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# Matrix metalloproteinases (MMP-3 and MMP-9) implication in the pathogenesis of inflammatory bowel disease (IBD)

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**Abstract**

Inflammatory bowel disease is a chronic disease, with unknown etiology, characterized by a sustained inflammatory cascade that gives rise to the release of mediators, capable of degrading and modifying bowel wall structure. The present study investigated changes of circulating metalloproteinases (MMP-3, MMP-9) and CRP levels in patients with ulcerative colitis and Crohn's disease, in order to contribute to the elucidation of pathogenesis. We have studied serum samples of 67 patients, of which 46 with ulcerative colitis (mean age 44.8 years) and 21 affected by Crohn's diseases (mean age 39.52 years), who were hospitalized in the Clinic of Gastroenterology of the Emergency County Hospital of Craiova, Romania. For the quantitative determination of MMP-3, MMP-9 and CRP, the ELISA technique was used. Both patients, with Crohn's disease and ulcerative colitis, showed increased production of studied immunomarkers, which were correlated with some clinical stages, indicating their involvement in the disease activity.

***Keywords***: metalloproteinases, inflammatory bowel diseases, ulcerative colitis, Crohn's disease.

## Introduction

Inflammatory bowel diseases (IBD), which include ulcerative colitis (UC) and Crohn's disease (CD) are idiopathic, chronic and remitting inflammatory diseases of the gastrointestinal tract. These disorders differ by clinical symptoms, endoscope findings, pathological characteristics and immunopathophysiology [1–4].

Although numerous investigations were performed, the etiology and pathogenesis of human IBD remains unknown. Experimental and clinical studies suggested that the initiation and pathogenesis of IBD are multifactorial, involving the intervention of genetic, environmental, immunological and infectious factors. The different mediators (cytokines, metalloproteinases) were suggested as possible participants in the pathogenesis of the inflammatory response of IBD [5, 6].

Matrix metalloproteinases (MMPs), a large family of enzymes with specificity for the various proteins of the extracellular matrix, are implicated in tissue remodeling processes and chronic inflammatory condition. In recent years, many studies aimed at understanding the involvement of MMPs in intestinal homeostasis, and their functions in IBD. More MMPs (MMP-1, -2, -3, -8, -9, -12) have been reported to be involved in the pathogenesis of colitis induced in a variety of animal models. Several studies have shown that elevated levels of MMPs correlates directly proportional to the activity of the disease [7–11].

This paper aims serum quantitative investigation of MMP-3, MMP-9 and CRP in patients with IBD compared with the control group, the establishment possible associations and correlations between these serological markers and certain stages of clinical activity, in order to understand their involvement in the pathogenesis of inflammatory disease.

## Materials and Methods

We performed a prospective study which included 67 patients diagnosed with IBD: 46 cases with UC (gender ratio 25 M/21 F, mean age 44.8 years) and 21 cases with CD (13 M/8 F, mean age 39.52 years) who were hospitalized in the Clinic of Gastroenterology of the Emergency County Hospital of Craiova, Romania, during the period October 2011–May 2014. The disease was diagnosed by physical examination, ultrasound studies of the abdomen, endoscopic studies of the alimentary tract, serial radiological studies, histopathological studies of mucosal specimens of large intestine and immunoserological tests.

Parallel, we used a control group of 30 patients with

mean age 45.16 years, hospitalized in the Clinic of Gastroenterology and what were diagnosed with functional constipation, dyspepsia and irritable bowel syndrome, patients unaffected by CD or UC.

For the quantitative determination of MMP-3, MMP-9 and CRP, was used ELISA technique, with Invitrogen Corporation (Camarillo, CA, USA) and INOVA kits. The values were expressed in pg/mL for interleukins and mg/mL for CRP. Blood samples were collected by venous puncture in a fasting state in the morning. Sera were obtained from clotted (30 minutes, room temperature) and centrifuged (15 minutes, 1500 rpm) of blood. Serum samples were stored at -80$^0$C until analysis.

### Statistical analysis

Patients' data management system and data processing was performed using Microsoft Excel, data analysis module. Statistical analysis was done using GraphPad Prism 5 and retaining only the results under the 5% for statistical significance. The significance of differences between groups was examined with a Mann–Whitney *U*-test or Kruskal–Wallis, when multiple comparisons were made. Correlation analysis between the immunomarkers concentrations studied and the degree of disease activity was conducted with Pearson's test with respect to data type and distribution. All tests were two-sided and *p*-values ≤0.05 were considered significant. All values were expressed as arithmetic mean (mean), accompanied by 95% confidence interval (95% CI).

