubated for 1 h at 25 °C. The conjugate (anti-calprotectin-AE) was purified by molecular exclusion chromatography using a Sephadex G-25 column (10 × 1 cm) pre-equilibrated with phosphate-buffered saline (PBS, pH 7.2). Fifteen 1 mL fractions were collected during elution, and protein content was determined by optical density at 280 nm using an Ultrospec 2100 Pro spectrophotometer (Amersham Biosciences).

2.3 Immunochemiluminescent Measurements

For chemiluminescent analysis, tissue fragments ($0.3 \times 0.3 \times 8 \times 10^{-4}$ cm) were incubated with 100 μL of anti-calprotectin-AE conjugate for 2 h at 4 °C, then washed in PBS. The samples were transferred to 1.5 mL polypropylene tubes containing 100 μL of PBS. Chemiluminescence was measured using a Turner BioSystems luminometer (single-tube module 9200-001). To initiate the reaction, 50 μL of 0.5% H_2O_2 + 0.1 N HNO_3 (Trigger A) was added to 50 μL of the sample, followed by 50 μL of 0.25 N NaOH (Trigger B) to induce alkalinization. Light emission was quantified as Relative Light Units (RLU) with a counting time of 5 s per sample.

2.4 Immunohistochemistry Study

Tissue samples were sectioned in triplicate and subjected to indirect immunostaining using monoclonal anti-calprotectin (S100A8/A9; BMA Biomedicals). Antigen retrieval was performed in 100 mM citrate buffer (pH 6.0) for 2 min after boiling in a pressure cooker. Sections were cooled to 35 °C and incubated with 3% hydrogen peroxide for 30 min at room temperature to block endogenous peroxidase. Samples were then incubated with anti-calprotectin antibodies (1:200 dilution) for 2 h at 37 °C. Detection was performed using the Reveal Polyvalent Free-Biotin DAB System (Biogen). PBS (100 mM, pH 7.2, with 150 mM NaCl) was used for all washing steps. For negative controls, the primary antibody was replaced with PBS. IImmunohistochemical slides were independently analyzed by two blinded pathologists to minimize observer bias. Discrepancies were resolved by consensus. Inter-observer agreement was assessed using the Cohen's κ coefficient, which yielded a value of 0.70, indicating substantial agreement.

2.5 Digital Histomorphometric Analysis

For quantitative analysis, three representative microscopic fields were selected per sample. Images at 400× magnification were captured using a Samsung CCBBW-410 video camera coupled to an Olympus BH-2 microscope and analyzed with MOTIC Image Plus 2.0 software. Histomorphometric analysis was performed using GIMP 2.8.18 software (GNU Image Manipulation Program). Calprotectin-positive areas were quantified in pixels per field (total field area = 12,234 μm^2).

2.6 Statistical Analysis

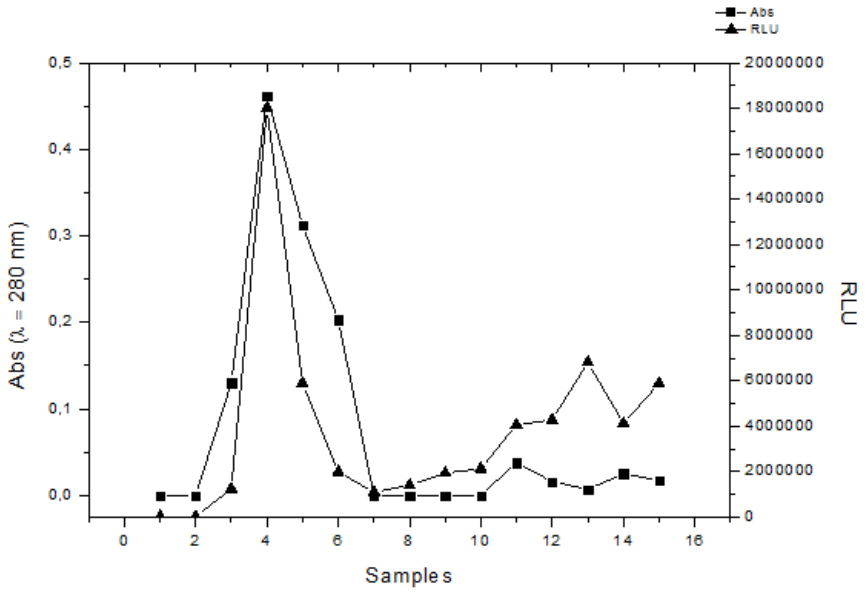
Data obtained from the immunochemiluminescence assay were expressed as mean ± standard deviation and analyzed using OriginPro 8.0 SR4 (OriginLab Corporation, Northampton, MA, USA). Immunohistochemistry data were analyzed using GraphPad Prism 7.0 (GraphPad Software, USA). Data normality was assessed using the Shapiro–Wilk test. Given the small sample size of some subgroups, non-parametric analyses were prioritized when normality could not be confirmed. Comparisons among groups were performed using one-way ANOVA followed by Tukey's post hoc test for parametric data, and Kruskal–Wallis followed by Dunn's test for non-parametric data. When applicable, Spearman's rank correlation was used to evaluate associations between

