

## Fecal Calprotectin for Assessment of Inflammatory Activity, Monitoring of Therapy and Prediction of Relapse in Ulcerative Colitis

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In patients in remission, average FCP levels did not differ from that in controls ( $p=0.704$ ), and at elevation above 250 mg/kg relapse of disease occurred in the next month.

**CONCLUSION:** FCP is non-invasive bio-marker for evaluation of the inflammatory activity in UC patients, for monitoring of the therapeutic effect and predicting disease relapses.

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**Key words:** Ulcerative colitis; Intestinal inflammation; Fecal calprotectin; Therapy; Relapse

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### ABSTRACT

**AIM:** To investigate the role of FCP as a non-invasive marker for assessment of inflammatory activity, monitoring of therapy and prediction of relapses in UC patients.

**METHODS:** The study included 37 patients with UC. The extent and clinical severity of UC was assessed by the modified Montreal classification. Endoscopic activity was assessed by the Mayo scoring system. The control study was performed in eleven patients after treatment. Fourteen of the patients were followed-up and tested to achieve complete remission. FCP was assessed by "Calpo Test" based on ELISA.

**RESULTS:** FCP levels in patients with total and left side UC were significantly higher than the relevant parameters of the patients with distal UC and controls ( $p<0.001$ ). FCP of patients with left side and total UC was similar ( $p=0.541$ ). FCP levels were increased significantly with an increase of clinical severity, but the differences between the cases with moderate and severe clinical activity were not statistically significant ( $p=0.096$ ). Comparing the results of EMS with the values of FCP showed a statistically significant correlation—higher values of EMS correspond with higher levels of FCP. After the treatment, significant reduction in FCP levels were found ( $p<0.0001$ ).

### INTRODUCTION

Ulcerative colitis (UC) is a chronic idiopathic disease characterized by recurrent episodes of diffuse inflammation of the large intestine's mucosa followed by periods of remission. In view of patient management, it is of great significance to determine the degree of inflammatory activity in colonic mucosa<sup>[1]</sup>.

The inflammatory activity in UC (and also in gut inflammation in general) could not be assessed directly by doctors or patients. For this reason, lots of methods have been developed to characterize the severity and the extension of the inflammation. Various indexes are used in clinical trials but their use in clinical practice is based on subjective patient complaints. For this reason, they cannot be very precise<sup>[2,3]</sup>. Some laboratory markers of inflammation are used in an attempt to get over this problem, such as erythrocyte sedimentation rate (ESR), granulocytes and platelets count in the peripheral blood, C- reactive protein (CRP), etc. Unfortunately, they have low specificity for gut inflammation and do not reflect adequately the disease activity<sup>[4,5]</sup>. In order to optimize the assessment of gut inflammation, some other tests and methods are used. The increase of intestinal permeability could be a surrogate marker of inflammation but this method had not been widely used in clinical

practice<sup>[6]</sup>. One of the most precise ways to localize inflammation in the gut is Indium111-leukocyte scanning and the count of Indium111-bounded leukocytes in the feces<sup>[7]</sup>. This method is unsuitable for outpatient practice because of the exposure to radiation, the four-day collection of the stool samples, and its high price.

Although non-invasive imaging tests like ultrasound, computed tomography (CT), magnetic resonance imaging (MRI) and roentgen-contrast imaging (enteroclysis, contrast enema) could be helpful in localizing and staging the intestinal inflammation, the following disadvantages could occur in some of them-suboptimal sensitivity and specificity, exposure to unacceptable dose of ionizing irradiation, and high price<sup>[3]</sup>.

Nowadays a gold standard for assessing the site, extension and severity of the intestinal inflammation is the endoscopy with biopsy<sup>[1]</sup>. However, endoscopic procedures are invasive, with a substantial risk of complications<sup>[8]</sup>. Such procedures can be painful and frequently require use under general anesthesia, which may be dangerous for the patient. Bowel preparation for colonoscopy is unpleasant and could be hazardous in some situations<sup>[3]</sup>.