### Histological and immunohistochemical study

For the positive and differential diagnosis of intestinal inflammatory disease, as well as for the evaluation of the disease evolution stage, there were performed colonoscopies and there were harvested samples of intestinal mucosa in all patients. The biological material was fixed immediately after harvesting in 10% formalin solution for 24 hours at room temperature and then included in paraffin. For the histological study, there were used the classical stainings with Hematoxylin–Eosin (HE) and PAS–Hematoxylin, and for the immunohistochemical study there were used the markers in Table 1.

**Table 1 –** ***Antibodies used in the immunohistochemical study***

| Antibody | Code | Clone | Antigen retrieval | Specificity | Dilution | Source |
|---|---|---|---|---|---|---|
| *CD20* | M0755 | L26 | Sodium citrate buffer, pH 6 | B-lymphocytes | 1:100 | Dako |
| *CD3* | A0452 | F7.2.38 | Sodium citrate buffer, pH 6 | T-lymphocytes | 1:100 | Dako |
| *CD68* | M0814 | KP1 | Sodium citrate buffer, pH 6 | Macrophages | 1:200 | Dako |

## Results

### The distribution of patients according to clinical and pathological entity and the severity of the clinical activity flare

Two widely accepted scores, the modified Truelove–Witts severity index (TWI) for UC and the Harvey–Bradshaw severity index (HBI) for CD were used to quantitate disease activity. Depending on the score obtained each patient was placed in the appropriate activity type. In our study, from a total of 21 patients with CD, seven (33.33%) were diagnosed with mild activity, nine (42.86%) with moderate activity and five (23.81%) patients with severe activity. At the UC group, 15 (32.61%) patients had mild, 22 (47.83%) experienced a moderate form, and nine (19.56%) showed a severe form of disease.

The distribution of patients according to clinical and pathological entity and the severity of the clinical activity flare, on the scores TWI and HBI obtained is shown in Tables 1 and 2.

**Table 1 –** ***Distribution of patients according to the severity CD flare activity***

| Activity | HBI (Harvey Bradshaw severity index) | No. of patients | % |
|---|---|---|---|
| *Mild* | 6.00 (5.24–6.75) | 7 | 33.33 |
| *Moderate* | 12.56 (10.67–14.44) | 9 | 42.86 |
| *Severe* | 18 (16.76–19.24) | 5 | 23.81 |

**Table 2 –** ***Distribution of patients according to the severity UC flare activity***

| Activity | TWI (Truelove–Witts severity index) | No. of patients | % |
|---|---|---|---|
| *Mild* | 3.20 (2.89–3.51) | 15 | 32.61 |
| *Moderate* | 7.23 (6.72–7.74) | 22 | 47.83 |
| *Severe* | 11.33 (10.18–12.49) | 9 | 19.56 |

### Serum concentrations of studied markers

Serum levels of MMP-3, MMP-9 and CRP were significantly higher in IBD patients than in controls ($p$<0.0001) (Figures 1–6).

(a) In patients with CD we obtained, for both matrix metalloproteinases, significantly higher concentrations compared with healthy controls ($p$<0.0001). Thus, for MMP-3 mean serum levels was 8.37 ng/mL (95% CI: 7.22–9.53) and for MMP-9 was 500.40 pg/mL (95% CI: 450.20–550.60). Control groups for the two metalloproteinases (MMP-3 and MMP-9) presented the following mean serum concentrations: 3.23 ng/mL (95% CI: 2.92–3.54), and respectively 112.20 pg/mL (95% CI: 104.30–120.10) (Figures 1 and 2).

CRP parameter had mean value 9.33 mg/mL (95% CI: 7.27–11.40) in CD patients serum, significantly higher levels than in controls, which presented 5.17 mg/mL (95% CI: 3.86–6.47), $p$<0.0001 (Figure 3).

(b) In patients with UC, MMP-3 mean levels was 5.34 ng/mL (95% CI: 4.40–6.27) and for MMP-9 was 608.30 pg/mL, (95% CI: 574.50–642.20), significantly higher than those of healthy controls (3.23 ng/mL, 95% CI: 2.92–3.54), $p$<0.0001 and respectively:112.20 pg/mL ( 95% CI: 104.30–120.10), p<0.0001 (Figures 4 and 5).

CRP had significantly higher serum levels (10.78 mg/mL, 95% CI: 8.06–12.96) in patients with UC than in controls, (5.17 mg/mL, 95% CI: 3.86–6.47), $p$<0.0001 (Figure 6).

