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Fecal sST2 correlates with the disease severity of ulcerative colitis

Fecesni sST2 korelira sa težinom ulceroznog kolitisa

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Abstract

Background/Aim. Ulcerative colitis (UC) is a chronic, relapsing inflammatory disease affecting the distal colon and rectum with complex pathogenesis and diagnosis, indicating the need for new diagnostic and prognostic markers. The aim of this study was to determine the fecal values of TNF-α, IL-17, IL-10 and soluble protein ST2 (sST2) in the patients with UC and their relationship with clinicopathological aspects. **Methods.** The samples of stool of 80 patients with UC were analyzed. Concentrations of TNF-α, IL-17, IL-10 and sST2 were measured by ELISA. **Results.** Concentrations of TNF-α, IL-17 and sST2 were significantly increased in the feces of patients with the higher endoscopic, clinical and total Mayo score, as well as in the patients with an intense crypt destruction, erosion of the mucous membranes, architectural changes, neutrophil infiltration and eosinophil infiltration. The local value of anti-inflammatory cytokine IL-10 in liquid fraction of feces was increased in the patients with an advanced endoscopic stage of UC. The moderate positive correlation between the fecal sST2/IL-17 and the clinical and histological parameters of disease severity and also the strong correlation between sST2 and IL-17 was also observed in the feces of patients with UC. The analysis of receiver operating characteristic (ROC) curves showed that the optimal cut-off value for sST2 of 624.0 pg/g allows the discrimination of clinical stages of UC. **Conclusion.** The increased fecal value of sST2 in the UC patients with a higher endoscopic, clinical and histological stage of disease may be considered as a sign of the disease severity. The fecal values of sST2 can be used as a valuable marker for UC severity.

Key words:
colitis, ulcerative; biomarkers; disease progression; feces; tumor necrosis factor-alpha; interleukin-10; interleukin-17; il1rl1 protein, human.

Apstrakt

Uvod/Cilj. Ulcerozni kolitis (UC) je hronična, relapsirajuća inflamacijska bolest koja zahvata distalni kolon i rektum sa kompleksnom patogenezom i dijagnozom, ukazujući na potrebu za novim dijagnostičkim i prognostičkim markerima. Cilj studije bio je utvrđivanje vrednosti TNF-α, IL-17, IL-10 i rastvorljivog proteina ST2 (sST2) fecesu bolesnika sa UC kao i njihovogodnosa sa klinikopatološkim aspektima bolesti. **Metode.** Analizirani su uzorci stolice 80 bolesnika sa ulceroznim kolitisom. Koncentracije TNF-α, IL-17, IL-10 I sST2 merene su korišćenjem senzitivnog ELISA (*Enzyme-linked immunosorbent assay*) testa. **Rezultati.** Koncentracije TNF-a, IL-17 i sST2 značajno su bile povećane u fecesu bolesnika sa većim endoskopskim, kliničkim i ukupnim Mayo skorom, kao i kod onih sa izraženim oštećenjem kripti, erozijom sluzokože, strukturnim promenama tkiva, neutrofilnom infiltracijom i eozinofilnom infiltracijom. Lokalna vrednost antiinflamacijskog citokina IL-10 u tečnoj frakciji fecesa bila je povećana kod bolesnika sa uznapredovalim endoskopskim stadijumom bolesti. Takođe je nađena umerena pozitivna korelacija između vrednosti sST2/IL-17 u fecesu i kliničkih i histoloških parametara težine bolesti, kao i snažna korelacija između vrednosti sST2 i IL-17 u fecesu bolesnika sa UC. Analizom ROC krive ustanovljeno je da granična vrednost za sST2 od 624,0 pg/g determiniše potencijalno veći rizik za razvoj klinički teže forme UC. **Zaključak.** Povećane vrednosti sST2 u fecesu bolesnika sa UC sa uznapredovalim endoskopskim, kliničkim i histološkim stadijumom bolesti može se smatrati znakom težine bolesti. Vrednosti sST2 u fecesu mogu se koristiti kao marker procene težine UC.

