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THE ROLE OF HISTOPATHOLOGY AND IMMUNOHISTOCHEMISTRY IN MONITORING OF PATIENTS WITH INFLAMMATORY BOWEL DISEASE

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I. GENERAL PART. STUDY OBJECTIVES

Inflammatory bowel diseases (IBD) represent a pathology of significant importance and contemporary relevance within the fields of gastroenterology, general surgery, and histopathology. IBD encompasses ulcerative colitis (UC) and Crohn's disease (CD), both of which are characterized by a chronic course marked by alternating periods of relapse and remission. Despite advances in research over the past decades focused on these conditions, current scientific data remain insufficient to enable an optimal and individualized medical approach tailored to each patient category and to the various phenotypes of inflammatory bowel disease.

As evidenced by both retrospective and prospective studies, relapse rates, the occurrence of complications, and progression to refractory or fulminant forms requiring urgent surgical intervention remain at significant levels. However, improvements in these parameters have been observed following the introduction of biological therapies with monoclonal antibodies, as well as newer agents such as anti-interleukin therapies, JAK inhibitors, and anti-integrin treatments. Overall, the endoscopic remission rates per molecule, analyzed individually, do not exceed 60–70% [1]. Therefore, it becomes increasingly necessary to develop research areas that can establish objective criteria and useful markers for assessing the prognosis and monitoring of patients with IBD. The importance of the chosen topic also stems from epidemiological data indicating a significant increase in incidence and prevalence over the past decades, including Romania [2], with a notable impact on the healthcare system. With regard to ulcerative colitis, up to 90% of patients will experience at least one relapse during their lifetime, and an early relapse, as well as the absence of sustained remission within the first two years of disease onset, are associated with an unfavorable prognosis [3]. According to previous studies, up to 25% of patients with ulcerative colitis will require a total colectomy due to disease refractoriness to biological therapy [4]. Regarding Crohn's disease, the rate of surgical interventions is significantly higher, although a slight improvement has been observed in recent decades. Thus, according to meta-analyses, the risk of surgery 10 years after diagnosis is approximately 40% [5], and after 20 years, 75% of patients will have undergone at least one surgical procedure. As previously mentioned, the rate of surgical interventions—which can serve as a surrogate marker for disease refractoriness to medical therapy—has not decreased significantly in recent decades, despite the advent of new therapeutic agents such as biological therapies. Among these, infliximab, an anti-TNF agent, has played a central role,

having been approved in 1998 for Crohn's disease [6] and in 2005 for ulcerative colitis [7]. Another argument supporting the relevance and importance of the chosen topic is the increased risk of developing dysplastic lesions and, subsequently, colorectal cancer (CRC) within 6 to 8 years from the time of diagnosis [8]. In this study, biopsy samples will also include the assessment of dysplastic changes and the immunohistochemical analysis of the p53 positivity index, as well as the Ki-67 proliferation index.

Thus, the main specific objectives of the project include:

1. Expanding current knowledge and correlations related to clinical-biological, endoscopic, histopathological, and immunohistochemical parameters involved in inflammatory bowel diseases, with the aim of deepening the understanding of their underlying mechanisms.
2. Elaborating on the relationship between intestinal inflammation and the development of dysplastic processes.
3. Enhancing the histopathological parameters used in the diagnosis and follow-up of inflammatory bowel diseases, in order to improve the criteria employed in scoring systems that assess lesion activity, treatment response, and remission.
4. Identifying the most specific imaging, histopathological, and immunohistochemical parameters for monitoring inflammatory bowel diseases.
5. Identifying population subgroups at increased risk of progression to severe disease forms, which require individualized strategies for monitoring and therapeutic escalation (step-up approach).

To achieve these objectives, an interdisciplinary and multicenter approach represents a key element, with the current study involving gastroenterologists and pathologists from tertiary centers specializing in the management of inflammatory bowel diseases (Bucharest Emergency Clinical Hospital, Fundeni Clinical Institute, Craiova Emergency Clinical Hospital).

In conclusion, the study proposes a comprehensive and integrated investigation—epidemiological, clinical-biological, endoscopic, histopathological, immunohistochemical, and statistical—of 46 cases of inflammatory bowel disease, through both retrospective and prospective analysis, in the context of patient monitoring and identification of prognostic markers and individualized therapeutic strategies. The results obtained from this study may contribute to refining the selection of biological therapies in inflammatory bowel diseases,

improving the endoscopic and histopathological evaluation criteria for lesions in order to enhance prognosis, and identifying specific patient subtypes that may require early therapeutic escalation or more aggressive monitoring protocols.

II. PERSONAL CONTRIBUTIONS

1. The importance of histologic remission in ulcerative colitis and the use of Nancy index in routine clinical practice

1.1 Introduction

Ulcerative colitis is a chronic inflammatory disease with a varying disease course, still bearing a high risk of surgery at 1, 5, or 10 years after diagnosis, despite a temporal decline in the need for surgery over the last six decades [5]. Since the 1980s, the main objective of clinical trials was the improvement of symptoms. In the following years, cumulative evidence led to the recommendation of some composite scores consisting of symptom resolution and mucosal healing as the primary endpoint [9]. In UC, patients with a Mayo Endoscopic Score (MES) 1 have a threefold risk of clinical relapse compared with MES 0 [10]. In 2007, D'Haens was one of the first authors to highlight histologic remission's importance by stating that it should be strongly considered as a secondary endpoint in clinical trials [11]. There is a lack of a standardized reporting method for histologic remission, which limits its clinical utility in daily practice. As demonstrated by Lemmens et al., there is a high degree of interobserver variability in histopathology reports, except for the extremes of the spectrum, i.e., inactive or severely active disease [12]. The aim of this review is to scrutinize the available data regarding the most recent and validated histologic scores developed for ulcerative colitis and the individual subcomponents to determine the optimum standardized and practical approach.

1.2 Histologic Scores in UC

Around thirty scoring systems were developed to assess histologic activity. Still, they are mainly used in research protocols, and their practical relevance in deciding how to manage patients' therapy is limited [13]. Only a few underwent formal content validation and using non-validated scoring systems may have hindered the development of a systematic microscopic response to treatment in UC.

The Geboes score (GS) was conceptualized more than 20 years ago as a histologic activity system that showed good reproducibility but modest agreement with the endoscopic grading system [14]. The Geboes score evaluates seven histological features of inflammatory bowel disease, but due to its complexity, it never adhered to daily clinical practice. Histologic remission is defined as continuous GS ≤6 or GS ≤2 (absence of epithelial neutrophils) [15]. One of the first studies to prospectively evaluate the association between Geboes grades and long-term outcomes in patients with UC was published in 2016 [16]. In this study, 179 patients with UC in clinical remission were enrolled; the baseline histologic grade was the only independent factor associated with clinical relapse over 12 months. A Geboes score >3.1 had a sensitivity and a positive predictive value of 74% and 40%, respectively, for clinical relapse. In a Geboes score of 0 or 1, the relative risk of clinical relapse was only 0.22 [17].

The Robarts Histopathological Index (RHI) was developed in 2017, and it is mainly derived from the GS. Even though RHI can be considered a simplified version of the GS, an analysis of three prospective cohorts showed that both scores are strongly correlated in their definitions of histological response, as 95% of patients classified as having histological response according to RHI also do so by the same criteria of the GS. There is a paucity of studies reporting the sensitivity of RHI in predicting clinical relapse when compared with GS. This gap in the literature was addressed by one prospective study published in 2022 [18], with 187 UC patients in clinical and endoscopic remission in whom rectal biopsies were performed and histologic remission was defined as RHI < 3. Only 43% of patients with both clinical and endoscopic remission also associated with histologic remission. The risk of relapse was lower in patients with histologic remission than in those with histologic activity. The cumulative risk of relapse in patients with histologic remission at 1 and 3 years was 20.7 (vs. 45% in patients with histologic activity) and 56.3%, respectively (vs. 67.8%) [18]. Surprisingly, in a post hoc analysis [18], there was no significant difference in the risk of relapse between patients who achieved histologic remission and those with histologic activity confined only to the lamina propria (neutrophil infiltrates in the lamina propria, but not in the epithelium).