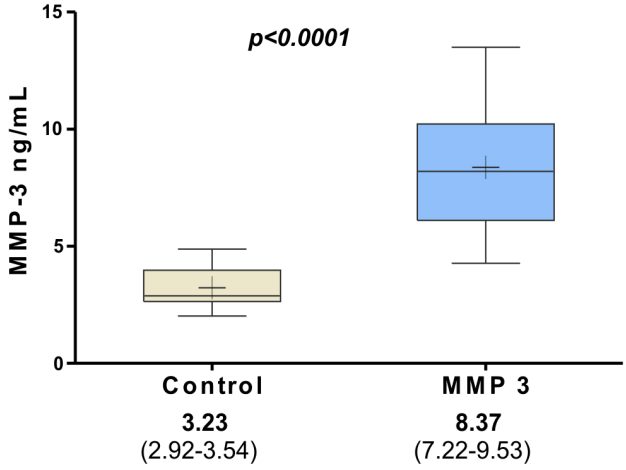


**Figure 1** – *Serum MMP-3 levels in CD-patients comparative with control group (p<0.0001).*

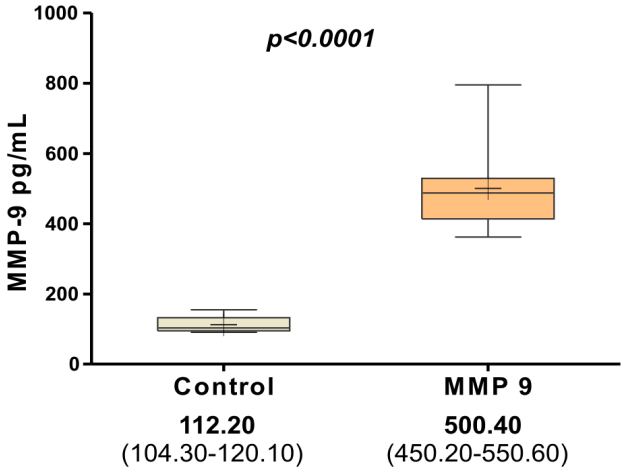


**Figure 2** – *MMP-9 in patients with CD comparative with control group (p<0.0001).*

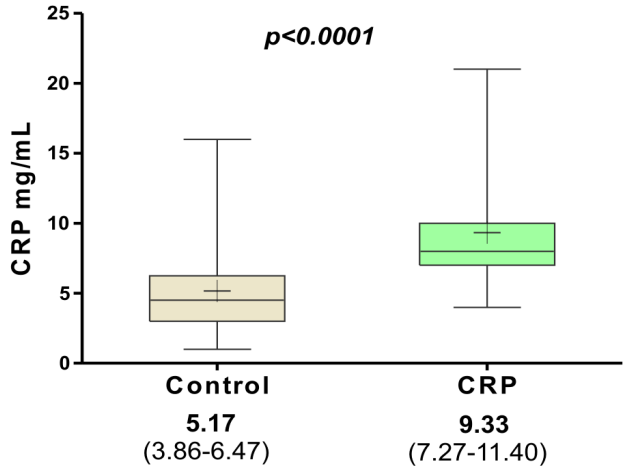


**Figure 3** – *Serum CRP levels for patients with CD, comparative control group (p<0.0001).*

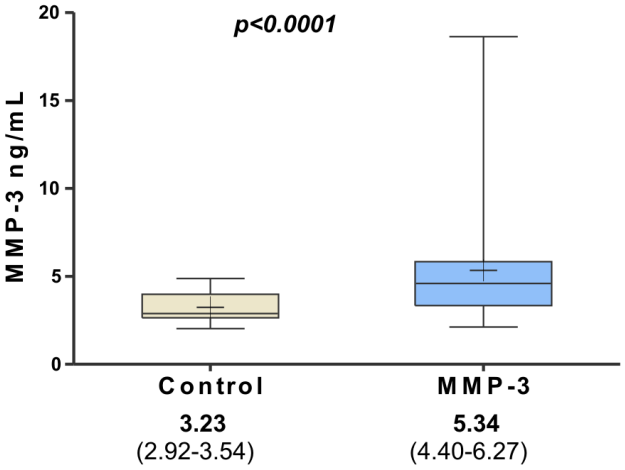


**Figure 4** – *Serum MMP-3 concentrations in patients with UC comparative with control group (p<0.0001).*

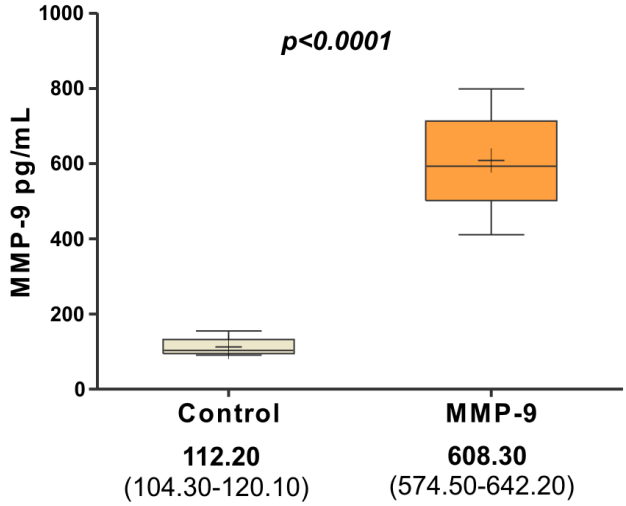


**Figure 5** – *Serum MMP-9 concentrations in patients with UC comparative with control group (p<0.0001).*

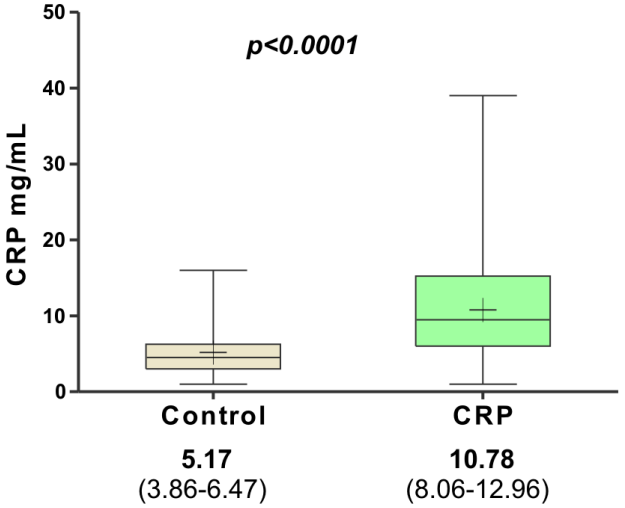


**Figure 6** – *CRP concentrations in patients' serum with UC comparative with control group (p<0.0001).*

### Associations between MMP-3, MMP-9, CRP and the disease activity

Comparing the concentrations of markers analyzed we identified significant associations between levels markers studied (MMP-3, MMP-9, CRP) and various stages of clinical activity for CD and UC.

(a) The associations in CD

MMP-3 serum values were similar in patients with moderate and severe clinical activity and significantly higher than those in the control group. Statistically significant differences between the concentrations of MMP-3 in serum of patients with severe and mild

activity ($p$=0.049), moderate activity and mild activity ($p$=0.008) were observed too. There were no statistical differences between the serum concentrations of CD patients with severe and moderate activity ($p$=0.898). In the case of serum levels of MMP-9 have been found a proportional increase with the stages of clinical activity, exceeding significantly statistic the control group. Statistically significant differences were observed between the serum MMP-9 concentrations of patients with severe and mild activity ($p$=0.049). CRP concentrations in serum of patients with CD were increased with the disease activity but the only statistically significant difference was between the group of patients with severe and mild activity ($p$=0.05). Levels of markers studied, distributed on CD patients according to clinical stages are shown in Table 3.