In the search for new, more accessible, informative and inexpensive tests for diagnosis and assessment of gut inflammation in the last years of the 20<sup>th</sup> century, more attention was paid to the bioactive markers<sup>[9]</sup>. Inflammation is characterized by the migration/activation of series of cells toward the site of infection or tissue damage. The main role in cell migration during inflammation belongs to neutrophil granulocytes. In cases of gut inflammation they pass into the feces and the same happens to the substances (especially proteins) contained in and secreted by neutrophils. The neutrophil proteins (surrogate markers) include calprotectin, lactoferrin, polymorphonuclear elastase, myeloperoxidase, etc.<sup>[10-12]</sup>. The finding and the quantitative assessment of these proteins in the feces makes them very suitable biomarkers for demonstrating, staging and following-up the gut inflammation. Calprotectin is the most widely applied neutrophil biomarkers in clinical practice. It is about 5% of total protein and 60% of cytosol proteins of neutrophils and is released from them during cell activation or cell death. Competing with various enzymes for limited quantities of zinc, calprotectin can suppress a great number of zinc-dependent enzymes and thereby kill certain microorganisms and other cells. It is found in six times greater concentrations in feces than in plasma<sup>[1,10,12]</sup>. In case of inflammation of the bowel wall neutrophils migrate toward and through it and thereby calprotectin goes into the feces. It resists metabolic degradation by intestinal bacteria and is stable in the stools for up to seven days<sup>[13]</sup>.

We aimed to investigate the role of fecal calprotectin (FCP) as a noninvasive marker for assessment of inflammatory activity, monitoring of therapy and prediction of relapses in patients with UC.

## METHODS

This prospective study enrolled 37 patients – 17 male and 20 female at an average age of 43.1 (19-64) years with a newly diagnosed UC. The patients were hospitalized in the Clinic of Gastroenterology, University Hospital Queen Joanna in Sofia for the period from August 2008 to August 2011. The diagnosis was made on the basis of generally accepted criteria. At the moment of the inclusion of the patients in this study, the extension of colitis and the severity of the clinical course of the disease were assessed by the first author using the modified Montreal classification of inflammatory bowel disease (IBD)<sup>[14]</sup>. Endoscopic activity was assessed by the Mayo endoscopic score<sup>[15]</sup>. Patients affected by neoplastic diseases of the colon and patients taking non-steroid anti-inflammatory drugs

(NSAIDs) for less than a month before the study were excluded. All of the UC patients were clinically and endoscopically active at the time of taking of stool samples for FCP. The results of these 37 samples recorded values of FCP at the time when the diagnosis of the disease was made. On the basis of this data, comparisons were made between values of FCP at different extension and severity of the disease. Subsequently, 14 patients with different extension and severity were followed-up and monitored until clinical, endoscopic and histological remission. Clinical remission (S-0) was defined as disappearance of symptoms - lack of subjective complaints and normal defecation without blood in the stool, and occurred at different time-points for different patients (from 2 to 6 weeks from initiation of therapy), depending mostly on the severity of the disease. Then, these patients were followed-up and controlled endoscopically around the 12<sup>th</sup> week of the initiation of therapy, when endoscopic and histological remission was documented. From this point until the end of the study, they were monitored clinically every month and provided samples for FCP examination. These 14 patients had serial FCP examinations. This group included patients living close to the hospital regions, who had the possibility of frequent visits to control examinations. The remaining 23 patients had only one study of FCP, made at enrolment.

Eight of the patients with active UC had ulcerative proctitis – E1, 15 of the patients had left colitis (distal from splenic flexure) – E2 and 14 had extensive colitis (proximal from splenic flexure) – E3. Clinical course was determined as severe (S3) in 16 patients, moderate (S2) in 15 and mild (S1) in 6 patients. Patients with mild UC were hospitalized due to requirements of the hospital protocol for inclusion in this study.

In 11 patients with severe disease, extension of colitis E2-3, severity S2-3 and endoscopic Mayo score 2-3, stool samples were collected again after 14 days of treatment with steroids, ciprofloxacin and metronidazole in the common dosage, with improvement of the symptoms. A second stool sample was taken in another 3 patients without improvement of the symptoms. All samples for FCP were taken before steroid and antibacterial treatment (if it was carried out). Twenty-one healthy volunteers (12 male and 9 female at an average age 40.8 (18-69) without any history of gastrointestinal symptoms and without any complaints at the moment of the sample taking were tested for FCP as a control group.

### Analysis of FCP

Calprotectin was analyzed in stool samples by means of “Calpo Test” based on enzyme linked immunosorbent assay (ELISA), made by Nova Tec Immunodiagnostica of Germany. All tested persons provided 1-5 grams of stool samples. The samples were immediately put into freezer at 20°C. Before analyzing, the samples were defrosted at room temperature followed by extraction of 100 mg feces from each sample in a buffer solution. A defined volume of supernatant was diluted 50 times and introduced into veritable determination of calprotectin. The reading was automatic against a calibration curve. The final result was present in milligrams per kilogram feces (mg/kg).

