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Investigation of inflammatory activity in ulcerative colitis

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Abstract

Inflammatory bowel diseases (IBDs), ulcerative colitis and Crohn's disease are lifelong disorders, characterized by the chronic inflammation of all or part of our digestive tract. Cytokines have an essential role in the pathogenesis of IBDs, because they control the inflammatory response, and the disequilibrium of pro-inflammatory/anti-inflammatory cytokines may lead directly to tissue destruction. Histopathologically, these diseases are characterized by the extent and the distribution of mucosal architectural abnormality, the cellularity of the lamina propria and the present cell types, but these features frequently overlap. We performed a prospective study, which included 46 patients diagnosed with ulcerative colitis (UC) (gender ratio 25 males/21 females, mean age 44.8 years) and 30 subjects, with similar demographic characteristics, which were selected from the patients investigated for other digestive disorders, unaffected by UC. Serological investigations were performed by quantitative determination of IL-17, IL-13, and CRP using ELISA sandwich technique. We have achieved significantly higher concentrations of IL-13, IL-17 and CRP in the serum of patients with UC, compared to the control group. We have found in our study correlations between ulcerative colitis activity and serum levels of interleukins, IL-13 and IL-17. Because IL-17 serum levels were significantly correlated with the disease severity and only cytokine had a significantly statistic correlation with high serum levels of CRP in UC patients, IL-17 can be considered an important progress inflammation marker of this disease.

Keywords: interleukin-13, interlukin-17, ulcerative colitis, inflammation.

Introduction

Inflammatory intestine diseases (IBDs) are lifelong idiopathic conditions and chronic diseases, characterized by the chronic inflammation of all or part of our digestive tract that primarily includes ulcerative colitis and Crohn's disease. Usually, it includes severe diarrhea, pain, fatigue and weight loss. IBD can be debilitating and sometimes leads to life-threatening complications [1–3]. These disorders differ in clinical symptoms, endoscope findings, histopathological characteristics and immunopathophysiology [2–4]. Ulcerative colitis (UC) is an inflammatory intestine disease that causes long-lasting inflammation and ulcers in the most inner lining of our large intestine (colon) and rectum. Although the disease has a variable distribution, it is limited to the distal intestine [4, 5]. The most common symptoms of UC are abdominal discomfort and blood or pus present in diarrhea [6]. In general, UC is a disease caused by a complex interaction of environmental, genetic, and immunoregulatory factors [5, 6]. Environmental risk factors, such as infectious agents, drugs, diets, and stress, are crucial to UC susceptibility [7]. The histological diagnosis of UC is based on four main feature categories: mucosal architecture, lamina propria cellularity, neutrophil granulocyte infiltration, and epithelial abnormality. Acknowledgement of the normal range of appearances of colorectal mucosa is necessary for optimal interpretation of biopsy samples [8]. Cytokines play an essential role in the pathogenesis of IBDs, because they control the inflammatory response, and the unbalance of pro-inflammatory/anti-inflammatory cytokines may direct to tissue destruction.

The objectives of this work were measuring of serum cytokines levels (anti-inflammatory IL-13 and pro-inflammatory IL-17) and of C-reactive protein, and identification of possible correlations between these serological markers and severity of colitis activity.

Materials and Methods

In our study, there were included 46 adult patients with UC (all patients old or newly diagnosed), hospitalized in the Clinic of Gastroenterology and investigated in the period between 2011 and 2014. Diagnosis was based on the medical history, clinical examination, lower gastrointestinal endoscopy and pathological examination of biopsy pieces. For this group, each case was included in the Montreal classification according to the lesion extent:

E1 – proctitis, E2 – left colitis, E3 – extensive colitis. To classify cases according to severity there was used the Truelove–Witts scale (TWI). Depending on the score obtained, every patient was placed in the appropriate activity type, as follows: TWI score ≤5, mild activity; TWI score = 5–9 moderate activity; TWI score ≥9 severe activity [9, 10].

In parallel, there was constituted a control group consisting of 30 subjects selected from patients hospitalized for other digestive disorders, unaffected by UC. Both groups were established based on inclusion and exclusion criteria. For every UC patient, there was developed a structured form, which included contact information, demographic data, family history and personal pathology, clinical manifestations, laboratory analysis, Montreal phenotypic classification.