**Table 3 –** ***The values for the serological markers, depending on the different stages of clinical activity in CD***

| Parameter (mean ± SD) | Severe activity | Moderate activity | Mild activity | Control group |
|---|---|---|---|---|
| *MMP-3 [ng/mL]* | 9.69 ± 1.40 | 9.88 ± 1.60 | 7.00 ± 2.27 | 3.23 ± 0.86 |
| *MMP-9 [pg/mL]* | 544.42 ± 189.30 | 531.90 ± 109.80 | 505.60 ± 67.91 | 112.20 ± 21.10 |
| *hs-CRP [mg/mL]* | 13.00 ± 4.80 | 9.00 ± 4.87 | 7.14 ± 2.12 | 5.17 ± 3.48 |

(b) The associations in UC

Increased serum MMP-3 levels were found in patients with UC, the highest values were in the patients with severe disease activity. Statistically significant differences have been observed between levels of MMP-3 in sera from patients with severe activity and mild activity ($p$=0.049), and between moderate activity and mild activity ($p$=0.0062). There were not statistical differences between the concentrations of patients with severe and moderate activity ($p$=0.4463). We were noticed values MMP-9 increased in direct proportion to the severity of flare activity of UC, the highest concentrations were in patients with severe disease activity. There have been evidenced significant statistic differences between concentrations of MMP-9 in serum of patients with severe activity and mild activity ($p$=0.0012) and moderate activity or mild activity ($p$=0.0026). Concentrations of MMP-9 did not reach statistical significance in patients with severe and moderate activity ($p$=0.3494). Analyzing the CRP levels in serum of patients in various stages of clinical activity of IBD, we noticed a proportional increase to the severity of the disease activity. We found in UC patients a significant statistic differences between CRP concentrations of patients with severe and mild activity ($p$=0.009). The values obtained for serum markers investigated in patients affected by UC and distributed according to clinical stage are shown in Table 4.