The Nancy Index (NI) analyses three characteristics of mucosal activity: acute, chronic inflammatory infiltrate and ulceration. With reference to NI, it was found that using only three grades of acute inflammatory cell infiltrate (absent, mild, or moderate to severe) was sufficient, while assessment of basal plasmacytosis as an index of chronic inflammatory infiltrate did not improve the index's sensitivity, being therefore considered futile [19]. This

stepwise evaluation based on the worst feature of each of the three characteristics is simple and practical, leading to ECCO's recommendation of using this score for routine clinical practice [20]. Histologic remission is defined as NI =0, while NI =1 defines histologic response [19]. Due to higher clinical applicability, the Nancy index was compared to the Geboes score to prove validity in a large cohort of more than 400 UC patients [21]. NI was demonstrated to be strongly correlated with GS, with 92% of patients considered to be in histologic remission and 99% of patients considered to have histologic activity by both scores [21].

1.3 Individual markers of histologic activity

The amplitude of the effect of different histologic markers on clinical outcomes has been the subject of a systematic review [22]. The absence of neutrophils in the lamina propria or epithelium predicted a lower risk of relapse or exacerbation, with an RR of 0.32 [22]. This is in contrast to the recently published post-hoc analysis of the review mentioned above, where neutrophils in the lamina propria did not influence the outcome [23]. Other features that predicted relapse/exacerbation were crypt abscesses, eosinophils in lamina propria, and chronic inflammatory cell infiltration. Conversely, the absence of basal plasmacytosis, basal lymphoid aggregates, and architectural crypt distortion were not associated with decreased relapse/exacerbation.

1.4 Conclusions

Taking into consideration the evidence presented above, we believe that a more practical approach would be beneficial from multiple perspectives. First, the homogeneity in histopathology reports that the implementation of activity scores consequently determines a better adherence in clinical practice and would support the use of histologic remission as a formal target. Secondly, the Nancy score seems to be the most simplistic approach, yet it has proven validity when compared to the more complex Geboes score. Though these scores perform well in defining histologic activity, only looking into the status of intraepithelial neutrophils also proved accurate in terms of long-term prognosis, and it represents the most accurate individual histologic marker for remission. Currently, we pledge for the simplified pathway in histopathology report through the use of Nancy Index or by characterisation of individual histologic parameters as we have proceeded in our studies that will be presented in the next chapters.

2. Pathology assessment in inflammatory bowel disease - prospective study from two referral centres

2.1 Introduction

Inflammatory bowel diseases (IBDs) possess a wide spectrum of clinical manifestations and disease severity. The update of STRIDE consensus has proposed a temporal approach with short, intermediate and long-term targets, with symptomatic response at the beginning and endoscopic healing at the end of the spectrum [24]. Histological healing is only asserted as a formal target, particularly for UC [24]. Endoscopic healing is arguably an inaccurate marker of histologic activity since mostly one-third patients with endoscopic remission do not simultaneously express microscopic healing [26]. At the same time, histologic grade has the strongest association with clinical relapse risk [16]. Consequently, some leading experts stated that histologic remission should be considered a critical end-point in clinical trials [11]. This study delves into characterization of histological samples in IBD patients both prior and during biologic treatment, aiming to assess the impact on clinical evolution, therapy response and management, as well as correlations with biochemical and endoscopic parameters.

2.2 Materials and methods

This is a prospective study from two referral centers in Romania assessing histological parameters of 40 biologic therapy-treated IBD patients in two stages of their disease course.

- In the first phase, biopsies were collected before introduction of biologic therapy (either de novo, or switch to another class due to primary/secondary loss of response).
- In the second phase, patients were reassessed after 6-12 months to compare histological findings. For a thorough evaluation, clinical, biochemical (complete blood count, C-reactive protein, fecal calprotectin) and endoscopic parameters were simultaneously gathered.

Patients were considered for enrolment when the disease course asserted introduction of biologic therapy - either escalation from conventional therapy (biologic-naive patients) or switch to another class due to primary/secondary loss of response (biologic-experienced patients). The histopathology report was addressed using the Nancy Index, which is recommended in daily practice due to its accessibility and accuracy [27, 28]. Therapy was

guided in accordance with currently available recommendations published by European Crohn's and Colitis Organization (ECCO) and British Society Guidelines BSG [29,30].

2.3 Results

2.3.1 Acute inflammation

All parameters of acute inflammation that were assessed (cryptitis, abscesses and ulcerations) had a lower incidence in the treated-patient group compared to the initial evaluation. The most significant improvement was observed for mucin depletion, with a 57.8% downgrade (p value 0.018) in the second-phase evaluation. It was followed by cryptitis (54.5%), ulcerations (41.1%) and abscesses (26.6%). Despite the notable improvement in histologic evaluation, the only parameter that reached statistical significance was mucin depletion (p value 0.018).

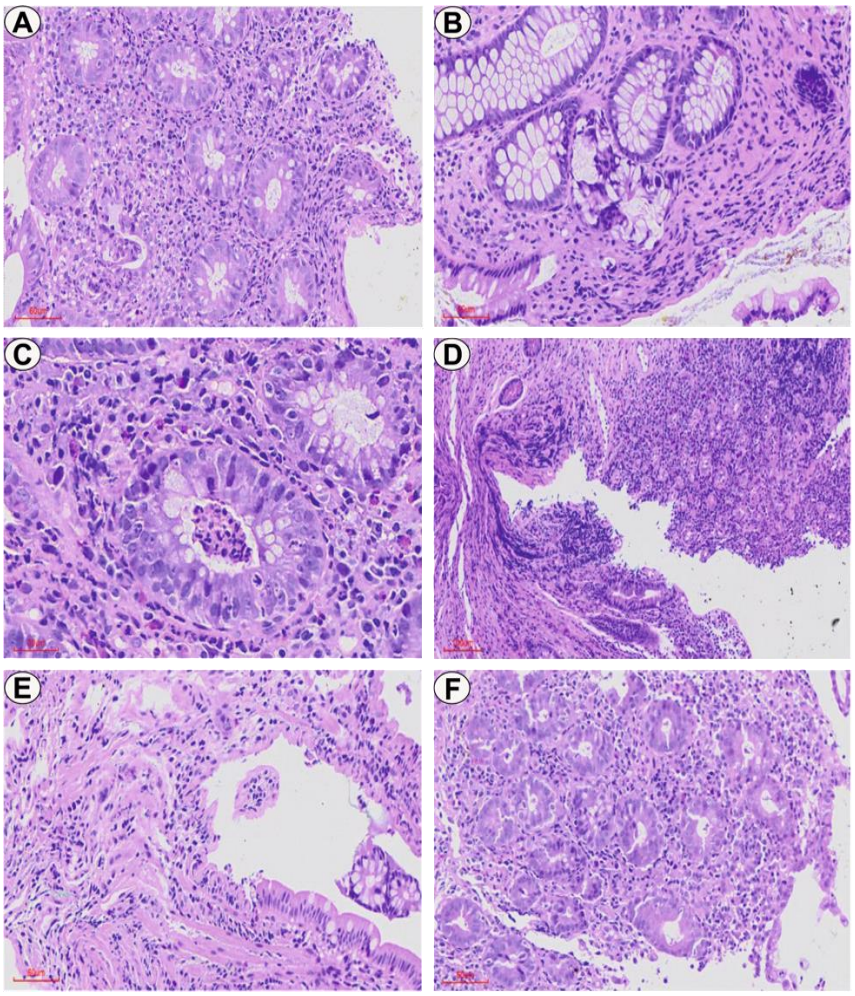

Figure 1. Acute inflammation features. A. UC, before treatment; cryptitis and cryptic abscesses, HE, x 200. **B**. UC, same patient after treatment (6 months); histologic improvement (cryptitis and abscess resolution), HE, x 200. **C.** UC, cryptic abscess, HE, x 400. **D**. CD, before treatment, ulceration, HE, x 100. **E**. CD, same patient after treatment (7 months); fibrosis, HE, x 100. **F**. UC, same treatment; mucin depletion and low-grade dysplasia, HE, x 200.