Ključne reči:
kolitis, ulcerativni; biološki pokazatelj; bolest, progresija; stolica; faktor nekroze tumora; interleukin-10; interleukin-17; il1rl1 protein, humani.

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Introduction

Inflammatory bowel disease (IBD) presents a chronic, relapsing and progressive inflammation of gastrointestinal tract [1]. The two most studied entities of IBD are the ulcerative colitis (UC) and Crohn`s disease (CD). UC is a chronic, relapsing inflammatory disease affecting the distal colon and rectum, limited to mucosa and associated with continuous, submucosal inflammation and the formation of ulcers [1–2]. The pathogenesis of disease is complex. The immune system seems to be the major mediator in it [3]. It is usually based on imbalance between pro- and anti- inflammatory cytokines in the intestinal mucosa and subsequent chronic inflammation [4–5]. The secretion of type 1 (TNF-α, IFN-γ), type 2 (IL-4, IL-10) and type 17 cytokines (IL-17) as well as a response to self-antigens in mucosa are predominant factors in the genesis and development of UC [6–9]. Although UC seems to be predominantly Th2 disease [10], recent data revealed an important role of type 1 and type 17 innate and acquired immune responses in the onset and progression of disease [11]. Local chronic inflammation, typical for UC, often leads to the clinical symptoms and signs such as diarrhea, rectal bleeding, abdominal pain, fever, anemia and body weight loss [2]. The disease activity can be determined by using the clinical and endoscopic scores, serum or fecal biomarkers [12–14]. The endoscopic findings of mucosa were classified as ones of the major parameters in estimating the disease severity [13–15]. A disadvantage of using the clinical and endoscopic scores for the disease severity evaluation is usage of invasive and costly procedures [15]. This has led to the need for noninvasive tests regarding the disease evaluation. There has been a sustained interest in the identification of state biomarkers for the UC severity [11, 15]. New markers should contribute to the prediction of prognosis.

The aim of the study was to compare the accuracy of selected biomarkers in assessing the disease severity. Concentrations of biomarkers TNF-α, IL-17, IL-10 and soluble ST2 protein (sST2) were assessed in the stool samples of patients with UC. We tested how reliably these biomarkers reflect the clinical and endoscopic scores and the histopathological characteristics of affected tissue. For more sensitive markers, we determined cut- off levels for the clinically and endoscopically estimated severe disease.

Methods

Ethic approvals

The study was conducted at the Center for Gastroenterology, Clinical Center of Kragujevac and the Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Serbia. The ethics approvals for this study were obtained from relevant Ethics Committees of the Clinical Center Kragujevac, Kragujevac, Serbia, and Faculty of Medical Sciences, University of Kragujevac, Serbia. All experiments were performed according to the relevant guidelines and regulations. The informed consent was obtained from all patients in writing.

Patients

Eighty patients, between 21 and 80 years of age diagnosed as the UC cases were recruited in this study. A diagnosis was made on basis of established clinical, endoscopic and histological criteria [16]. The study did not include the patients with no well-defined pathology, no adequate clinical document available, or with previously diagnosed coexisting cardio-pulmonary, renal, hepatic, allergy and rheumatic disease, who were treated with anti-inflammatory drugs. The stool samples were taken before the surgery and stored at -80°C.

Disease activity index

The clinical activity of the disease was evaluated according to the data available from the patients' records. Three clinical variables were graded: frequency of evacuation, amount of blood in the stool, and a physician's global assessment. The Mayo clinical subscore 0 was defined as remission, 1–3 as mildly active disease (I), 4–6 as moderately active disease (II), and ≥ 7 as severely active disease (III) [17]. The endoscopic findings were scored according to the Mayo endoscopic subscore, graded as normal (0), mild (I), moderate (II), or severe (III) disease activity. Finally, the full Mayo Score was calculated on basis of four parameters: stool frequency, rectal bleeding, endoscopic evaluation and a physician's global assessment [18]. The score 0–I was defined as remission and the score II–III as active disease [19].

Histological activity

The histological activity was scored according to the Geboes Score (GS), considering the presence of architectural changes, neutrophils, eosinophils, crypt destruction and erosion of the mucous membranes [20].