**Table 4 –** ***The values of serological markers according to different stages of clinical activity of UC***

| Parameter (mean ± SD) | Severe activity | Moderate activity | Mild activity | Control group |
|---|---|---|---|---|
| *MMP-3 [ng/mL]* | 7.08 ± 3.76 | 5.89 ± 3.34 | 3.60 ± 1.17 | 3.23 ± 0.83 |
| *MMP-9 [pg/mL])* | 683.50 ± 93.61 | 646.13 ± 101.20 | 519.19 ± 17.39 | 112.20 ± 21.10 |
| *hs-CRP [mg/mL]* | 16.11 ± 10.06 | 10.86 ± 6.47 | 7.17 ± 5.20 | 5.17 ± 3.48 |

## Correlations between serological markers and scores indices

Profile serological study of patients diagnosed with IBD, continued with another principal goal, to establish correlations between serological markers investigated (MMP-3, MMP-9, CRP) and TWI, HBI scores. Using the software GraphPad Prism 5, we calculated the Pearson's correlation coefficient and demonstrated the presence of correlations between serum levels of MMP-3, MMP-9 and activity of CD (Table 5) or of UC (Table 6).

**Table 5 –** ***Correlations between investigated serological markers and HBI in CD patients***

| Marker | MMP-9 | HBI | CRP |
|---|---|---|---|
| *MMP-3* | $r$=0.554* | $r$=0.276* | $r$=0.016 |
| | $p$=0.037 | $p$=0.049 | $p$=0.943 |
| *MMP-9* | | $r$=0.608* | $r$=0.246 |
| | | $p$=0.039 | $p$=0.282 |
| *HBI* | | | $r$=0.398* |
| | | | $p$=0.047 |

*r*: Pearson's correlation coefficient; *Statistically significant correlation.

**Table 6 –** ***Correlations between investigated serological markers and TWI in UC patients***

| Marker | MMP-9 | CRP | TWI |
|---|---|---|---|
| *MMP-3* | $r$=0.248 | $r$=0.209 | $r$=0.344* |
| | $p$=0.095 | $p$=0.162 | $p$=0.029 |
| *MMP-9* | | $r$=0.425* | $r$=0.308* |
| | | $p$=0.003 | $p$=0.037 |

*r*: Pearson's correlation coefficient; *Statistically significant correlation.

In CD patients, the following findings were noted: MMP-9 concentrations correlated positively with HBI, the score obtained in the evaluation of CD activity ($r$=0.608, $p$=0.039); no significant correlations were observed between concentrations MMP-9 and CRP ($r$=0.246, $p$>0.05). Serum levels of MMP-3 were correlated better with indices of disease evaluated for this entity (Table 5).

The concentrations of MMP-9 serum were correlated with all the indices evaluated for CU patients but not with the levels of MMP-3 serum. Serum levels of MMP-9 showed significant correlation with TWI score ($r$=0.308, $p$=0.037) but MMP-3 levels were statistically correlated only with the number of points obtained in the evaluation of disease activity by TWI score ($r$=0.344, $p$=0.029) (Table 6).

## Histological and immunohistological aspects of intestinal inflammatory disease

In UC, the histological study highlighted the presence of an abundant inflammatory infiltrate in the chorion of the large intestine mucosa, formed of neutrophil granulocytes, lymphocytes, plasmocytes and macrophages heterogenously located among the glandular crypts. The presence of the inflammatory infiltrate determined the disorganization of the glandular device (Figure 7). The immunohistochemical study highlighted the high presence of T-lymphocytes (Figure 8), with a tendency of nodular organization, rare B-lymphocytes (Figure 9), and relatively numerous macrophages (Figure 10).

In CD, the histological study highlighted a granulomatous inflammation, extended both in the mucosa and

submucosa of the digestive tract (Figure 11). The inflammatory infiltrate was sometimes extremely intense, which led to the rearrangement of the mucosa and submucosa structure, with the alteration of the mucosa glandular architecture. Also, there was observed a hypertrophy associated with hyperplasia of lymphoid follicles in the digestive tract wall. The immunohistochemical examination highlighted the presence of numerous T-lymphocytes at the granuloma periphery, in its structure and in the inflammatory infiltrate of the mucosa chorion (Figure 12). B-lymphocytes were absent in the granuloma structure, but they were also present at its periphery, in the inflammatory infiltrate of the chorion and in the lymphoid follicles present in the mucosa (Figure 13). The macrophages were present in a high number both in the granuloma structure and in the inflammatory infiltrate developed among the Lieberkühn glands (Figure 14).

## Discussion

The intestinal inflammatory disease (UC and CD) are severe disease, with an ever-growing incidence and prevalence [12, 13]. A study performed by Molodecky *et al.* (2011) [14] showed that the highest annual incidence of UC in Europe was of 24.3 in 100 000 persons and in North America was of 19.2 in 100 000 persons, while in Asia and Middle East the incidence was only of 6.3 in 100 000 persons. For CD the highest annual incidence was recorded in North America with 20.2 in 100 000 persons, followed in Europe with 12.7 in 100 000 persons, while in Asia and Middle East the incidence was of 5.0 in 100 000 persons. The highest prevalence values recorded in Europe for IBD (UC, 505 in 100 000 persons; CD, 322 in 100 000 persons) and North America (UC, 249 in 100 000 persons; CD, 319 in 100 000 persons). According to some relatively new data, IBD affects more than 2.2 million persons in Europe and over 1.6 million people in the USA.