2.3.2 Chronic inflammation

Basal plasmacytosis and metaplastic changes improved considerably during follow-up evaluation, with a decrease of 83.3% and 64.2% respectably. Both parameters reached statistical significance. A similar significant decline (27.5%) was not observed in the assessment of lymphoid aggregates. Granulomas were detected in the same number of CD patients. Acute and chronic inflammatory parameters that reached statistical significance are depicted in Figure 3.

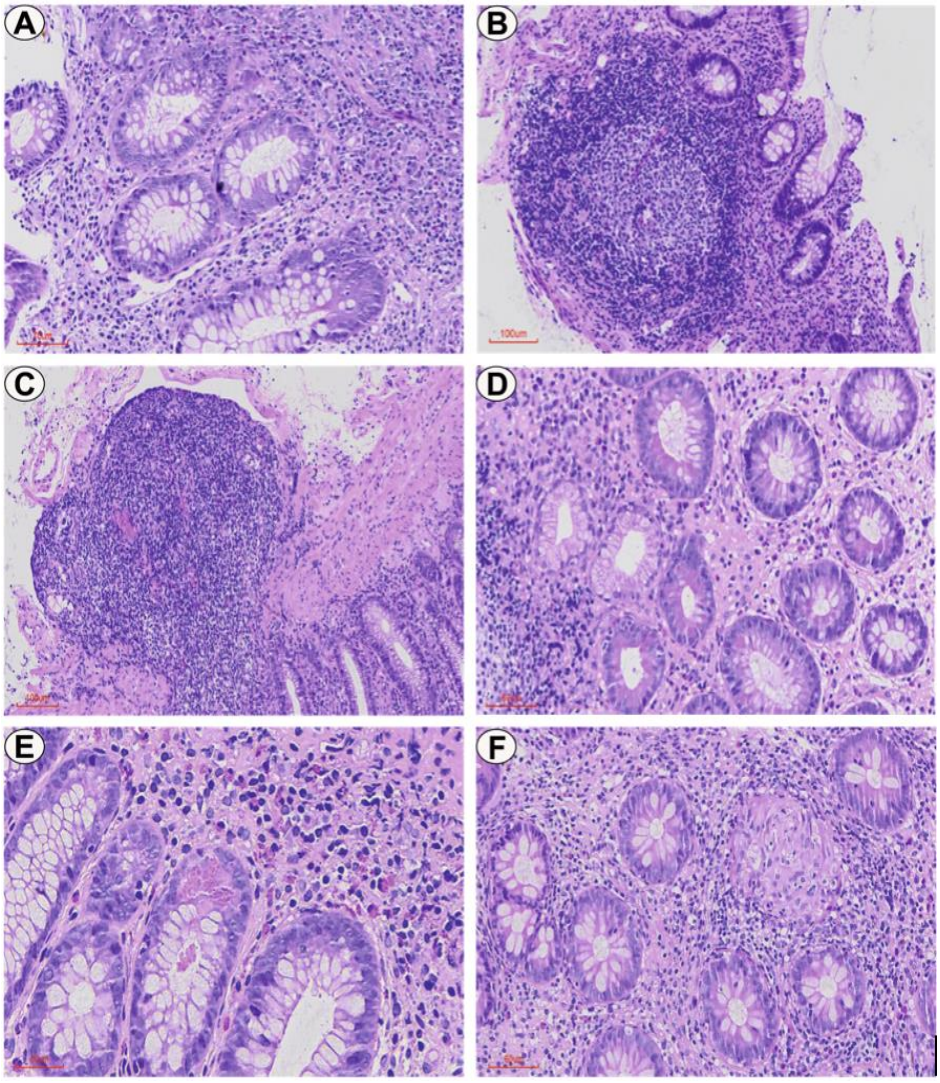

Figure 2. **Chronic inflammation features. A**. UC, basal plasmacytosis, HE, x200. **B-C**. UC, lymphoid aggregates, HE, x100. **D**. CD, postoperative status, Paneth metaplasia (ileo-colic anastomosis), HE, x 200. **E**. UC, Paneth metaplasia, HE, x 400. **F**. CD, granuloma and architectural distortion, HE, x 200.

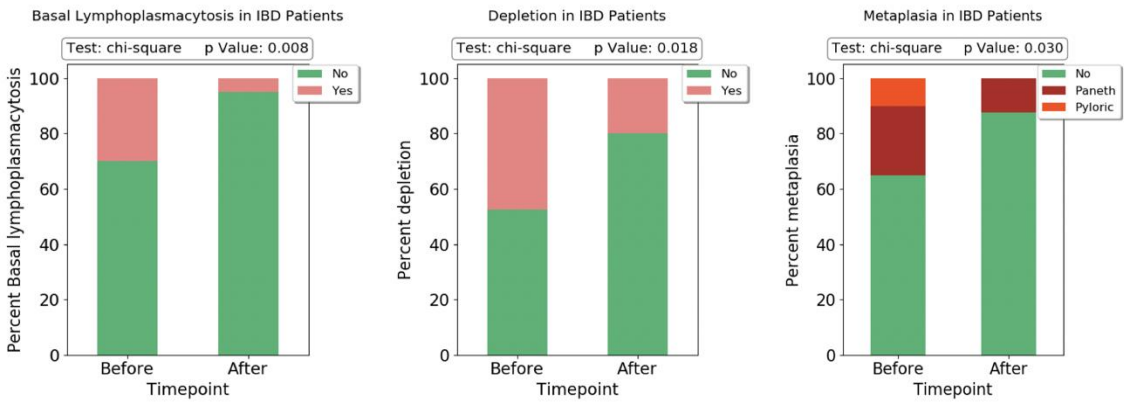

Figure 3. Histopathological features with statistical significance (p<0.05) - basal plasmacytosis, mucin depletion, metaplastic changes.

By univariate analysis, presence of lymphoid aggregates, mucin depletion, and ulcerations indicates a strong positive association with treatment response (OR >4). On the opposite side, patients with a diagnosis of IBD >5 years and previous biologic therapy are the most susceptible to treatment non-response. (OR 0.1 and 0.23, respectively) (Figure 4).

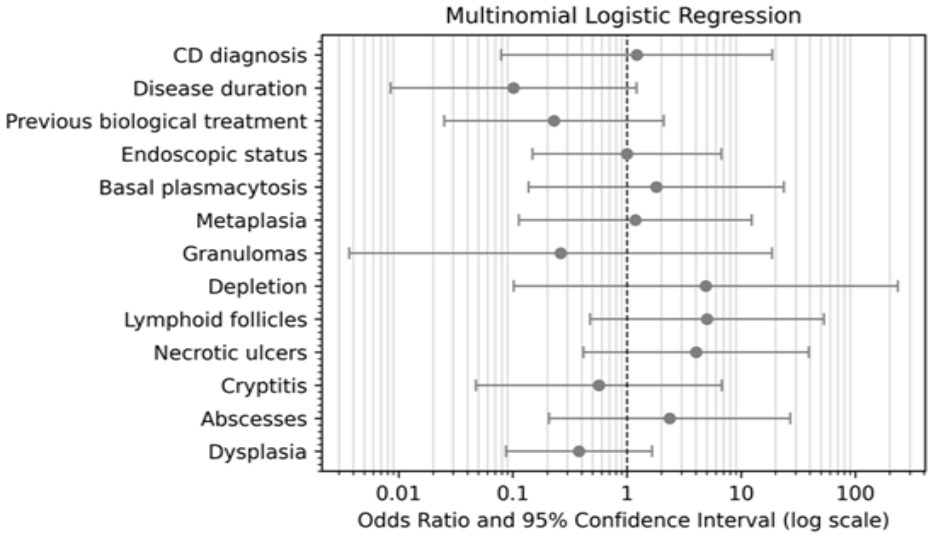

Figure 4. Forest plot expressing odds ratio for biologic therapy response

2.3.3 Nancy index

Blinded to clinical or endoscopic data, the GI pathologist divided patients in 2 groups, "responders" and "non-responders". Patients were classified as "responders" in case of improvement of at least 2 histological parameters. Statistical analysis highlights the accuracy of NI ≥1 in defining treatment-responders, while Nancy grade 3 and 4 is associated with lack of response in IBD (Figure 5). Nancy Index is the most accurate (88%), sensitive (92%) and specific (88%) parameter in identifying treatment-responders. Endoscopic

activity has a reasonable sensitivity (86%), but lacks specificity (60%). At the other side of the spectrum, clinical activity (HBI in CD patients and cMS in UC, respectively) lacks both sensibility and specificity (36% and 66.6%, respectively). Quite surprisingly, fecal calprotectin (FC) had a less-than-expected sensibility (60%).