### Statistical methods

The value quoted as normal for fecal calprotectin was <50 mg/kg of stool (the baseline value of the manufacturer). For data analysis the following statistical methods were used: descriptive statistics for tabular and graphical presentation of results; Kolmogorov-Smirnov test and Shapiro-Wilk test for normality of the distributions of the studied variables; nonparametric tests of Mann-Whitney to

**Table 1** Values of fecal calprotectine analysed by ELISA Calpo Test in patients with ulcerative colitis.

Group of patients	N	ELISA FCP					Percentiles	
		Mean	SD	Median	Min	Max	25 <sup>th</sup>	75 <sup>th</sup>
Active UC	36	1289.2	793.1	1113	93.1	2530	694.0	2122.5
UC-S1	5	141.1	67.5	120.6	93.1	258.5	96.8	195.8
UC-S2	15	1276.7	684.5	1108.0	338.0	2402.0	730.0	2018.0
UC-S3	16	1659.7	665.9	1699.0	682.0	2530.0	971.3	2314.3
UC-E1	7	214.9	142.1	133.0	93.1	461.0	100.4	338.0
UC-E2	15	1469.8	673.7	1233.0	529.0	2428.0	872.0	2228.0
UC-E3	14	1632.9	639.7	1640.5	891.0	2530.0	949.8	2198.0
UC-before therapy	11	1798.5	562.7	2018.0	891.0	2472.0	1233.0	2176.0
UC after therapy	11	737.1	439.0	945.0	214.0	1397.0	265.0	1103.0
UC in remission	10	42.2	19.1	38.5	18.3	77.8	24.5	57.5

verify the hypotheses of a difference in two independent samples; Wilcoxon signed-rank test for verification of the hypotheses in two related samples; Analysis of variance / ANOVA/ ; Post Hoc Tests of Tamhane; and ROC analysis. The obtained results were assessed as statistically reliable in threshold level of significance  $p < 0.05$ .

**Ethics approval**

The study was approved by the Ethics Committee of University Hospital Queen Joanna in Sofia, Bulgaria. Written informed consent was obtained from all patients and control subjects.

**RESULTS**

The average value of FCP in the control group was 38.3 mg/kg (3.3-68.0) with a standard deviation (SD) of 17.9 and median 36.2. In the present study, we accepted an increased value above  $1.96 \times SD + \text{mean value}$  (38.3), e.g. values above 73.4 mg/kg. All of the controls were below this value (Table 1).

The average value of FCP in the patients with clinical and endoscopic activity ( $n=37$ ) was 1289.2 mg/kg (93.1-2530) which was many times higher than the values of FCP in the controls ( $p < 0.0001$ ).

**Results by location**

In 8 of the patients with active UC the disease was localized in the rectum (E1). The average value of FCP in this group was 214.9 mg/kg (93.1-460) which was significantly different from the controls ( $p < 0.001$ ) (Figure 1). The average value of FCP in the patients ( $n=15$ ) with left-sided colitis (E2) was 1469.8 mg/kg (529-2428) which was significantly higher than the controls ( $p < 0.0001$ ) and than E1 group ( $p < 0.001$ ) (Figure 1). The average value of FCP in patients with extensive disease (E3) was 1632.9 mg/kg (891-2530) which was significantly different from the controls ( $p < 0.0001$ ) and from E1 group ( $p < 0.001$ ). This average value was higher than the average value of FCP in E2 group but had no statistical significance ( $p=0.5412$ ) (Figure 1).

ROC analysis of FCP values by extension of the disease found that sensitivity and specificity of FCP for distinguishing E-1 (proctitis) from E-2 and E-3 (extensive colitis) were optimal (both 100%) at value of 495 mg/kg. Because of the fact that optimal values for sensitivity and specificity were reached at this cut-off level, we did not represent this result graphically.