All these statistical data, as well as the clinical symptoms that are quite severe in some cases and the possibility of malignant degeneration [15] motivate the interest of various research teams in the study of these diseases.

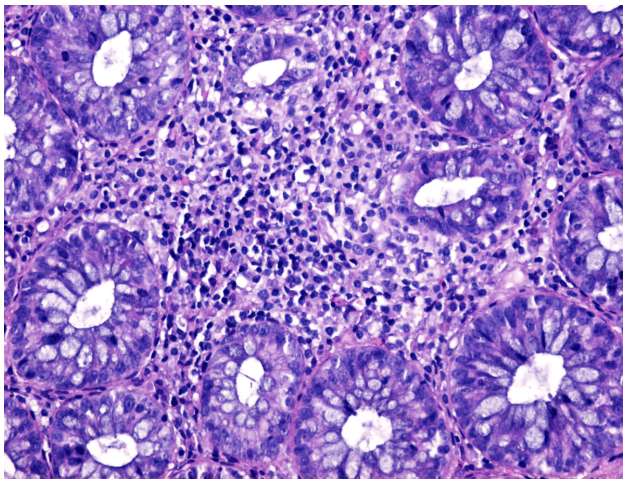

**Figure 7** – ***Image of large intestine mucosa from a patient with ulcerative colitis where we may observe the presence of an abundant inflammatory infiltrate in the chorion, formed of neutrophils, lymphocytes, plasmocytes and macrophages. HE staining, ×200.***

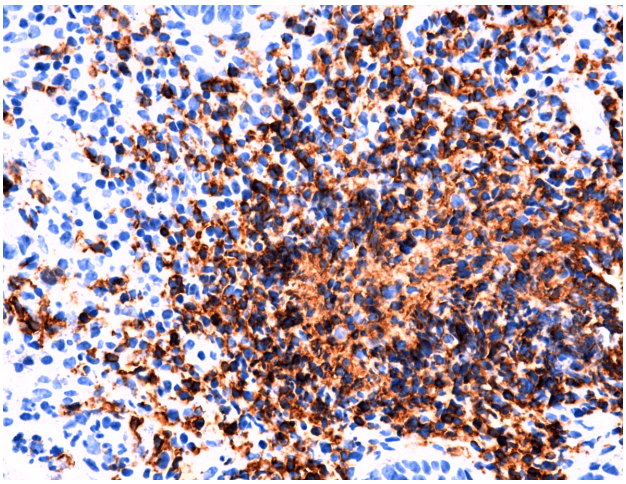

**Figure 8** – ***Inflammatory infiltrate rich in T-lymphocytes, with a tendency of nodular organization, present in the chorion of the colon mucosa. Immunomarking with anti-CD3 antibody, ×400.***

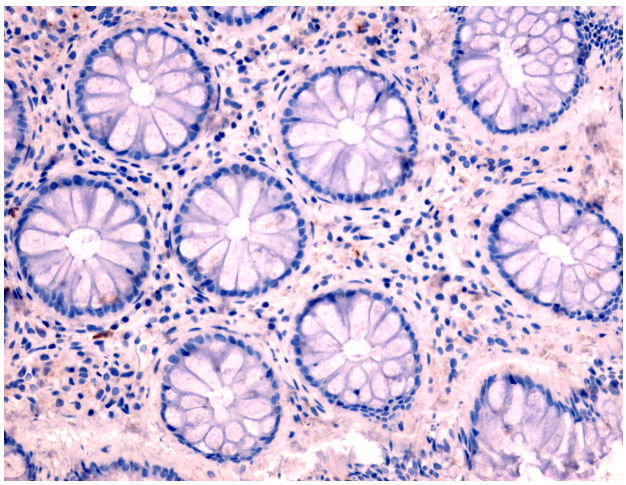

**Figure 9** – ***UC, mild form with rare B-lymphocytes disseminated among the intestinal crypts. Immunomarking with anti-CD20 antibodies, ×200.***

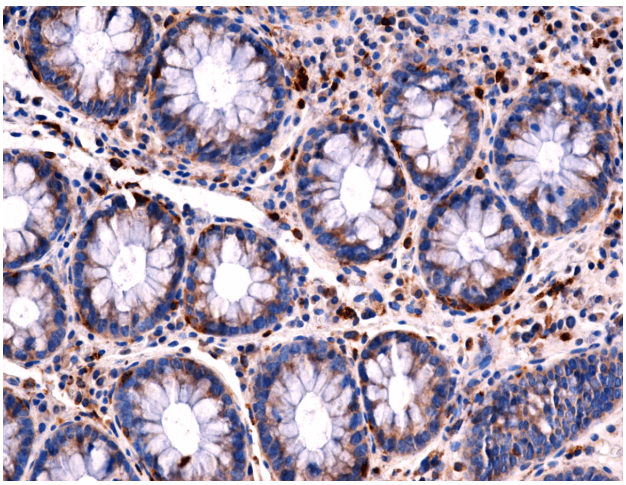

**Figure 10** – ***UC mild form, with numerous macrophages present in the inflammatory infiltrate of the lamina propria, peri-glandulary. Immunomarking with anti-CD68 antibody, ×200.***