Sampling and biological material

For determining the presence and concentrations of the serological markers, there were collected blood samples by venous puncture in the morning. The serum samples were obtained from clotted (30 minutes, room temperature) and centrifuged (15 minutes, 1500 rpm) blood. The serum samples were stored at -80^{0}C until analysis.

The serological investigations were performed by quantitative determination of IL-17, IL-13, and CRP using the ELISA sandwich technique with Invitrogen Corporation (Camarillo, CA, USA) and INOVA kits. The values were expressed in pg/mL for interleukins and mg/mL for CRP. To identify the associations between the concentrations of these serological disease markers and different UC clinical phenotypes, we used the data provided by the Montreal classification.

Statistical analysis

The information obtained was stored in Microsoft Excel files and then underwent a statistical processing in order to analyze the relationship between the clinical and laboratory data of patients. The management of patient data and the data processing was performed by using Microsoft Excel with XLSTAT suite for MS Excel and the statistical analysis was performed using statistical software indicated for scientific calculations, GraphPad Prism 5 and IBM SPSS Statistics 20.0. The significance of differences between groups was examined with a Mann–Whitney *U*-test or Kruskal–Wallis, when multiple comparisons were made. The correlation analysis between IL-13, IL-17 and CRP concentration and the degree of disease activity was conducted with Pearson's test regarding the data type and distribution. All tests were two-sided and *p*-values ≤0.05 were considered significant.

Histological and immunohistochemical study

The harvested biopsies from the UC patients were immediately fixed in 10% formalin solution for 24 hours and included in paraffin, using the classical histopathological protocol. The sectioning of the biological material was performed in the Microm HM350 rotary microtome. For the histological study there were used the classical stainings with Hematoxylin–Eosin (HE) and trichromic Goldner–Szekely (GS).

For the immunohistochemical study, there were performed sections with a 4 μm thickness that were collected on poly-L-Lysine blades, after which they were kept in a thermostate at 37^{0}C for 24 hours in order to increase the adherence of the biological material to the slide blade. Then, there followed the deparrafinization and hydration of the histological sections, after which the biological material was incubated for 30 minutes in a 3% oxygenated water solution (hydrogen peroxide). For the antigen unmasking, the sections were boiled in the microwave oven, in a solution of sodium citrate, pH 6, for 21 minutes. After boiling, there followed the stage of blocking the unspecific sites in 2% fat-free milk, for 30 minutes. The sections were incubated with primary antibodies for 18 hours in the refrigerator at 4^{0}C, and the next day there was applied the biotinylated secondary antibody (αMs/αRb) for 30 minutes, followed by the passing into HRP Streptavidin for 30 minutes. The signal was detected with 3.3'-diaminobenzidine (DAB) (Dako) followed by contrasts with Hematoxylin, alcohol dehydration, xylene clarification and fixing of the blades in a DPX environment (Fluka).

In our study, we used the following markers: CD20 (M0755, L26 clone, Dako) for highlighting B-lymphocytes, CD3 (A0452, F7.2.38 clone, Dako) for highlighting T-lymphocytes and the CD68 antibody (M0814, KP1 clone, Dako) for highlighting the macrophages.

Results

In our study, we evaluated a total of 46 adult patients clinically and endoscopically diagnosed with ulcerative colitis. Of these, 25 (54.35%) were males and 21 (45.65%) females. The age mean at the time of diagnosis was 44.8-year-old, the average duration of the disease evolution 4.48 years and prevalent location of lesions (Montreal classification) was left colitis (E2) (71.74%) (Table 1).

Table 1 – ***Demographic and clinical characteristics of the study population***

Characteristics	Values
Age mean at the time of diagnosis	44.80 years
Gender ratio	25 M (54.35%) / 21 F (45.65%)
The average duration of the disease evolution	4.48 years
Location prevalent lesions (Montreal classification)	Left colitis (E2) – 71.74%

The distribution of patients according to clinical and pathological entity and the severity of the disease clinical activity on the TWI score obtained is shown in Table 2; thus, in acute disease activity the mean TWI score was 3.20 and had proportionally higher values in moderate and severe activity (Table 2).