**Results by severity**

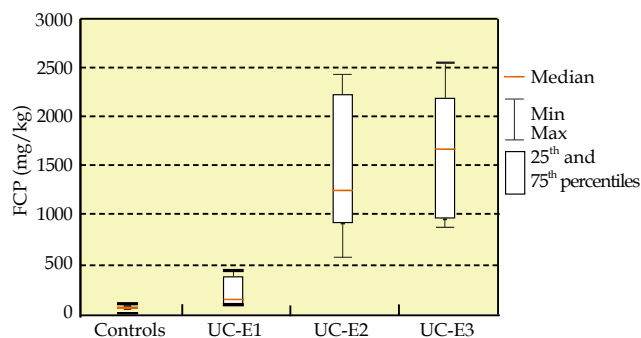
In our study we had just 6 patients with mild disease (S1), whose average value of FCP was 141 mg/kg (93.1-258.5), which was significantly higher than that of the controls ( $p < 0.001$ ). However, the number of subjects in this group was too small to perform correct statistical analysis. In moderate ulcerative colitis – S2 ( $n=15$ )

the average value of FCP was 1267.7 mg/kg (338-2402) which was significantly higher than the controls ( $p < 0.0001$ ) and than the S1 group ( $p < 0.01$ ). In 16 patients with severe disease (S3) the average value of FCP was 1632.9 mg/kg (682-2530) which was significantly higher than the controls ( $p < 0.0001$ ) and mild – S1 disease ( $p < 0.01$ ) but not significantly higher than moderate – S2 disease ( $p=0.0969$ ) (Figure 2).

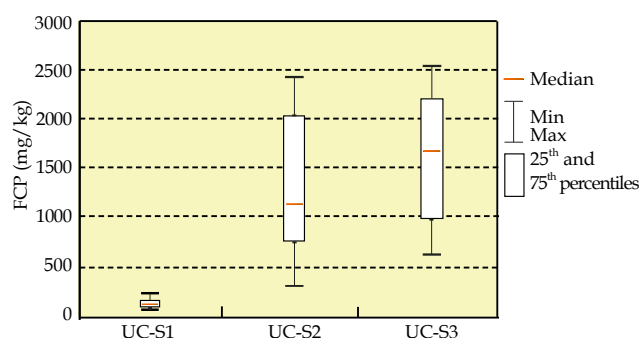
Of all 37 patients, just 4 had endoscopic Mayo score (EMS) 1 - and four were with E-1 location. Thirteen had EMS 2 (4 of them with E-1 and 9 with E-2 or E-3). The remaining 20 patients had EMS 3 – all of them were with extensive colitis. Performance of Analysis of variance (ANOVA) showed statistically significant correlation between FCP and EMS,  $F(2;34) = 20.01, p < 0.001$ . Higher values of EMS correspond with higher levels of FCP. The additional Post Hoc Tests of Tamhane showed statistically significant differences in average values of FCP between score 1 and score 2 ( $p < 0.01$ ), score 1 and score 3 ( $p < 0.0001$ ), and between score 2 and score 3 ( $p < 0.0001$ ) (Figure 3).

**Results over time with therapy**

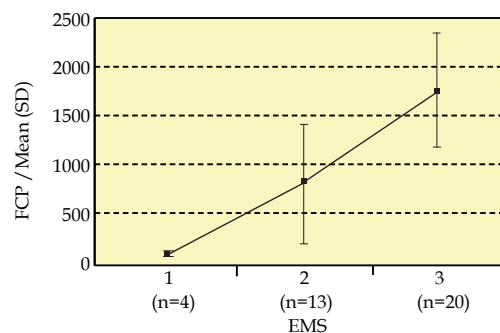
In 11 patients with active and extensive UC classified as E2-3,



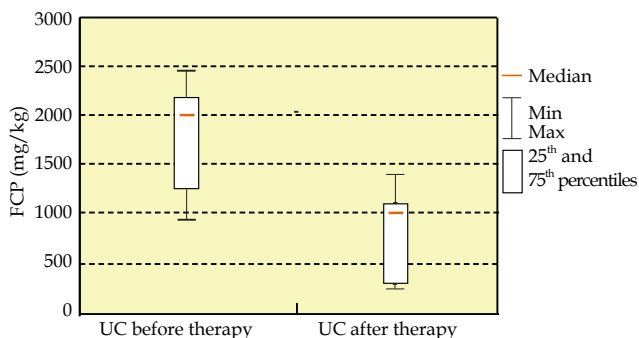
**Figure 1** Values of FCP in controls and patients with UC depending on extension of disease - E.