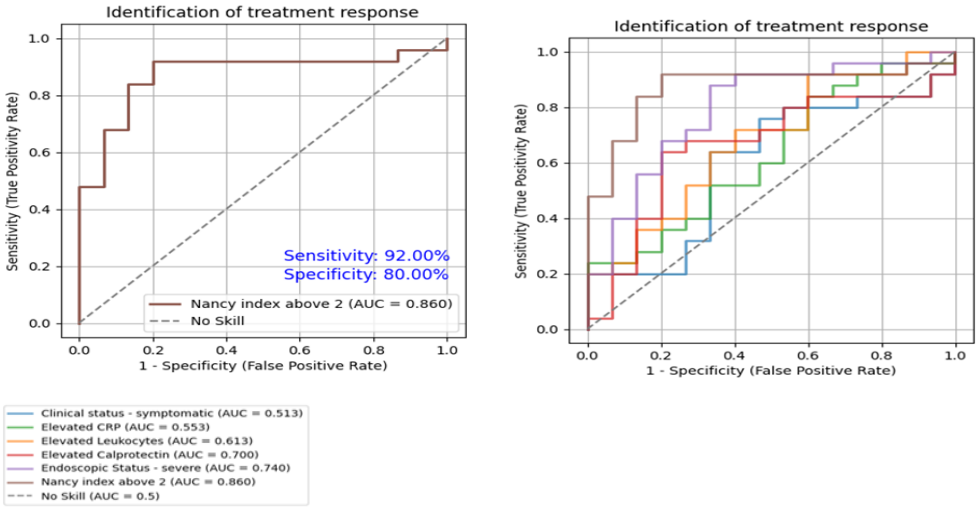

Figure 5. Logistic regression analysis, Sn. and Sp. of Nancy Index, and comparison with clinical, biochemical and endoscopic parameters

2.4 Conclusions

There are several reasons that justify our option to expand the histologic assessment to individual acute and chronic inflammatory parameters, beyond a single histopathological score. In our study, basal plasmacytosis was a feature that improved considerably after treatment, particularly in UC patients (present in 55% samples before biologic therapy vs 10% during follow-up). Reduced lymphoid aggregates sizes during treatment was also observed in treatment responders, finding in line to another cohort of patients treated with Vedolizumab in a study conducted by Herrerias *et al* [31]. Mucin depletion is another feature overleaped by histological scores, despite that mucus abnormalities are described as causative agents in IBD in a recent study [271]. According to the group of Villanacci, complete restoration of mucin expression should be considered a histological marker of mucosal healing [32]. In our cohort, the presence of mucin depletion was significantly reduced in samples obtained after treatment (40% vs 95%). According to the univariate analysis, the two major factors associated with treatment *non-response* were a long history of IBD diagnosis (>5 years) and failure to a previous biologic therapy. Conversely, factors associated with treatment *response* were a high burden of mucin depletion, ulcerations and lymphoid aggregates in initial samples (before therapy).

In our opinion, improvement of individual histological parameters should be implemented as long-term targets in future guidelines, together with implementation of Nancy Index for evaluation of both UC and CD patients.

3. Immunohistochemistry analysis in inflammatory bowel disease - is interleukin-10 underestimated?

3.1 Introduction

The scientific background behind the expression of cytokines in inflamed mucosa has evolved over the last decades. Interleukins are a type of cytokine produced by macrophages, T lymphocytes, neutrophils, mast cells and epithelial cells [33,34]. Cytokines, such as TNF-α, IL-1β and IL-6, can drive tissue damage [35-37], whereas IL-10 is typically recognized as an anti-inflammatory molecule [34, 38, 39]. The aim of this study was to prospectively assess the immunohistochemical parameters of the main inflammatory cytokines involved in the pathophysiology of IBD. Vigorous research has not previously been conducted in prospective trials on the molecular expression of these cytokines in different phases of disease (before and during treatment) to assess the impact of medical therapy. Simultaneously, there is a remarkable paucity regarding immunohistochemical and histological correlations in biopsy samples from IBD patients.

The current research addresses these important gaps in the literature while also aiming to identify a subset of high-risk patients.

3.2 Materials and methods

This was a prospective study assessing the immunohistochemical (IHC) parameters of 46 IBD patients treated with biologic therapy at Bucharest Clinical Emergency Hospital at two stages of their disease course. A cohort of 10 non-IBD patients (with a normal colonoscopy report) underwent a similar IHC analysis to increase statistical power. In order to improve the accuracy of immunohistochemistry evaluation, a semiquantitative integrated optical density (IOD) analysis was performed for IL-1β, IL-6, TNF-α and IL-10, since manual interpretation of IHC and the reproducibility of scoring systems are highly subjective [40]. IOD was computed as pixel area x mean intensity. The Ki-67 proliferation index and p53 positivity index were computed as positive epithelial cells/total epithelial cells. In each

case, positive cells from five different microscopic fields were counted from the ‘hot-spot’ staining areas. The final mean value was used for the analysis.

3.3. Results

3.3.1 TNF-α immunohistochemistry analysis

The IHC markers were observed in epithelial cells (enterocytes, mucin-producing cells), within normal, reactive, and dysplastic epithelia, as well as in stromal elements.The highest mean IOD TNF-α staining was observed in samples with dysplasia, abscesses, mucin depletion and basal plasmacytosis (Figure 6).

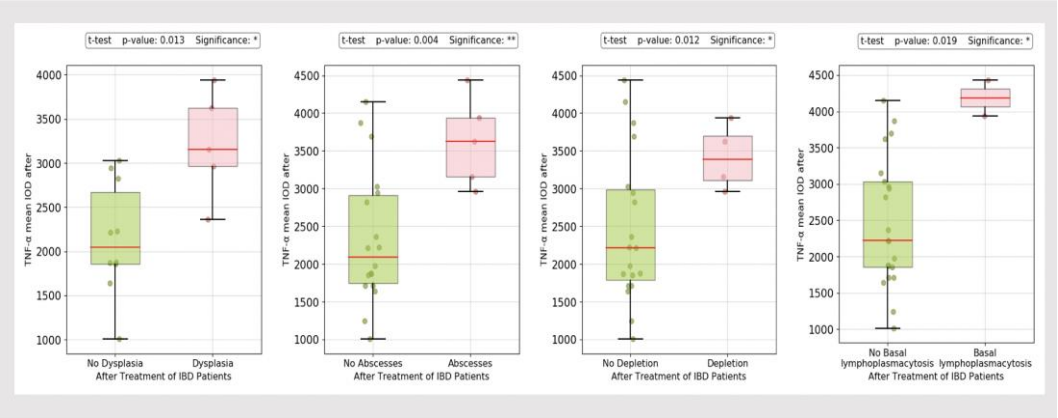

Figure 6. 3. TNF-α mean IOD in IBD patients (dysplasia, abscesses, mucin depletion and basal plasmacytosis)

Our group assessed the TNF-α mean IOD before and after treatment in responder versus non-responder patients. Responders were defined as those with a decrease of ≥3 points in the Mayo score (UC patients) and a decrease of 2 points in the Harvey Bradshaw Index (HBI) score from baseline values (CD patients), according to definitions previously used in other clinical trials [41,42]. Relatively surprisingly, the comparison of TNF-α staining between the two groups did not lead to statistically significant differences, despite non-responders exhibiting a slightly higher TNF-α mean IOD before treatment in UC patients. In CD patients, the difference in IHC staining was almost negligible. The post-treatment values were relatively similar in CD (Figure 7).