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

	Activity	TWI	Patients	%
Truelove–Witts scale (the severity of flare activity)	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 levels of the UC studied parameters (IL-13, IL-17and CRP)

The serum values of IL-13 (60.14 pg/mL, 95% CI: 51.35–68.93), IL-17 (42.11 pg/mL, 95% CI: 38.36–45.87) and CRP (10.78 mg/mL, 95% CI: 8.06–12.96) were significantly higher in patients with UC than in control patients (IL-13, 26.16 pg/mL, 95% CI: 24.43–27.89, $p<0.0001$; IL-17, 34.08 pg/mL, 95% CI: 30.22–37.94, $p<0.005$; CRP, 5.17 mg/mL, 95% CI: 3.86–6.47, $p<0.0001$) (Figures 1–5).

Serum concentration markers according to different stages of UC clinical activity

Comparing the concentrations of the analyzed markers, we found that their values differ in the subgroups of patients with mild, moderate and severe serum activity (Table 3).

Table 3 – ***Concentrations of serological markers according to different stages of clinical activity***

Parameter (mean ± SD)	*Severe activity*	*Moderate activity*	*Mild activity*	*Controls*
IL-13 [pg/mL]	61.81 ± 22.48	64.62 ± 31.15	57.40 ± 33.29	26.16 ± 4.64
IL-17 [pg/mL]	42.32 ± 5.32	47.76 ± 10.83	34.48 ± 15.42	34.08 ± 10.33
CRP [mg/mL]	16.11 ± 10.06	10.86 ± 6.47	7.17 ± 5.20	5.17 ± 3.48

All IL-13 values of patients were increased compared to the control values, emphasizing the highest ones in the moderate activity subgroup. Serum IL-17 patient levels were higher than those in the control group; the highest IL-17 values were identified in the patients with moderate clinical activity. CRP presented the highest values in UC patients with severe clinical activity.

The IL-17 values were increased especially in the patients with type E3 lesions.

We also found a significantly higher CRP in the patients with extensive colitis (E3) as compared to left colitis patients (E2) (15.63 mg/mL *vs.* 9.48 mg/mL, $p=0.0378$) (Figure 6).

Correlations between the UC investigated parameters

The profile serological study of the patients diagnosed with inflammatory intestine disease, continued with another primary goal, namely to establish correlations: between the investigated serological markers, interleukins and CRP, and between serological markers and the TWI scores (severity of disease activity).

Using the GraphPad Prism 5 software, we calculated the Pearson correlation coefficient for establishing the presence of the correlations between the serum levels of interleukins, CRP and UC activity in adults (TWI) (Table 4).

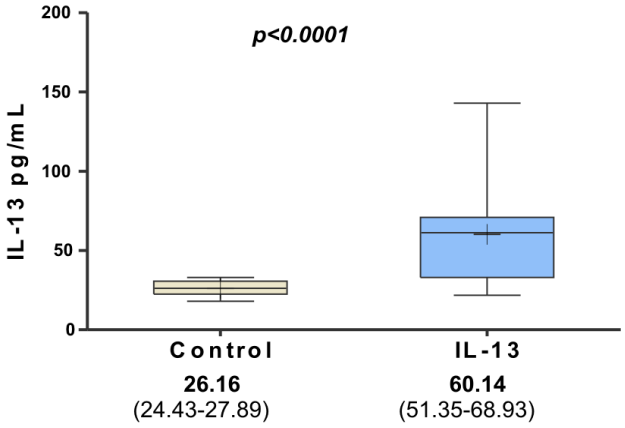

Figure 1 – ***Serum IL-13 concentrations in patients with UC, comparative with control group.***