Measurement of TNF-α, IL-17, IL-10 and sST2 in feces

Stools (1–10 g) were collected in the sterile containers and weighed. They were divided into 1 g aliquots and then emulsified in 5 mL of protease inhibitor cocktail (SIGMA, P83401), diluted 1:100, and centrifuged for 5 minutes at 400 G, at 4°C, as previously described [21, 22]. The supernatant fluid was collected and stored at -80°C until ELISA.

The fecal concentrations of cytokines were measured, as described [9], using the sensitive enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN,) specific for human cytokines according to the manufacturer's instructions. Briefly, the 96-well plates were coated with capture antibody, overnight. The plates were washed with a washing buffer (0.05% Tween-20 in PBS) and incubated with blocking buffer (1% bovine serum albumin in PBS) for 1 hour at room temperature. The serum/fecal samples, or standard recombinant TNF-α/IL-17/IL-10/sST2 were introduced to the plates for 2 hours before the application of biotinylated detection antibody for 1 hour at room temperature. After introduction of streptavidin peroxidase for 1 hour, the plates were developed with substrate reagent for 20 minutes. The reaction was stopped by adding 4 mol/L sulfuric acid, and the absorbance was read at 495 nm by a microplate reader. We measured the exact concentration of the men-

tioned biomarkers by intrapolation of a standard curve made by a series of well-known concentrations as per manufacturer's instruction. The values of measured cytokines are presented as pg/g of feces.

Statistical analysis

The statistical analyses were performed using the SPSS 20.0 software. The results were reported as mean and standard error (SE). The statistically significant difference between the means of two groups was determined using the Student's *t*-test for the independent samples if the data had normal distribution, or the Mann-Whitney *U*-test for data without normal distribution. The Pearson's correlation evaluated the possible relationship between the cytokines and disease severity and progression in the patients with UC. The strength of correlation was defined as negative, or positive, weak (-0.3 to -0.1 or 0.1 to 0.3), moderate (-0.5 to -0.3, or 0.3 to 0.5), or strong (-1.0 to -0.5 or 1.0 to 0.5). The *p*-value of 0.05 was considered statistically significant.

Results

Eighty patients, between 21 and 80 years of age diagnosed and histologically confirmed as UC were recruited in this study. There was no significant difference in gender distribution. The clinical and pathologic characteristics of these patients are presented in Table 1. We have assessed the concentration of pro- and anti-inflammatory cytokines as well as sST2 in the fecal liquid fraction of all patients. The patients with UC were classified into two groups based on the endoscopic, clinical and total Mayo score, respectively: I – scores 0 and 1; II – scores 2 and 3. The representative images of endoscopic Mayo subscores in patients with UC are shown in Figure 1 A and B. Further, the patients were divided according to the histological characteristics (crypt destruction, erosion of the mucous membranes, architectural changes, neutrophil infiltration and eosinophil infiltration) into two groups: I: scores 0 and 1; II: scores 2 and 3. We analyzed the values of previously defined markers of interest between the defined groups.

Table 1

Baseline characteristics of patients

Parameters	Values
Gender (male/female), n	46/34
Age (years), mean (range)	50 (21–80)
Endoscopic score (0/I/II/III), n	0/41/26/13
Clinical score (0/I/II/III), n	0/43/24/13
Mayo score (0/I/II/III), n	0/43/23/14
Crypt destruction (0/I/II/III), n	6/38/14/22
Erosion of the mucous membranes (0/I/II/III/IV), n	20/21/10/14/15
Architectural changes (0/I/II/III), n	0/38/22/20
Neutrophil infiltration (0/I/II/III), n	7/32/13/28
Eosinophil infiltration (0/I/II/III), n	17/25/20/18

Fecal concentrations of TNF-α, IL-17 and IL-10 are associated with the clinical activity, endoscopic and histo-pathologic characteristics of ulcerative colitis

Our evaluation revealed a significantly higher level of TNF-α in the group of patients with the advanced endoscopic, clinical as well as total Mayo score ($p < 0.05$), Figure 2A. We also noticed a significant increment of fecal level of TNF-α in the group of patients with an intense crypt destruction, erosion of the mucous membranes, architectural changes, neutrophil infiltration and eosinophil infiltration ($p < 0.05$), Figure 2B.