calprotectin levels and clinicopathological variables. Statistical significance was established at $p < 0.05$.

3. Results

The study population consisted of three groups: Crohn's disease (CD, n = 9), ulcerative colitis (UC, n = 30), and controls (n = 6). The mean ages were 39 ± 7 years for CD and 44 ± 8 years for UC. Female predominance was observed in both groups (55% in CD and 75% in UC). None of the participants had a history of cancer, although a positive family history in first-degree relatives was reported in seven patients with CD and nineteen with UC. Alcohol consumption was identified in 44% of CD and 29% of UC patients. Smoking or a history of smoking for more than two years was present in 55% of CD and 33% of UC cases. The anti-calprotectin antibody was successfully conjugated with acridinium ester (AE). Molecular exclusion chromatography (Sephadex G-25) confirmed the conjugate formation, with overlapping protein and chemiluminescent peaks corresponding to the fourth elution fraction (Figure 1).

Figure 1. Chromatographic profile of the conjugate (AntiCalprotectin-AE) on a column of Sephadex G-25 (10 x 1cm). Elution performed with 10 mM phosphate buffer in 0.15 M NaCl (PBS), pH 7.2. Fractions (1ml samples) were collected and absorbance and chemiluminescence measured.



Quantitative analysis demonstrated that calprotectin levels were significantly elevated in IBD tissues compared with controls (Figure 2). The mean relative light unit (RLU) values were 308,184 ± 69,199 (Range 112,846–590,203) for CD, 271,687 ± 65,669 (Range 101,235–658,948) for UC, and 93,644 ± 2,226 for controls. All measurements were performed in triplicate, with a coefficient variation between 24.2% and 38.9%. Statistical analysis confirmed a significant difference in calprotectin chemiluminescence between IBD and control tissues ($p < 0.05$, Tukey's post-hoc test), while no significant difference was observed between CD and UC groups ($p > 0.05$).

Immunohistochemical evaluation revealed cytoplasmic calprotectin expression predominantly within infiltrating polymorphonuclear leukocytes and macrophages in inflamed mucosa (Figure 3). Although visual staining intensity was modest compared with chemiluminescent signal amplitude, the quantitative histomorphometric analysis demonstrated significantly higher calprotectin-positive area in IBD tissues than in controls (Figure 4).

Figure 2. Comparison between the immuchemiluminescence of Crohn's disease (CD, n = 9), ulcerative colitis (UC, n = 30) and normal anorectal (n = 6) labeled with AntiCalprotectin-AE. Statistically significant variations were observed among RLU values of both inflammatory bowel disease (IBD) and normal anorectal.