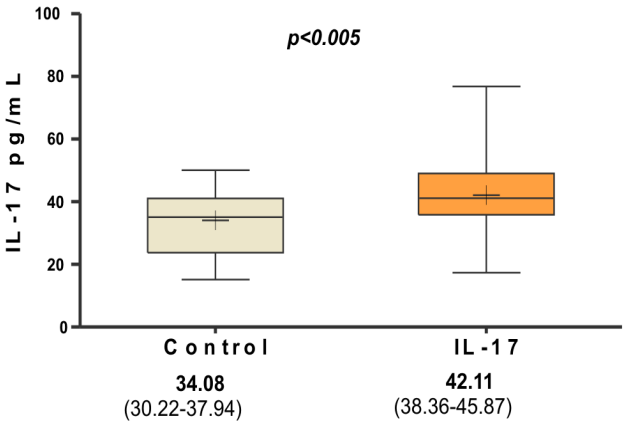

Figure 2 – ***Concentrations IL-17 in patients' serum with UC comparative with control group.***

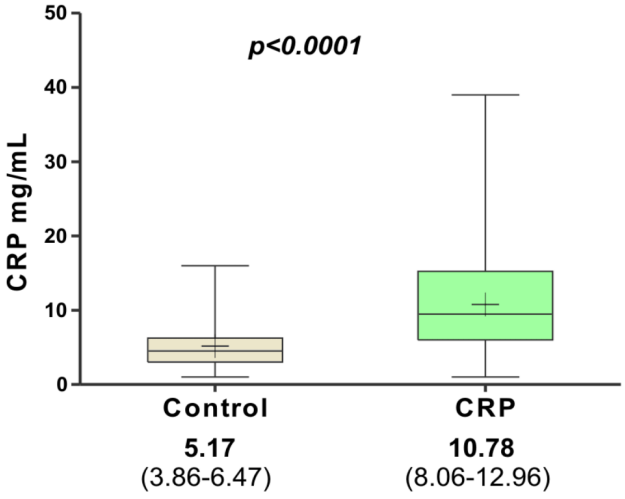

Figure 3 – ***Concentrations CRP in patients' serum with UC comparative with control group.***