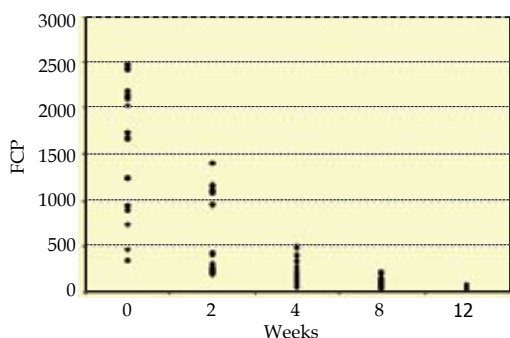
**Figure 2** Values of FCP in patients with UC depending on severity of disease - S.



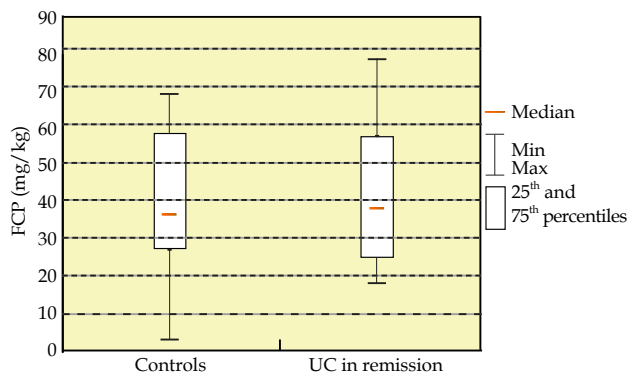
**Figure 3** Analysis of variance (ANOVA) of FCP values according to endoscopic Mayo score.



**Figure 4** FCP values in the group patients /n=11/ with extensive and severe UC before and after the 14<sup>th</sup> day of therapy.



**Figure 5** FCP values in dynamics in all patients /n=14/ who were followed up and reached complete remission.



**Figure 6** Values of FCP in controls and in patients with UC in remission.

S2-3 and endoscopic Mayo score 2-3, we calculated the average value of FCP at 1798.5 mg/kg (891-2472) before the treatment with methylprednisolone, ciprofloxacin and metronidazole. Fourteen days after the start of therapy we found that if the clinical activity had been decreased to S0-1 and endoscopic activity to Mayo 1-2, the corresponding average value of FCP would have been decreased to 737.1 mg/kg (214-1397) and the difference from the initial mean value of FCP would have been statistically significant ( $p < 0.001$ ) (Figure 4).

In 3 of the patients with severe disease –S3 and lack of improvement in the course of therapy, the very high initial values of FCP did not decrease considerably. Because of the small number of subjects we did not calculate average values and statistical significance.

Fourteen of the patients with active UC were followed-up to full clinical, endoscopic and histological remission – S0 and Mayo score 0. Moreover, except for the first one, they had provided samples for FCP examination at the 2<sup>nd</sup>, 4<sup>th</sup>, 8<sup>th</sup> and 12<sup>th</sup> week. (Figure 5)

The average value of FCP in the remission group was 42.2 mg/kg (18.3-77.8) and did not differ significantly from the controls

( $p = 0.7037$ ) (Figure 6).

In six of the patients with UC in remission we observed relapse of the disease in line of supportive treatment with 5-aminosalicylic acid (5-ASA). The assessment of the samples provided by these patients during their visit before the relapse occurred (in absence of any clinical symptoms) showed that the FCP values rose above 250 mg/kg. In the next few days (up to one month), e.g. the time of the next visit, a clinical and endoscopic relapse occurred. The patients who did not relapse had FCP values similar to the values found in documentation of remission during the control tests. Because of the small number and the different extension/severity of the disease in these patients we did not perform statistical analysis.

## DISCUSSION

One of the possible methods for assessing the inflammation discussed in the recent years is the analysis of neutrophils' infiltration in the intestinal mucosa and their transmigration to the lumen<sup>[16]</sup>. In patients with active ulcerative colitis FCP values vary from 200 to 20 000 mg/kg<sup>[8,17,18]</sup>. Many authors argue that FCP levels are more closely related to histological evaluation than to endoscopic images, suggesting that this biological marker is more sensitive than the endoscopy in the assessment of IBD activity<sup>[8,12]</sup>. Several studies demonstrate that higher levels of inflammation are associated with elevated FCP showing a significant correlation between FCP and severity of inflammation<sup>[8]</sup>. It is believed that FCP better reflects the disease activity in UC than in Crohn's disease<sup>[18,19]</sup>.