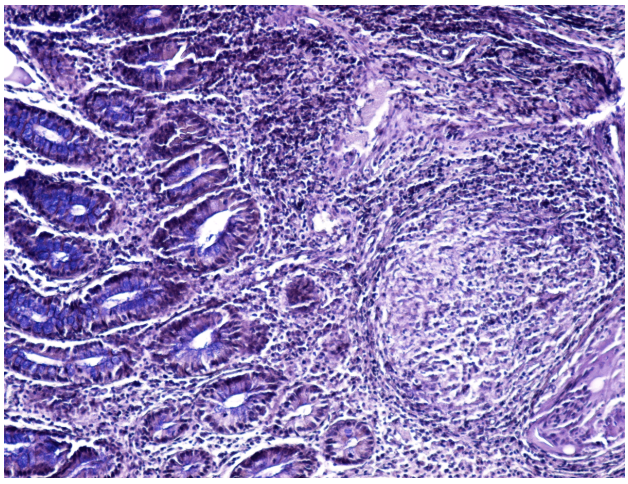

**Figure 11 –** ***Granulomatous inflammation in a CD patient. There is observed the presence of an abundant inflammatory infiltrate in the mucosa, the rearrangement of the microscopic structure of the mucosa and submucosa and the development of a granuloma in the deep side of the mucosa and submucosa. HE staining, ×100.***

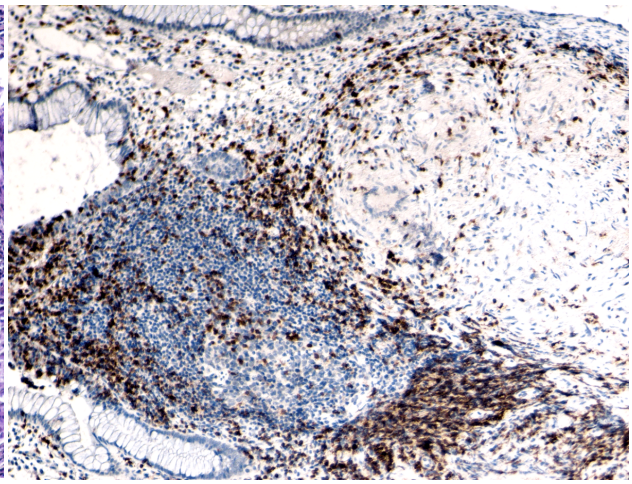

**Figure 12 –** ***Microscopic image of CD with numerous T-lymphocytes in the inflammatory infiltrate at the granuloma periphery as well as in its structure. Immunomarking with anti-CD3 antibody, ×100.***

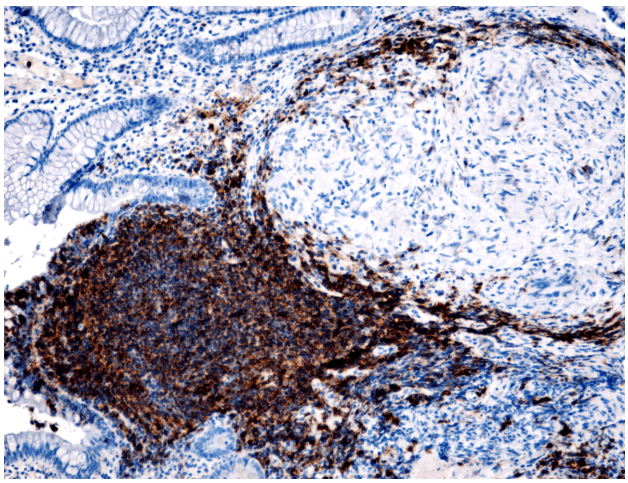

**Figure 13 –** ***B-lymphocytes present at granuloma periphery in the inflammatory infiltrate and at lymphoid follicle level in the mucosa. Immunomarking with anti-CD20 antibodies, ×100.***