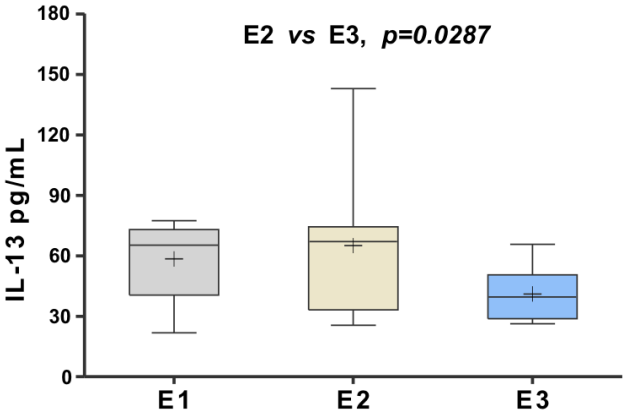

Figure 4 – ***Concentrations of IL-13 in functions of lesions extent (E).***

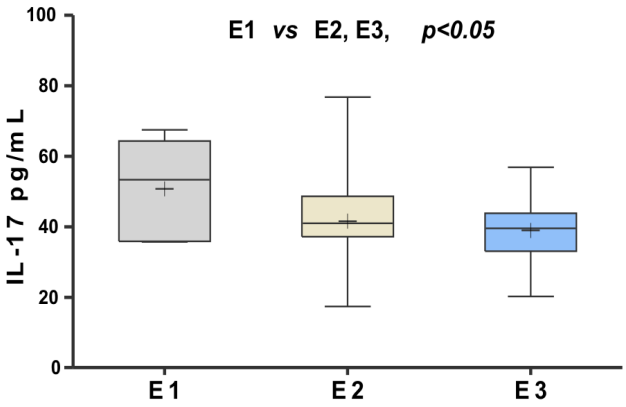

Figure 5 – ***Serum IL-17 levels in functions of lesions extent (E).***

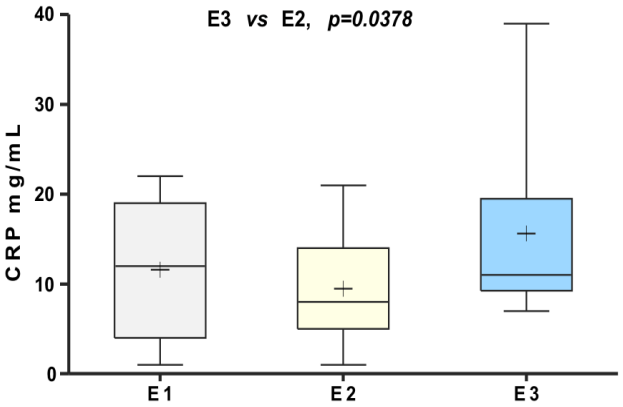

Figure 6 – ***Serum CRP levels in functions of lesions extent (E).***

Table 4 – ***Correlations between serological markers evaluated in UC***

Markers	IL-17	CRP	TWI
IL-13	*r=0.354**	*r*=0.108	*r*=0.093
	p=0.015	*p*=0.473	*p*=0.538
IL-17		*r=0.588**	*r=0.312**
		p=0.00001	*p=0.034*
CRP			*r*=0.223
			p=0.136

These correlations were observed between disease indices evaluated in UC: a weak correlation between the concentrations of the IL-13 and IL-17 cytokines (r=0.354, p=0.015); IL-17 was the only cytokine that correlated positively and highly statistical significant with the serum levels of CRP in UC patients (r=0.588, p=0.00001), IL-17 levels also correlate with the number of points obtained in the evaluation of disease activity by TWI score (r=0.312, p=0.034) (Table 4).

The histopathological study of large intestine mucosa biopsies allowed us to remark the presence of an inflammatory infiltrate in the lamina propria of the mucosa, variable in quantity, more or less abundant according to the severity of the disease (Figures 7 and 8). In mild forms, the inflammatory infiltrate was in moderate quantity, mainly formed of lymphocytes, distributed in a higher quantity under the covering epithelium. In medium and severe forms of the disease, the inflammatory infiltrate appeared disorganized, the Lieberkühn glands having different shapes and sizes. The inflammatory infiltrate almost entirely occupied the thickness of the colon mucosa, it was formed of polymorphonuclear leukocytes, neutrophils, lymphocytes, plasmocytes and macrophages, and sometimes it wend beyond the mucosa muscles, reaching the submucosa. Trichromic Goldner–Szekely staining allowed us to remark the presence of a very developed network of blood capillaries and of some high quantities of fibrilary collagen in the lamina propria (Figure 9).

The immunohistochemical study showed the presence of numerous T- and B-lymphocytes, diffusely disseminated or with a tendency of nodular accumulation (Figures 10 and 11) and of a high number of diffusely disseminated macrophages in the lamina propria (Figure 12). The blood vessels were represented by quite a developed network of blood capillaries with a continuous epithelium (Figure 13).

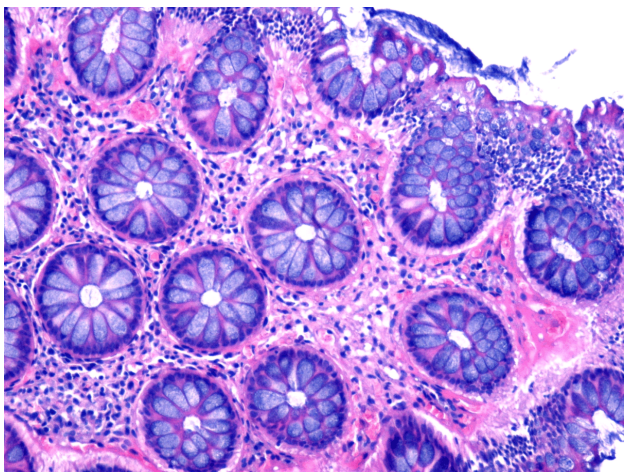

Figure 7 – ***Image of ulcerative colitis, mild form, with a moderate inflammatory infiltrate in the lamina propria, mainly formed of lymphocyes diffusely distributed among the Lieberkühn glands. HE staining, ×200.***