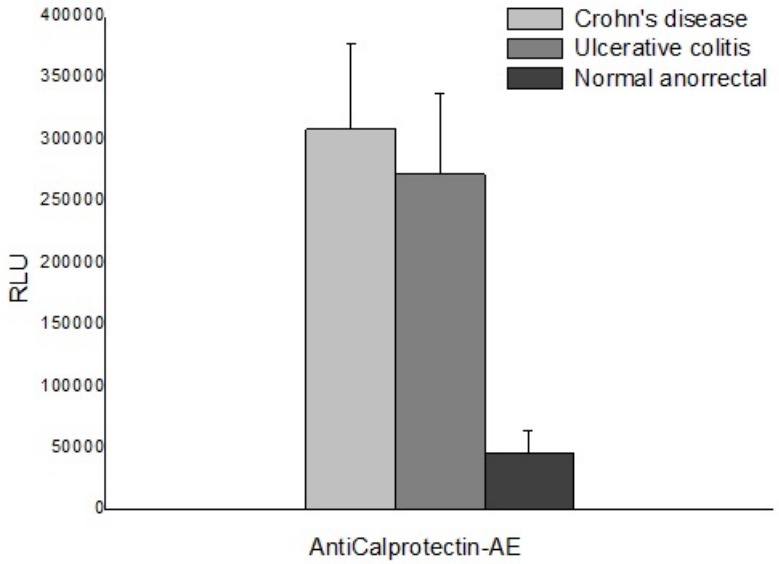


Figure 3. Immunoexpression of calprotectin in patients with inflammatory bowel disease (Magnification 400x).

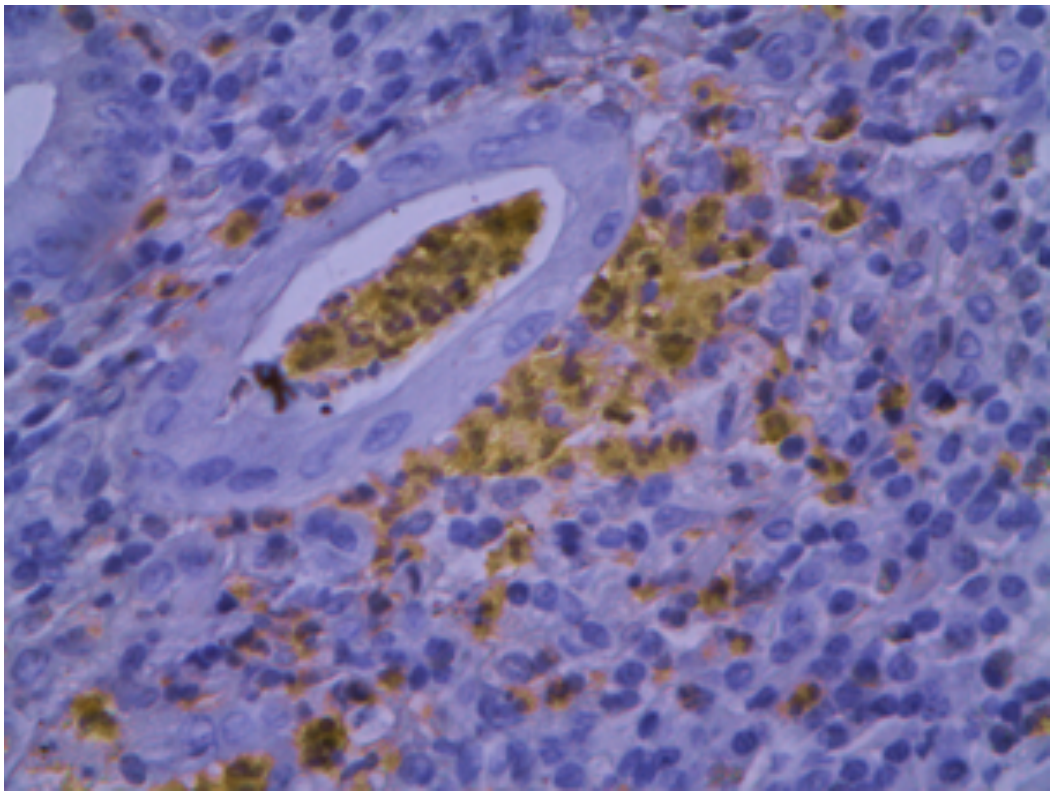
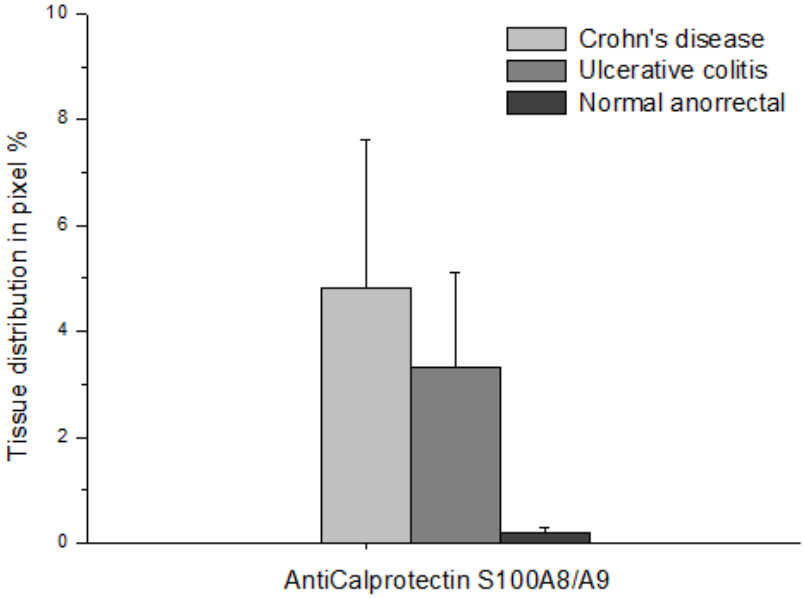