Neither a similar comparison in the subgroup of patients treated with anti-TNF- α therapy (Infliximab and Adalimumab) nor significant post-treatment variation was observed (Figure 8).

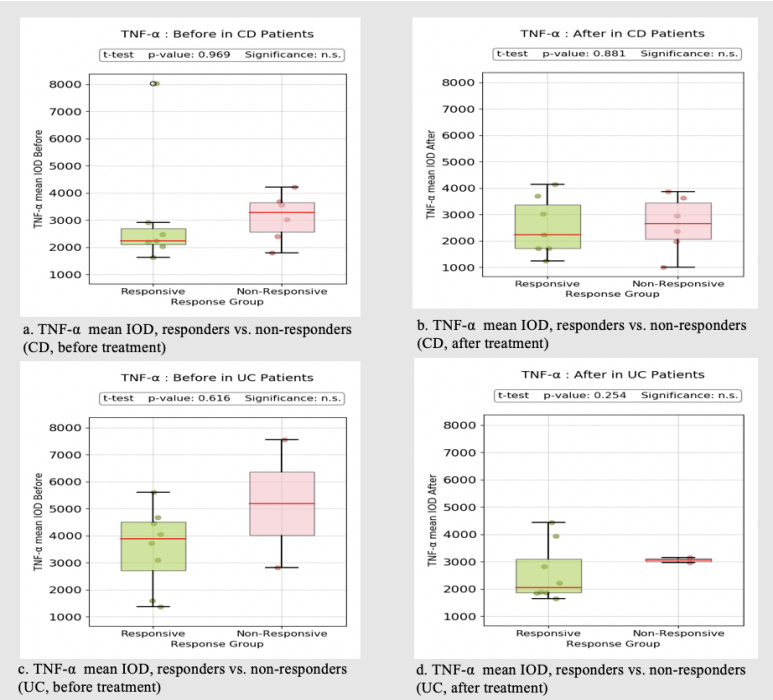

Figura 7. TNF-α mean IOD before and after treatment

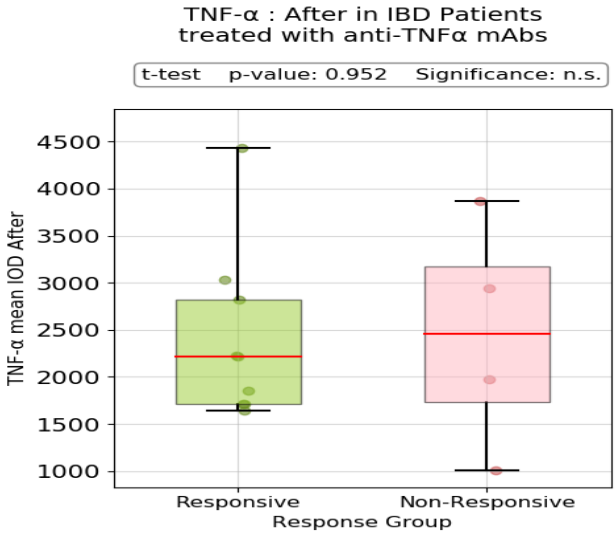

Figura 8. Post-treatment TNF- α mean IOD in patients treated with anti-TNF-α therapy (responders vs. non-responders)

There is a clear positive correlation (R = 0.42, p-value = 0.04) between fecal calprotectin and pre-treatment TNF-α mean IOD (Figure 9, left). A similar relationship is observed between post-treatment TNF-α and dysplasia grade (R = 0.55, p-value = 0.006) (Figure 9, right).

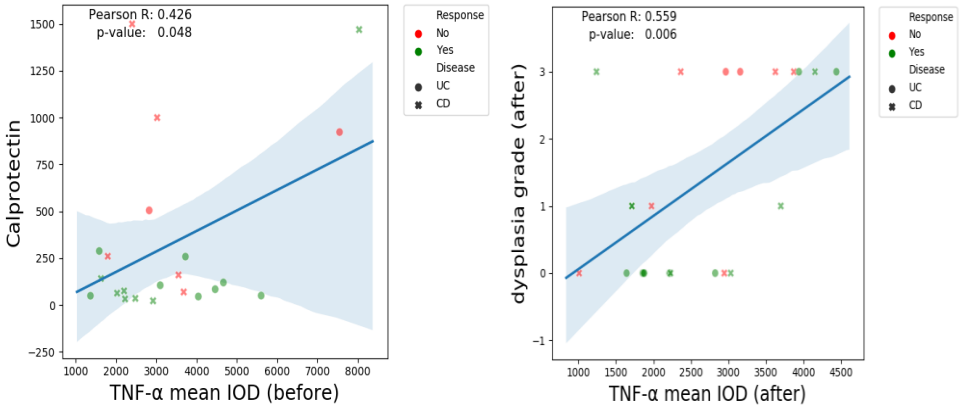

Figura 9. The Pearson correlation coefficient. Left - TNF-α mean IOD (before treatment) and FC. Right - TNF-α mean IOD (after treatment) and dysplasia grade

3.3.2 Interleukin-1β and IL-6 immunohistochemistry analysis

The mucosal expression of IL-1β and IL-6 was relatively steady between the initial and post-treatment evaluation, without major discrepancies. The mean IL-1β post-treatment IOD values tended to be slightly higher in non-responder CD patients compared to responders, but there was only a subtle difference (Figure 10). At the same time, the IL-6 IOD values remained relatively steady between pre- and post-treatment evaluations in both UC and CD patients (Figure 11).

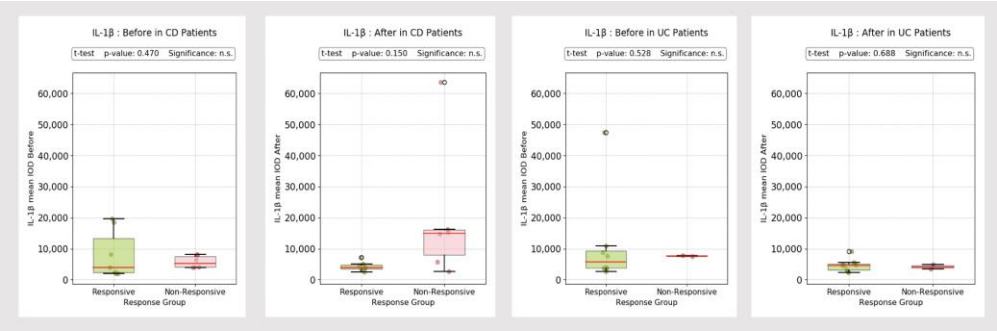

Figure 10. IL-1β mean IOD (control group vs. IBD group)

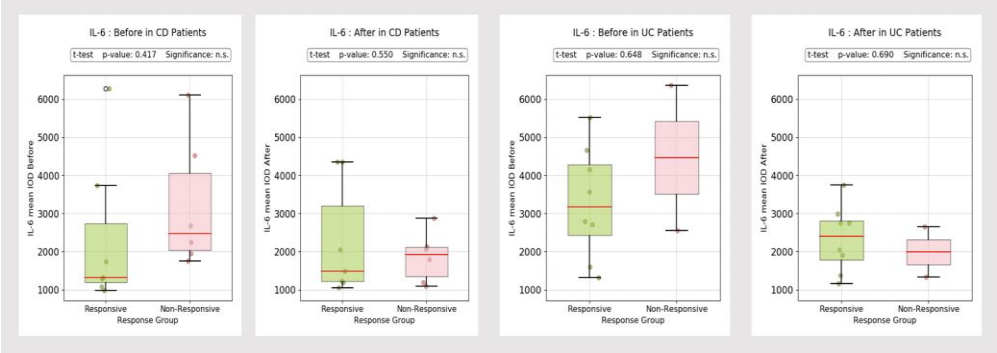

Figure 11. IL-6 mean IOD (control group vs. IBD group)

3.3.3 Interleukin-10 immunohistochemistry analysis

The IL-10 mean IOD values were remarkably higher in the control group compared to both UC and CD patients (p-value = 0.001) (Figure 12).