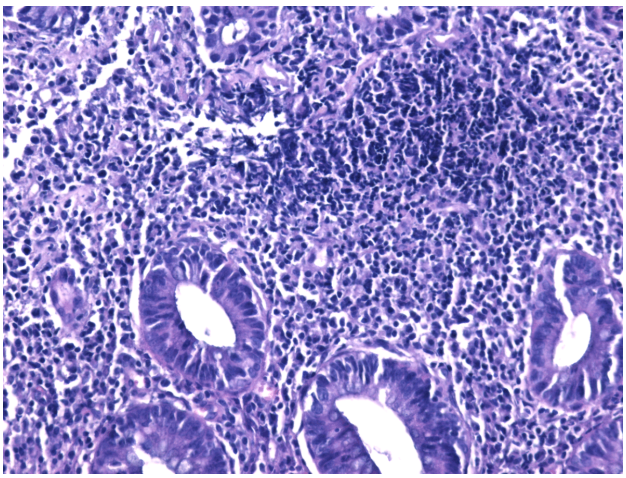

Figure 8 – ***Ulcerative colitis, medium form, characterized by the deformation of Lieberkühn glands, disorganization of the glandular device, by the development of an abundant inflammatory infiltrate, formed of neutrophils, lymphocytes, plasmocytes and macrophages. HE staining, ×200.***

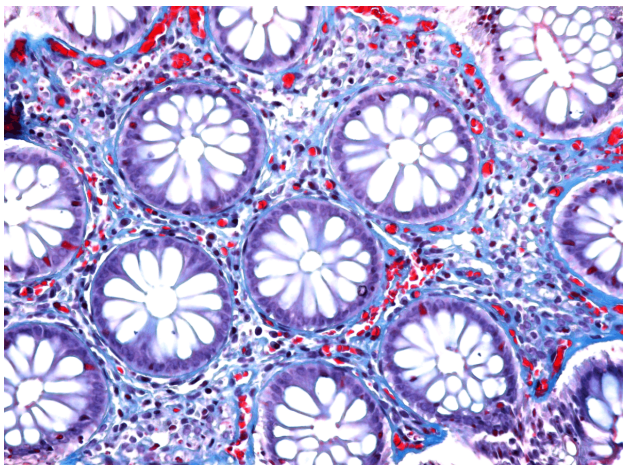

Figure 9 – ***Colon mucosa with Lieberkühn glands in transverse section, among which there may be observed the presence of a moderate inflammatory infiltrate, numerous blood capillaries and a high quantity of fibrillary collagen. Trichromic GS staining, ×200.***

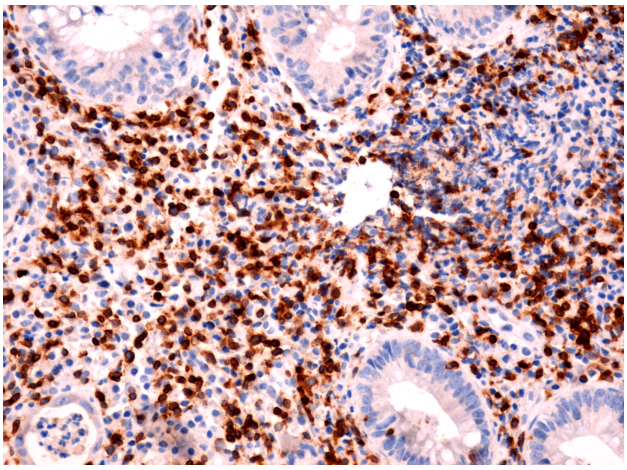

Figure 10 – ***Microscopic image from an area of colon mucosa, from a case of moderate ulcerative colitis, where there is highlighted the presence of an abundant inflammatory infiltrate, mainly formed of T-lymphocytes. Immunomarking with anti-CD3 antibody, ×200.***