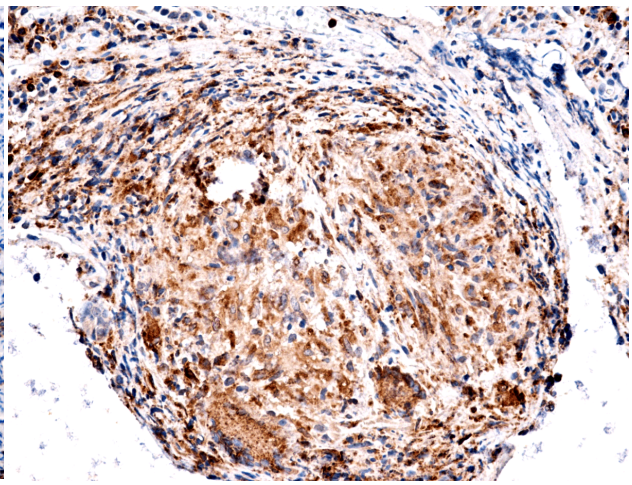

**Figure 14 –** ***Numerous macrophages present in the granuloma structure in a CD case. Immunomarking with anti-CD68 antibodies, ×100.***

The etiopathogeny of these diseases is still unknown, but most of the studies showed that these are multifactorial diseases in whose triggering may be involved environment factors, bacterial infections, immune and genetic factors [16–18].

The involvement of MMPs in inflammatory processes was demonstrated by studies in animal models that induced CD or UC, and in the lines and cell culture [19–21]. These studies add numerous histological investigations, which demonstrated correlation of MMPs in tissue affected by the degree of inflammation IBD. So far demonstrated changes of concentrations of MMP-3 and MMP-9 in histological samples collected from different regions affected by inflammation of the digestive tract, fact which lead to the ideea that these proteases could be considered the main metalloproteinases involved in the development of IBD [7–9, 22, 23]. Baugh *et al.* (1998) showed increased levels of MMP-1, MMP-2, MMP-3, MMP-9 in tissue samples affected by inflammation as compared to samples taken from unaffected IBD patients and to healthy subjects [23]. Similar results were presented by Louis *et al.* [22], von Lampe *et al.* [4] and Pedersen *et al.* [6].

Our study is an analysis that aims at a serological investigation of MMPs and CRP of patients with IBD and the establishment of possible associations and correlations between these serological markers and certain pathological phenotypes. The concentrations found by us for immunomarkers investigated in IBD patients, comparative with those found in control group are in accordance with the literature [7, 24, 25].

Specialized studies have confirmed the key role of MMPs in remodeling extracellular elements of connective tissue and in influencing migration pathways of immune cells in modulating of inflammatory process. It was found that the imbalance between synthesis and degradation of MMPs leads to inadequate tissue remodeling, which can be completed with the formation of ulcers in the mucosa.

It has been shown that MMP-9 and MMP-3 are two key enzymes involved in the degradation of intestinal tissue during CD and UC [9, 23]. In experimental animal models has been observed that the inhibition of these MMPs can result in the attenuation of inflammation in the intestine [19, 20]. Also, these metalloproteinases are involved in the release and activation of several biologically active molecules that amplify pathological processes, making it possible diagnostic and therapeutic target [26–28].

We found for CRP levels a proportional increase to the severity of flare of disease activity. These data for CRP are comparable to those determined in patients with active as against inactive forms of IBD [26]. CRP have been used for diagnostic and differential diagnostic purposes in inflammatory bowel disease but it is still far from ideal. The highest concentrations of serum CRP were found in patients with severe activity, these being more frequent in CD group compared to UC group, fact reported and by other authors [29]. Because IBD are chronic diseases characterized by a relapsing-remitting clinical behavior and dominated by intestinal inflammation that with time develops into a disabling disease, it is important to monitor the severity of inflammation before and after medication. The markers such as CRP, together fecal biomarkers (calprotectin and lactoferrin), are useful to analyze the inflammatory state of IBD and to identify patients requiring further investigations [30]. The lack of significant correlation between the two proteinases (MMP-3 and MMP-9) and CRP could be a proof of the superiority of these metaloproteinases sensitivity over standard marker [22].

Regarding the correlations between metalloproteinases studied and assessment of disease, activity indices have noted that MMP-3 better correlated in patients with UC and MMP-9 to those affected by CD. We did not notice that both MMPs submit correlation between their concentrations in serum. The data obtained in our study are consistent with previous literature [31–34].

The histological and immunohistochemical study helped us to establish a positive and differential diagnosis of intestinal inflammatory lesions.

## Conclusions

The serum concentrations of the studied immunomarkers (MMP-3, MMP-9 and CRP) were statistically higher in IBD patients compared to those found in the control group and were associated with different stages of disease evolution. The results of our investigations indicate the presence of correlations between the activity of inflammatory bowel disease and serum levels of matrix metalloproteinases. In the group of patients with CD, serum levels of MMP-3 were correlated better with indices of disease (HBI and CRP) evaluated, while in patients with UC, was achieved better correlation with MMP-9. We can consider the dosage of serum matrix metalloproteinases as an alternative diagnostic or additional at CRP, ESR and other indicators of inflammatory disease, used in diagnosis of IBD.

**Author contribution**

All authors have contributed equally to the present work.

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