The results of our study show a link between FCP and the extension and the severity of disease assessed by the Montreal classification. Several studies show relationship between FCP levels and severity of UC<sup>[19,20,21]</sup>. However, the relation of FCP levels to the extension of the disease has not still been studied enough. In patients with rectal involvement only (E-1) the inflammatory process is localized within a relatively small area of the intestine. FCP values in these patients are significantly lower than values in patients with extensive disease (E 2-3). Moreover, in our study FCP demonstrates optimal sensitivity and specificity to distinguish proctitis from extensive colitis at a cut-off point of 495 mg/kg. Certainly, we ourselves deal with some criticism of this result, mainly due to the small number of patients with E-1 in this study. Nevertheless we conclude that FCP levels above that quoted in patients suspected to have UC suggest extension of the disease. With regard to the severity of the disease course, patients with mild disease (S-1) have much lower values of FCP than patients with moderate and severe courses of disease. This is demonstrated convincingly and in tracking the effect of therapy administered in a group of 11 patients where values of FCP decreased significantly after 14 days of treatment. Similar results are reported by other authors<sup>[1,22]</sup>. It is striking that there was no significant difference between FCP levels in patients with extensive disease E-2 and E-3, although values in the subgroup E-3 are higher. The same applies to severity – the values in subgroup S-3 are considerably higher than those in S-2, but did not reach a statistically significant difference. A possible explanation of this fact is that by more active inflammation, reflected in the severe course of disease, the levels of FCP may be affected by a number of factors: very rapid transit through the gut, changes in secretion and absorption, different amounts of blood in the feces, etc. On the other hand, the results of our study demonstrate significant difference in FCP levels according to the endoscopic activity of the disease, evaluated by the Mayo scoring system. We do not undertake to comment on the above statements that the FCP is more closely related to histology than to the endoscopic findings, we also do not

aim to investigate their sensitivity and specificity. However, our data convincingly demonstrates that high FCP values correspond to high EMS, and that the reduction of endoscopic activity results in reduced values of the FCP. The practical benefit of this statement would be more widespread use of FCP in monitoring the effects of treatment and in decreasing the number of control endoscopies. The average values of FCP in our study are among the highest reported so far. The majority of patients in our study had severe and extensive disease which explains their very high FCP values.

The values of the FCP in patients in remission in our study did not differ significantly from the values of calprotectin in the healthy controls. In a large study of Schoepfer *et al.*<sup>[20]</sup>, the average value of FCP in inactive UC is 42.0 mg/kg which is obviously very similar to the average value we found (42.2 mg/kg). The values by other authors are similar, too<sup>[23,24]</sup>. Some patients in remission may have low intestinal inflammation. Their FCP values are higher but usually do not exceed 150 mg/kg<sup>[23,24]</sup>. In our study we show the dynamics of decrease of FCP values in the group of patients that achieved complete remission. The figure illustrates that this decrease is pronounced during the first 4 weeks, then it is more supple in the next 4 weeks. At the 8<sup>th</sup> week follow-up patients were practically in clinical remission, but as is seen from the figure many of them continued to have slightly elevated FCP. In objectifying the complete remission (clinical, endoscopic and histological) our follow-up patients had reference or close to reference values of FCP.

In UC patients, the periods of remission were usually interrupted by a relapse of the disease. Most of the patients with stable IBD had a low-grade inflammation. It is considered that relapse can occur only when the inflammatory process reaches a critical force/critical levels. Since inflammation is a continuous process, direct assessment of the levels of inflammatory activity may provide a quantitative measurement of pre-symptomatic threatening clinical relapse<sup>[6]</sup>. The clinical application of this knowledge is the prediction of relapse. This may allow targeted treatment of earlier stages with a few side effects for prevention of relapse.

In our series, we followed 6 patients who showed clinical relapse within 1 month after serial examination and submission of samples for FCP. Since the samples were frozen, we found later (after the workout of samples) that the six values were above 250 mg/kg. Similar values have been reported by other authors<sup>[23,24]</sup>. The patients who did not relapse had low values of FCP in the control test (similar to those found in the documentation of remission), which proves that normal values of FCP in patients in clinical remission correlate with the term “mucosal healing”.

The introduction of rapid tests for quantitative examination of FCP allows timely response and enhancing the treatment strategy in patients in remission with elevated FCP determined by control studies. Thus, there is an opportunity to prevent relapse.

As a conclusion, FCP is a noninvasive, easy and reliable biomarker for assessment of inflammatory activity in patients with UC and for monitoring of the ongoing therapy. Extremely valuable for clinical practice is the possibility that the elevated FCP in patients in remission serves as a predictor of disease relapse; thus treatment can be targeted in order to prevent relapsing.

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