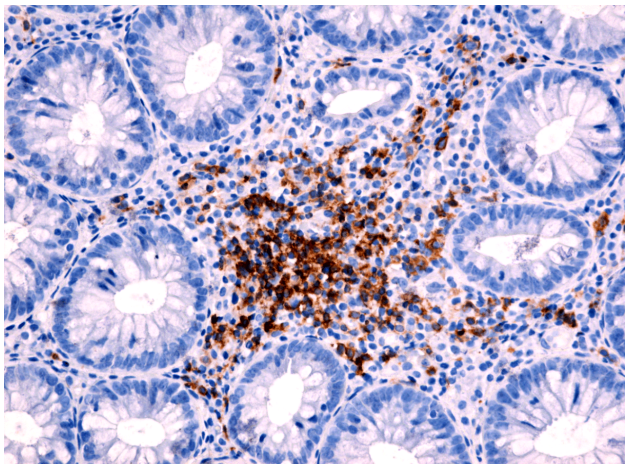

Figure 11 – ***B-lymphocytes with a tendency to nodular organization, in a case of ulcerative colitis. Immunomarking with anti-CD20 antibody, ×200.***

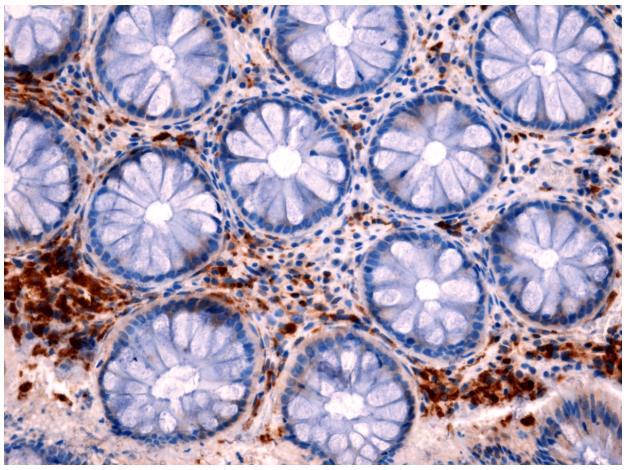

Figure 12 – ***Colon mucosa with ulcerative colitis, mild form, with numerous diffusely disseminated macrophages in the lamina propria. Immunomarking with anti-CD68 antibody, ×200.***

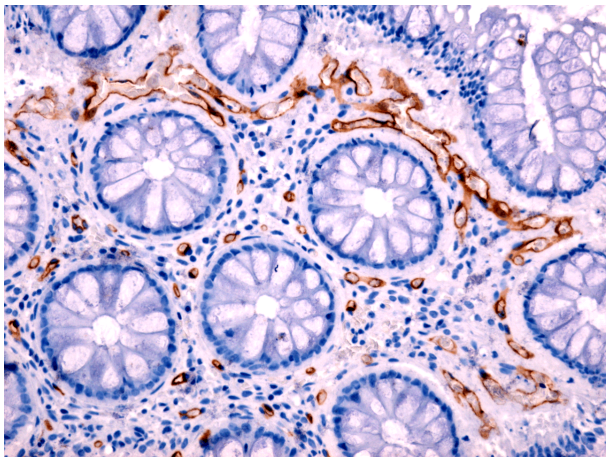

Figure 13 – ***Well-developed blood capillary network, in a case of mild ulcerative colitis. Immunomarking with anti-CD34 antibody, ×200.***

Discussion

Ulcerative colitis is a disease whose annual incidence varies between 1 and 20 cases in 100 000 persons and the prevalence varies between 8 and 246 in 100 000 persons [11]. The geographical distribution of the disease varies from one country to another and from one region to another. The etiopathogenic mechanisms of the disease are not known, but most of the studies indicate an alteration of the immune mechanisms.

Dysregulation of mucosal immune response in pathogenesis of inflammatory bowel disease is expressed by a dysregulated T-helper (Th) cell response that plays a major role in causing chronic intestine inflammation [12, 13].

In our study, by comparing the two interleukins serum levels, we observed the increasing trend of IL-13 and IL-17 serum levels with a maximum value in the group of patients with moderate ulcerative colitis activity and a slight further decline in the group of patients with severe activity. IL-17 showed statistically significant differences when comparing the serum activity in the patients with severe *vs.* mild activity (p=0.0483) and between moderate *vs.* mild activity (p=0.0065). There were no statistical differences between the serum levels of the patients with severe *vs.* moderate activity (p=0.2577). The IL-13 serum levels did not show statistical differences when they

were compared among different subgroups of patients (patients with severe *vs.* moderate activity, mild *vs.* moderate activity, severe *vs.* mild activity ($p>0.05$).