Figure 4. Percentage of immunoreactivity (in pixel) of the calprotectin per field captured (total area 12,234 μm²).



The mean pixel density per field was significantly greater in inflamed tissues ($p < 0.05$, Shapiro–Wilk = 0.9167), confirming the quantitative correlation between immunohistochemical signal and inflammatory status. Morphometric data are summarized in Table 1. No qualitative differences in staining patterns were observed between CD and UC, suggesting a shared inflammatory pathway involving neutrophil-derived calprotectin.

Table 1: Morphometric data of calprotectin immunoexpression in tissues of anorectal patients with Crohn's disease, ulcerative colitis and controls.

	Crohn's Disease		**Ulcerative Colitis**		**Control**	
	Pixel	**μ**	**Pixel**	**μ**	**Pixel**	**μ**
Minimum	15647	4,13 x106	7186	1,9 x106	53	14022,74
Maximum	87233	2,3 x107	57942	1,5 x107	543	143666,9
Average	37975	10 x106	26411,8	6,9 x107	192,8	51019,8

*Total area of the capture field = 12,234 μm²

4. Discussion

The prevalence of inflammatory bowel disease (IBD) in Western countries has reached approximately 0.5% of the general population, and incidence rates continue to rise in newly industrialized regions, suggesting a global epidemiologic transition of these disorders [19]. Populations with high prevalence, such as those in North America and Europe, show a greater frequency of IBD between the second and fourth decades of life, affecting men and women equally. The age and sex distribution observed in our cohort are consistent with these recent epidemiological findings [19, 20].

Several environmental and behavioral factors have been associated with IBD onset or progression. Smoking has been linked to an increased risk of Crohn's disease (CD), whereas smoking cessation, but not active smoking, has been associated with ulcerative colitis (UC) [20, 21]. Dietary factors, alcohol consumption, psychological stress, sleep disturbances, and low vitamin D levels have also been implicated in disease susceptibility [21]. Consistent with previous studies, a considerable proportion of our IBD patients reported smoking and alcohol use. The association between IBD and colorectal cancer is

well established, particularly in UC, which involves continuous mucosal inflammation confined to the colon. This pattern likely contributes to the higher incidence of colitis-associated carcinoma in UC compared with CD [22].