The patients with advanced endoscopic, clinical and total Mayo score revealed significantly higher IL-17 in feces in comparison to the patients with lower endoscopic and clinical scores ($p < 0.05$), Figure 3A. IL-17 was higher in the stool of patients with an intense crypt destruction, erosion of the mucous membranes, architectural changes, neutrophil infiltration and eosinophil infiltration ($p < 0.05$), Figure 3B.

As shown in Figure 4, the UC patients with higher endoscopic, clinical and total Mayo score appear to have a higher fecal level of IL-10, although this difference reached a statistical significance for endoscopic score only ($p = 0.006$).

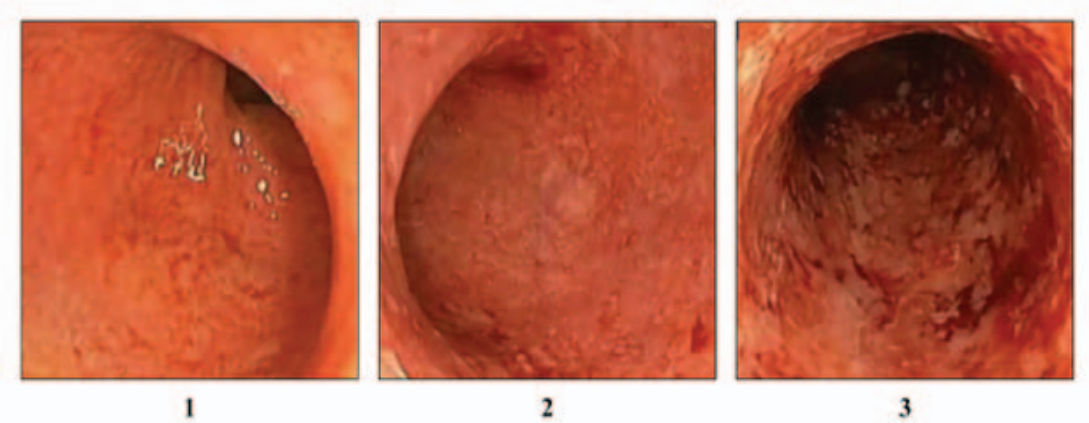

Fig. 1 – Mayo 1: mild activity (erythema, decreased vascular pattern, mild friability); Mayo 2: moderate activity (marked erythema, lack of vascular pattern, friability, erosions); Mayo 3: severe activity (spontaneous bleeding, large ulcerations).