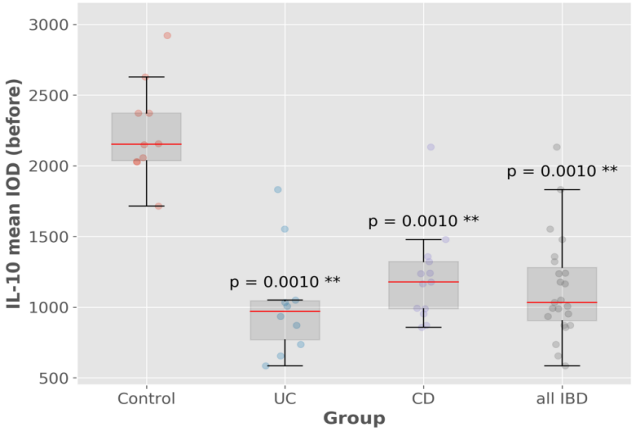

Figure 12. Mean IL-10 IOD values (control group vs IBD)

In the responder group, the post-treatment IL-10 mean IOD was significantly higher compared to the index evaluation in both CD and UC patients (p-value = 0.001). In nonresponders, the IOD values were relatively similar in CD patients before and after treatment, whereas the IOD values were slightly higher in UC patients, despite not reaching statistical significance (p-value = 0.08) (Figure 13). The pre-treatment IL-10 mean IOD values were significantly lower in responder versus non-responder IBD patients, with a p-value < 0.001 (Figure 14). Notably, pre-treatment IL-10 levels positively correlate with the Nancy Index after treatment (p-value < 0.001), and may therefore predict failure of biologic therapy (Figure 15).

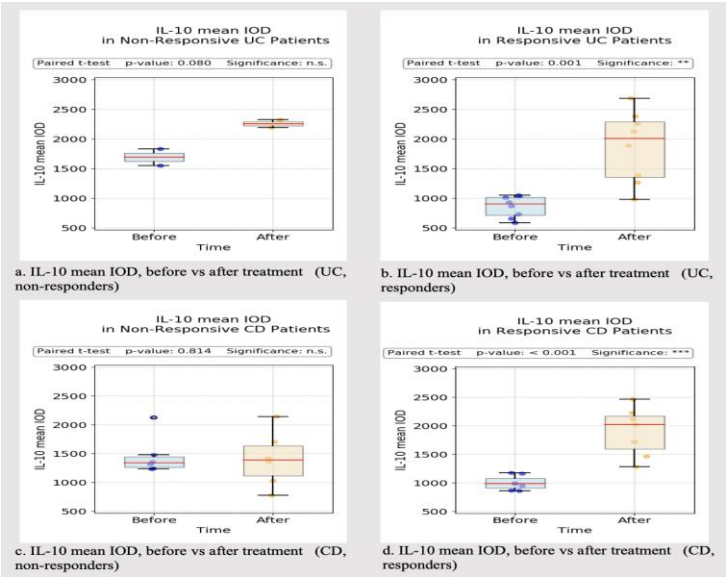

Figure 13. IL-10 mean IOD values (responder vs. non-responder IBD patients)

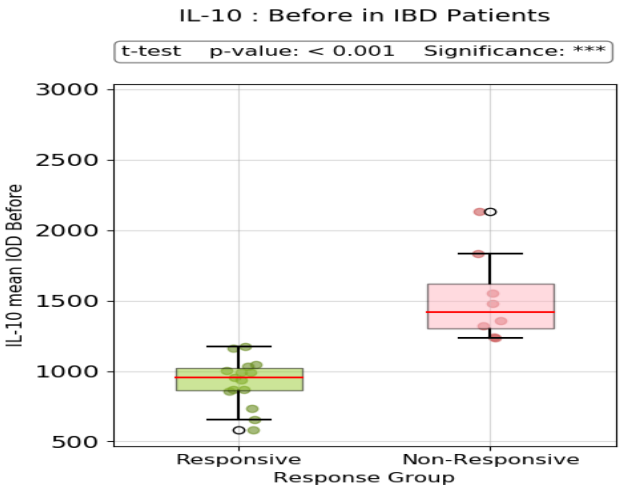

Figure 14. Pre-treatment IL-10 mean IOD values (responder vs. non-responder IBD patients)

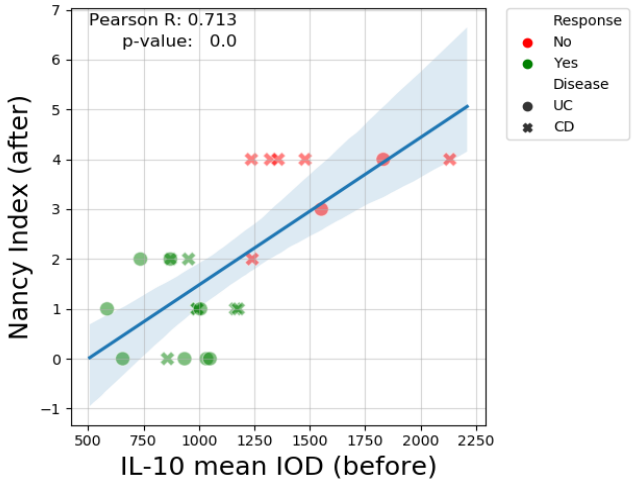

Figure 15. The Pearson correlation coefficient, pre-treatment IL-10 and post-treatment Nancy Index

3.4 Conclusions

TNF-α is a central cytokine in IBD pathophysiology and anti-TNF-α therapy reduces inflammation and aids mucosal healing [43]. In our study, UC patients had higher TNF-α expression compared to CD patients. There is no clear data comparing the level of mucosal TNF-α in UC versus CD patients, but prior analysis has suggested that TNF-α levels in the inflamed mucosa before treatment could predict treatment response, whereas post-treatment levels could be considered an index of the efficacy of anti-TNF-α treatment [44,45,46]. In our study, TNF-α expression was significantly higher in UC compared to CD patients. The highest IOD was observed in patients with dysplasia, abscesses, mucin depletion and basal plasmacytosis. Non-responders had higher pre- and post-treatment TNF-α expression in both

UC and CD compared to responders. On the contrary, the same analysis conducted in the subpopulation treated with anti-TNF-α therapy (Infliximab and Adalimumab) did not reveal a substantial difference in TNF-α expression between responders and non-responders. IL-1β and IL-6 are important cytokines for both intestinal inflammation and colorectal cancer [47]. In our cohort, we observed a positive correlation between IL-6 and TNF-α mean IOD values. The assessment of IL-1β quantified similar pre- and post-treatment values in UC, whereas post-treatment CD values were higher in non-responders.

Contrary to the previously mentioned interleukins, IL-10 exerts immunosuppressive effects on dendritic cells and macrophages by inhibiting their role to stimulate effector T cells [48,49]. The control group had markedly elevated levels of mucosal IL-10 expression compared to the IBD patients. In the IBD group, non-responder patients had higher pre-treatment levels compared to responders. The post-treatment IL-10 expression in this category was relatively similar in CD and only slightly elevated in UC, although this was without statistical significance (p-value =0.08). Conversely, in the responder group, both UC and CD patients had very low pre-treatment IL-10 immunopositivity and a noticeable elevation in post-treatment IOD values (p-value =0.001). There was a statistically significant difference (p-value < 0.001) in pre-treatment IL-10 mean IOD values between responders and non-responders in IBD patients. A very interesting observation can be made when assessing the relationship between pre-treatment IL-10 IOD values and post-treatment Nancy Index. There was a clear positive correlation (Pearson R value = 0.71) between these two indices, supporting the idea that high IL-10 immunohistochemical staining before treatment predicts failure of biologic therapy. Practically, a prolonged disease course is associated with weak IL-10 expression in post-treatment biopsy samples, which, at least in our study, is a feature of non-responsiveness.