Calprotectin (S100A8/A9) is a cytosolic protein abundantly expressed in activated neutrophils and monocytes. Acting as a calcium- and zinc-binding molecule, it functions as a damage-associated molecular pattern (DAMP) released during cellular stress and inflammation [23]. Numerous clinical studies have confirmed the diagnostic and prognostic value of calprotectin in IBD, particularly in fecal assays that reflect neutrophilic inflammation in the intestinal lumen [24,25]. Fecal calprotectin has been shown to correlate more strongly with histological indices than with endoscopic scores, suggesting that its increase may precede visible mucosal lesions [24]. Brazilian studies have reported mean fecal calprotectin concentrations ranging from 405–686 μg/g in IBD patients, values significantly higher than those observed in functional bowel disorders [26, 27]. Walsham and Sherwood [28] proposed an algorithmic approach for differential diagnosis, in which fecal calprotectin values above 150 μg/g are suggestive of IBD or colorectal carcinoma.

While fecal and serum calprotectin quantifications have been extensively validated, tissue-based detection remains less explored. In our study, the use of acridinium ester–conjugated antibodies enabled the detection of calprotectin directly in anorectal tissue extracts from IBD patients. The immunochemiluminescent method demonstrated high analytical sensitivity, with mean relative light unit (RLU) values of 308,184 for CD and 271,687 for UC, both significantly higher than those of control tissues. These findings highlight the potential of this method for detecting minute quantities of antigen and reinforce its analytical stability.

The immunohistochemical analysis showed cytoplasmic calprotectin expression predominantly in polymorphonuclear leukocytes and macrophages within inflamed mucosa, consistent with previous descriptions of its cellular localization [29]. Although visual staining intensity appeared modest, the quantitative morphometric analysis confirmed significantly higher calprotectin-positive areas in IBD compared with controls. These results validate the histopathological relevance of calprotectin as a marker of active mucosal inflammation and complement the chemiluminescent quantification.

This study has some limitations that should be acknowledged. The sample size, particularly in the control group, was relatively small and may have reduced statistical power. Additionally, post-mortem control tissues could be subject to partial protein degradation despite the short post-mortem interval and standardized fixation procedures. Another limitation is the lack of correlation with clinical activity indices, such as the Crohn's Disease Activity Index (CDAI) or the Mayo Score, which could further clarify the relationship between tissue calprotectin and disease severity. Future studies with larger cohorts and clinical correlation are warranted to validate these preliminary findings.

Although our findings demonstrate that immunochemiluminescent detection of tissue calprotectin is feasible and analytically robust, its clinical application remains exploring. For translation into routine diagnostic practice, further standardization is required, including inter-laboratory reproducibility, cost-effectiveness analyses, and validation against established biomarkers such as fecal calprotectin ELISA and endoscopic scoring systems. Nonetheless, the method may represent a valuable complementary tool for research on mucosal inflammation, potentially improving the accuracy of histopathological assessments in IBD.

In summary, this study provides evidence that calprotectin can be quantitatively detected in anorectal tissues of patients with Crohn's disease and ulcerative colitis using immunochemiluminescence, corroborated by histomorphometric immunohistochemical analysis. These findings support the role of tissue calprotectin as a marker of local inflammatory activity and reinforce the potential of chemiluminescent immunoassays for sensitive detection of mucosal biomarkers in IBD.

5. Conclusion

Immunochemiluminescent detection enabled quantitative assessment of calprotectin (S100A8/A9) in anorectal tissue samples from patients with inflammatory bowel disease, showing statistically significant differences compared with control tissues. The results demonstrated strong agreement between chemiluminescent and immunohistochemical analyses, supporting the role of calprotectin as a reliable marker of local mucosal inflammation. Although further studies are needed to validate these findings in larger cohorts and to correlate them with clinical activity indices, the method represents a promising approach for sensitive tissue-based biomarker evaluation in Crohn's disease and ulcerative colitis.

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Conflicts of Interest: The authors declare no conflict of interest.

Supplementary Materials: None.

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