IL-17 was considered the target of intensive research in autoimmune diseases. Increased IL-17 serum levels in UC patients might be explained through the synergistic activity of IL-17/IL-23 axis and pro-inflammatory cytokines, causing a severe clinical outcome in IBD patients. The prolonged excretion of blood in stool caused by an inflammatory process, causing iron metabolism disorder and anemia, may elucidate the inverse correlation between hemoglobin and IL-17 serum levels in UC patients [14–16].

Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution [17].

In time, we observed a phenotypic progression of the disease in newly diagnosed patients with left ulcerative colitis by weight loss, double weight extensive colitis and cases of proctitis. In terms of lesion localization, we observed that left colitis prevailed in UC patients. We found significantly higher concentrations of IL-17 in UC patients with extension to the left colon. Additionally, the IL-17 serum levels were significantly correlated with the severity of UC, suggesting that serum IL-17 might be closely related to the inflammatory process of this disease. Related to the extent of the E lesion and interleukins levels, we mention that IL-13 presented higher concentrations in E2-type lesions and IL-17 increased especially in patients with type E3 lesions.

We also found the CRP had significantly higher values in patients with extensive colitis (E3) as compared to patients with left colitis (E2). Analyzing the CRP levels in serum of patients in various stages of UC clinical activity, we found an increase in the direct proportion to the severity of disease activity, with the highest concentrations in patients with severe activity, similar data being observed by other authors, as well [18]. Only the serum concentrations in the patients with severe and moderate activity were significantly higher *vs.* the control group ($p<0.001$). Statistically significant differences were observed between the group of patients with severe *vs.* mild activity ($p=0.0094$) and between moderate *vs.* mild activity ($p=0.0481$). Between serum levels CRP of patients with severe *vs.* moderate activity there was not reached any statistical significance threshold ($p=0.1913$).

In establishing the diagnosis and management of UC, the histopathological examination plays an important part, but it always should be interpreted in correlation with the clinical, endoscopic and immunological examination. The histopathological diagnosis requires the knowledge of the morphological characteristics of UC, as there may exist a series of atypical pathological changes that may lead to misinterpretations [19, 20].

In our study, the histopathological examination highlighted changes of the Lieberkühn glands architecture, associated with the presence of an inflammatory infiltrate of various degrees, in the lamina propria of the colon mucosa. According to some authors, when the disease is at its highest activity, there may be highlighted neutrophils in the intestinal epithelium and glands (crypts), taking aspects of glandular abscesses; in the remission stage, the neutrophils are absent from the mucosa, although the chronic inflammation continues [21]. We identified the presence of a chronic infiltrate, mainly formed of T-lymphocytes, but there have been also identified B-lymphocytes, macrophages and plasmocytes. These aspects may be due both to the clinical forms of the disease and to the fact that our patients followed different treatments before undertaking the endoscopy and biopsy of large intestine mucosa. Some studies have identified, in long, severe, chronic forms of UC, the presence of plasmocytes in the inferior third of large intestine lamina [22, 23]. These histopathological aspects could make the distinction with infectious colitis [24].

We consider that the UC severity is mainly determined by the clinical symptoms, which most often correlate with the laboratory tests, the endoscopic and histopathological aspects. Still, the histopathological measurement of the inflammation degree is an approximately one. Still, the histopathological examination brings essential data regarding the severity of the disease and the risk of malignant degeneration [25].

Conclusions

We have achieved significantly higher concentrations of IL-13, IL-17 and CRP in the serum of UC patients compared to the control group. Our study showed the existence of a phenotypic association with serological markers analyzed in the serum of UC patients. In the UC patients with extension to the left colon (E2), there were found significantly higher IL-17 concentrations. Because the IL-17 serum levels were significantly correlated with the disease severity and was the only cytokine that correlated positively and significantly with high serum levels of CRP in UC patients, IL-17 can be considered an important marker of this disease inflammation progress.

Author contribution

All authors have contributed equally to the present work.

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