Ultimately, high pre-treatment interleukin-10 expression is associated with failure of biologic therapy, histological inflammatory activity and longer disease duration. It serves as a useful tool to identify a high-risk subset of IBD patients and guide a more intensive monitoring strategy.

4. Surgery for inflammatory bowel disease in the era of biologic therapy - a multicentric study in Romania

4.1 Introduction

Inflammatory bowel diseases (IBDs) are represented by idiopathic conditions characterized by chronic and dysregulated immune activation within the gastrointestinal tract in genetically susceptible individuals, with an accelerating incidence worldwide. Although biologic therapy has become a key component in the management of the disease since the approval of infliximab by the FDA in 1998, surgery is still required in almost half of patients at 10 years after the diagnosis and about one-third of patients require a second operation within 5 years after the first [50]. It is important to note that repeated surgical interventions are usually required in Crohn's disease (CD) because the objective is to treat the complications (e.g., intractable fibrotic stricture) whereas in ulcerative colitis (UC), it is frequently a consequence of a medical therapy failure leading to fulminant colitis. As noted by Wong et al., there is conflicting data regarding the role of biologic therapy in reducing the postoperative recurrence and the need for secondary surgical interventions [51]; there are studies where biologic therapy improved endoscopic recurrence [52], whereas other studies did not show any superiority of biologics [53]. It is therefore critical to analyse and attain a clear understanding of the factors responsible for the unacceptably high rate of surgical interventions despite the therapeutic advances that have been achieved in the last two decades. Thus, we aimed to assess the factors associated with the risk of surgery in IBD patients

4.2 Materials and methods

This is a multicenter retrospective cohort study of patients with IBD from three tertiary care hospitals in Bucharest, Romania (Fundeni Clinical Institute, Emergency Clinic Hospital and St. John Emergency Hospital). All data were retrospectively collected from the medical records of the patients (hospitalized between January 2017 and June 2021) and were kept anonymous and in accordance with the Declaration of Helsinki. The inclusion criteria for the study enrolment were patients older than 18 years with a diagnosis of either Crohn's disease or ulcerative colitis who underwent a surgical intervention related to the IBD. From a total of 540 IBD identified cases, 56 patients who underwent surgical interventions in the aforementioned period were ultimately included. ECCO (European Crohn's and Colitis

Organisation), BSG (The British Society of Gastroenterology) and ACG (American College of Gastroenterology) guidelines were followed in terms of the IBD management

4.3 Results

More than half (66%) of our patients were diagnosed between 20 and 40 years. This observation could confirm the results of prospective studies showing that young age at diagnosis and a low pre-treatment IL-10 mean IOD values [372] are a poor predicting factor for IBD evolution and also for surgery interventions [373]. Noticeably, 10.37% (56/540) of the previously diagnosed patients with inflammatory bowel disease required at least one surgical intervention during their disease course. Most of the patients (45 patients, 80.35%) had surgical interventions due to CD and only 11 patients (19.64%) had interventions that were related to UC.With regard to the disease phenotype, almost half of the surgical CD patients (22/45 patients, 48.8%) had an ileocolonic involvement (L3 Montreal), followed by colonic (16 patients, 35.5%) and ileal phenotypes (7 patients, 15.5%). The most common behavior of CD was the stricturing subtype (55%), followed by a penetrating disease (45%); nearly one-third of the latter subgroup had an associated perianal disease. As expected, the majority of patients who underwent surgery in the UC subgroup had an extensive (pancolitis/E3 Montreal) involvement (7/11 patients, 63.6%). Only one patient had a limited disease (proctitis). Similarly, there is data from the Swiss IBD Cohort Study proving an OR of 2.5 for surgery in UC patients with extensive disease [54] On the contrary, a favorable outcome was observed in patients with histopathological features of mucin depletion, ulcerations and lymphoid aggregates [55].

The mean age for the surgical interventions was 35.1 ±13.3 years. With a mean age of an IBD diagnosis of 32.6 ± 13.3 years, there was an interval of less than 3 years between the diagnosis and the timing of the first surgical intervention. Furthermore, half of the surgical procedures occurred in the first year of diagnosis. Another important finding in our study was that among the surgical interventions, more than half (51.8%) were performed in an urgent setting whereas 48.2% were elective. The most common indication for surgery was an intestinal obstruction (44%); stenoses of the terminal ileum and ileocecal valve were the most frequent obstruction sites (64%), followed by sigmoid colon (16%), ileocolonic (12%) and jejunum (4%). Multiple stenosis sites were noted only in 1 patient (4%). Perforation, fulminant colitis and fistula/abscess formations were the second most common complications that required a prompt surgical intervention, accounting for a total of 40%.

All perforations (8/8) were recorded in CD, in both stricturing (B2) and penetrating (B3) phenotypes. Two cases out of eight perforations were iatrogenic; one perforation occurred during a follow-up colonoscopy and one case after the therapeutic balloon dilation of a transverse colon stricture in a patient known with ileocolonic CD. In both cases, a segmental colectomy with a stoma formation was performed and the postsurgical outcome was favorable. Fulminant colitis was reported in eight UC patients. It is important to mention that most patients (6/8) had already initiated an anti-TNF biologic therapy before the complication occurred. The surgical management implied either a subtotal colectomy or a proctocolectomy with an ileostomy. The mortality rate was 1.8%. One death was reported due to generalized peritonitis secondary to an inflammatory mass perforation in the terminal ileum of a 62-year-old female with known CD. We observed that patients with biologic therapy before surgery had a lower mean age at the time of IBD diagnosis ($p = 0.005$) and also at the time of surgery ($p = 0.005$). Moreover, we identified a significantly higher number of patients with CD and no biologics before surgery who required an intervention; the opposite was observed in UC, where most patients were refractory to biologic therapy and underwent surgery ($p = 0.016$). Surprisingly, there were no statistically significant differences between the three groups in terms of the IBD phenotype, extraintestinal manifestations, type of surgery and postoperative complications.

We also compared the aforementioned variables between the CD and UC patients (Table 1). The statistically significant finding ($p < 0.001$) was that the IBD phenotype with an ileocolonic involvement in CD (L3) and extensive UC (E3) was associated with a high risk of surgery in both categories. The time of the biologic initiation, the type of surgery (elective/emergency) and the postoperative complications did not seem to influence the rate of surgical interventions between the two groups.

Table 1. Comparison of CD and UC subgroups

Variables	CD (n=45)	UC (n =11)	p-value
Gender (M/F (%))	51.1/ 48.9%	54.5/ 45.5%	0.838
Age (mean ± SD)	32.44 ± 14.41	33.55 ± 8.11	0.809
Age at surgery (mean ± SD)	35.09 ± 14.48	35.64 ± 8.04	0.905
Smoking (yes/no)	13.3/ 86.7	0/ 100%	0.334

Biologic treatment (yes/no)	80/ 20%	90.9/ 9.1%	0.667
Time of biologic initiation (< 3 months / > 3 months)	41.7/ 58.3%	60/ 40%	0.475
IBD phenotype			
L1/L2/L3	15.5/ 35.5/ 48.89%	/	<0.001
E1/E2/E3	/	9.1/ 27.3/ 63.6%	
Extraintestinal manifestations (yes/no)	22.2 / 78.8%	9.1/ 90.9%	0.434
Type of surgery (elective/emergency)	48.9/ 51.1%	45.5/ 54.5%	0.883
Early postoperative complications (yes/no)	20/ 80%	18.2/ 81.8%	0.631
Long-term postoperative complications (yes/no)	12/33 (26.7/73.3%)	36.4/ 63.6%	0.711

4.4 Conclusions

There are a few possible observations that might explain the lower surgery rate in our study. First, it is important to mention that the timeframe of the observations was shorter in our cohort (60 months) compared with the previously mentioned studies. Second, there was a good response to infliximab for fistula healing (>70% patients obtained a fistula closure in a median time of 12 weeks). Third, in around one-fifth of cases, a top-down medical approach was favored, with the early initiation of biologic therapy in patients with extensive disease suggestive of a potentially aggressive disease course. There are data that indicate that the early introduction of biologic therapy may improve the disease outcome through a faster remission, reducing the use of corticosteroids and reducing or delaying the need for surgery. Nevertheless, a high rate of surgical interventions in the first year of diagnosis was observed; half of the surgical interventions were performed in this time interval. In view of this concern, patient data were analyzed; a young age of diagnosis, ileal involvement and signs of stenosis were the main factors that seemed to contribute to the fulminant disease evolution. A high rate of postoperative complications, primarily ostomy-related, was observed in our study, which could be explained by the fact that almost half of the surgical interventions (48%) were performed in an urgent setting (<48–72 h from the index emergency department presentation). Compared with other studies, this rate of urgent setting surgery was significantly higher than the other evidence we found. In our retrospective study, only 28.57% patients had biologic therapy initiated before surgery

compared with 53.5% patients after surgery, which brought into question whether an earlier biologic therapy would have been beneficial to patients to an even greater extent. Both pre- and postoperatively, infliximab and adalimumab were the most frequent biologic therapies used. According to AGA (American Gastroenterological Association) guidelines, the early initiation of biologic therapy within 8 weeks after surgery is considered to be safe and beneficial [56]. The present study showed that biologic therapy did not have a significant statistical impact on the type of surgery (emergency vs. elective) and had no influence in terms of early or long-term postoperative complications.

In conclusion, the management of patients with inflammatory bowel disease has become increasingly complex and challenging, requiring a multidisciplinary approach. The unpredictable pattern evolution of the disease and the struggling encountered by clinicians to achieve long-term remission has fueled the development of novel molecules and it is critical to acknowledge the poor prognostic factors leading to a potentially aggressive disease behavior and last but not least, the importance of the proper indications and timing of biologic therapy and surgery in order to ensure the best possible care for patients.

5. Conclusions and personal contributions

5.1 Conclusions

Inflammatory bowel diseases have seen the most significant therapeutic progress among gastrointestinal pathologies over the past two decades, benefiting from the emergence of numerous molecules acting concertedly on key cytokines involved in the immunogenic pathway. In parallel, therapeutic targets have become increasingly stringent, with the benefit of histological remission being intensely debated.

The induction and maintenance of disease remission remain essential components of therapeutic management, alongside monitoring strategies. A reduction in relapse rates has been noted when using serial determinations of inflammatory markers (calprotectin and C-reactive protein), with therapy escalation (step-up) applied upon their elevation, even in the presence of clinical remission. The absence of microscopic remission serves as a marker of incomplete healing, predisposing patients to a loss of therapeutic response. Conversely, the cost-effectiveness and potential adverse effects associated with exposure to successive lines of biologic agents—should complete histological remission be used as the threshold for

therapeutic switching—remain questionable. Consequently, a more nuanced approach has emerged, focusing on the concept of 'histological improvement,' through individualized and time-based comparisons of inflammatory histopathological parameters. We consider this to be an optimal compromise in terms of long-term prognosis, and it represented one of the central topics addressed in this thesis. Certain parameters, such as basal plasmacytosis, are not included in standard histological scores, yet have demonstrated a clear association with the risk of clinical relapse. In our study, basal plasmacytosis, lymphoid aggregates, and mucin depletion were the histopathological changes showing the most significant improvement following biological therapy in patients with both clinical and biological response. We also advocated for the simplification and standardization of histopathological reporting, given the considerable global discrepancies in current practice. The Nancy histological score is, in our perspective, a practical tool which, despite its simplicity, has demonstrated validity when compared to the Geboes score and meets the necessary criteria for routine use in both ulcerative colitis and Crohn's disease.

The absence of endoscopic and histological remission contributes to persistently high surgical intervention rates. As such, we aimed to conduct one of the few studies in Romania investigating the clinical, biological, and therapeutic characteristics of the patient subpopulation refractory to biological therapy. Most surgical interventions were performed within the first year after diagnosis, with diagnostic latency and delayed initial medical assessment—especially in cases with insidious onset—being discussed as possible explanations. No causal relationship was identified between biological therapy and postoperative complications; rather, these were more closely associated with surgeries performed under emergency conditions.

Another major focus of this doctoral thesis was the dynamic alteration in the immunohistochemical expression of key cytokines involved in IBD, as well as the identification of prognostic markers. The immunoexpression of inflammatory cytokines was assessed both before and after biological therapy, with comparative analyses also conducted between responder and non-responder patient subgroups. The immunohistochemical analysis of IL-10 revealed significantly higher expression levels in the control group compared to IBD patients. Within the IBD cohort, non-responder patients showed significantly higher pre-treatment expression levels than those who responded to therapy. Among responder patients, very low IL-10 immunopositivity was observed pre-treatment, followed by a marked increase in IOD (integrated optical density) values post-treatment. Thus, a high level of IL-10 prior to initiating therapy constitutes a negative prognostic factor

for disease evolution in IBD patients. We also identified a positive correlation between the pre-treatment IOD value of IL-10 and the post-treatment Nancy index, thereby confirming the negative prognostic role of elevated IL-10 immunoexpression. Additionally, a prolonged disease course was associated with low post-treatment IL-10 IOD levels, which, in our study cohort, characterized the absence of therapeutic response. Current data indicate that a long disease duration is a negative predictive factor in both Crohn's disease and ulcerative colitis, and IL-10 expression may be one of the underlying immunopathogenic explanations.

5.2 Personal contributions

- Our study provided an in-depth immunohistochemical analysis of inflammatory markers with the aim of prospectively evaluating the impact of biological therapy during treatment and identifying prognostic or predictive markers of treatment response. The mean IL-10 levels at baseline assessment (prior to the initiation of biologic therapy) were significantly lower in patients who demonstrated a clinical and biological response compared to non-responders. Thus, elevated pre-treatment expression of interleukin-10 is associated with failure of biological therapy, histological inflammatory activity, and a longer disease duration. IL-10 expression may therefore serve as a valuable tool for identifying a high-risk subgroup of IBD patients who require early initiation of biological therapy and more intensive surveillance strategies. These findings may be extrapolated in future multicenter studies to identify novel prognostic markers of treatment response.
- In the current study, overexpression of the p53 positivity index and the Ki-67 proliferation index was observed in patients with dysplasia, abscesses, and mucin depletion, with a significant difference between treatment responders and non-responders. We propose that patients exhibiting marked immunohistochemical reactivity for p53 and Ki-67 should follow a strict surveillance protocol, even in the absence of other traditionally recognized high-risk factors for colorectal cancer (such as extensive colitis or a family history of CRC).
- The complexity of current histological scoring systems represents a barrier to implementing histological remission as a long-term therapeutic goal. In our view, the Nancy index is more easily applicable in routine clinical practice. The study focusing on histopathological analysis before and after biological therapy confirms the accuracy of the Nancy index in evaluating treatment response, yielding the highest scores in terms of accuracy, sensitivity, and specificity.

- We advocate for the widespread implementation of the concept of *histological improvement*, as it represents, in our opinion, a fair compromise between long-term prognosis and the risks of overexposure to multiple lines of biologic therapies in patients who do not strictly meet the criteria for complete histological healing. In the current research, certain individual histological features—such as mucin depletion, lymphoid aggregates, and ulceration—emerged as predictors of treatment response. This confirms the importance of prospective evaluation of individual histological parameters, which may lead to a more practical and easily implementable approach in general clinical practice.
- The absence of endoscopic or histological remission increases the risk of surgical interventions. For this reason, we found it necessary to analyze a cohort of patients with a history of surgery. We identified a significantly higher number of Crohn's disease patients without preoperative biologic therapy who required surgery; conversely, in ulcerative colitis, most patients were refractory to biologic treatment and subsequently underwent surgical intervention. However, there was no significant difference in biologic therapy administration between the pre- and postoperative periods. No influence of biologic therapy was observed with regard to IBD phenotype, extraintestinal manifestations, or type of surgery (emergency vs. elective). Postoperative complications also tended to be more closely associated with emergency procedures; biologic therapy had no statistically significant impact.

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