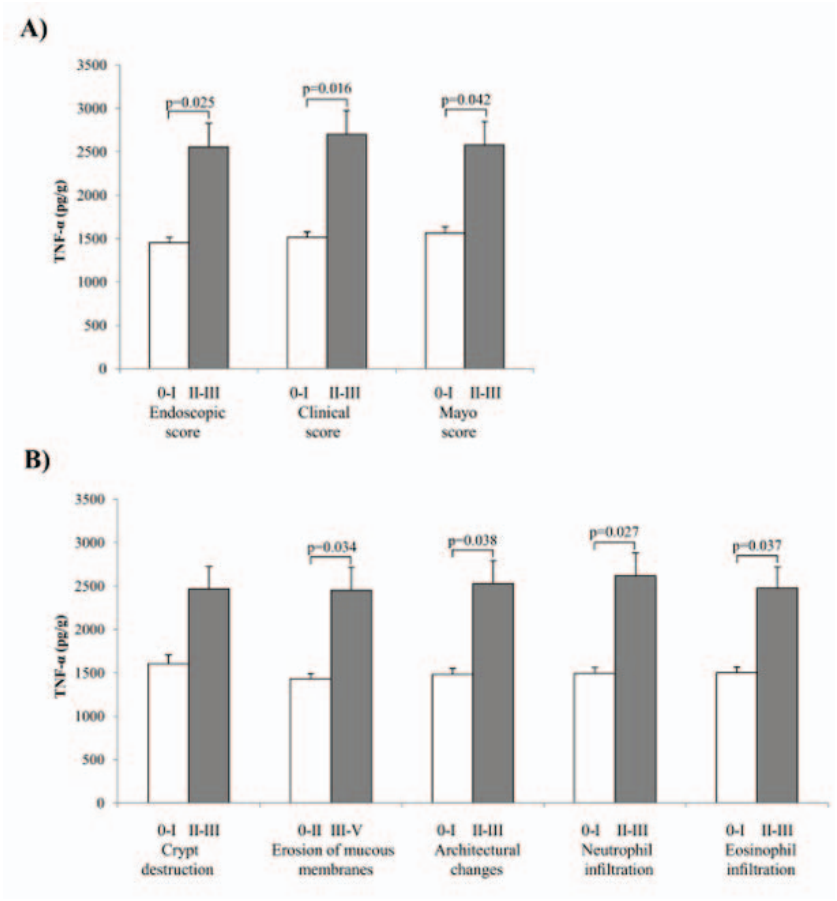

Fig. 2 – Distribution of fecal levels of TNF-α in the groups of patients with different Mayo and histological scores.
A) The patients with ulcerative colitis were divided into two groups, based on the endoscopic, clinical and total Mayo score. A fecal level of TNF-α was determined by ELISA; B) The patients with ulcerative colitis were divided into two groups, according to several histological characteristics group I – scores 0–I; group II – scores II–III.
A statistical significance was tested by the Mann–Whitney Rank Sum test.

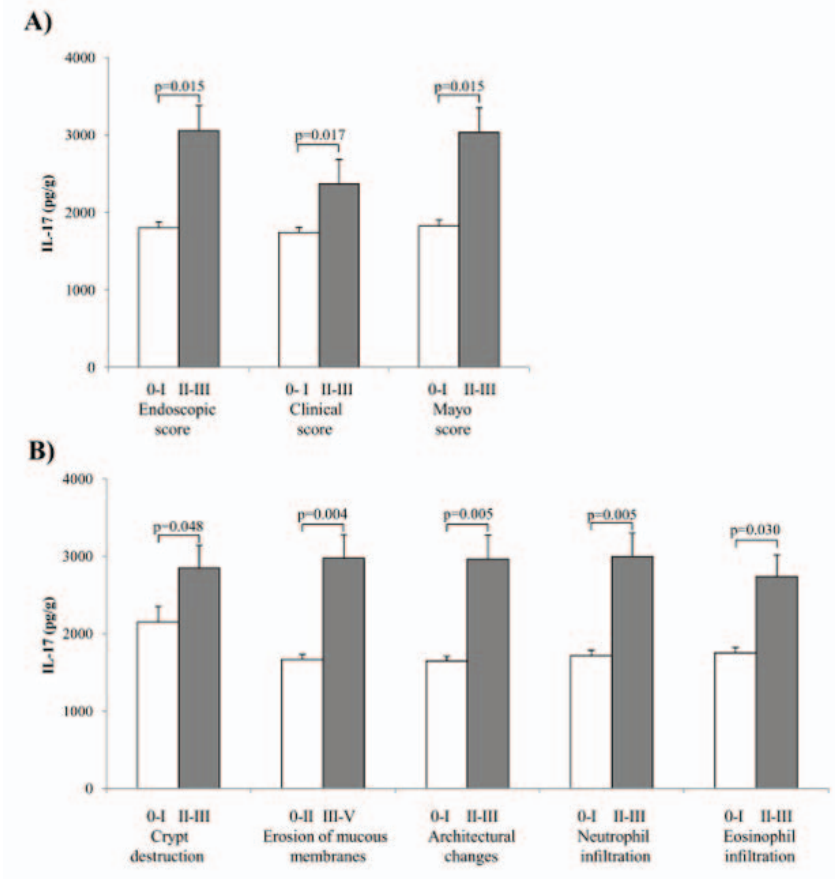

Fig. 3 – The analysis of fecal IL-17 in the ulcerative colitis patients according to the endoscopic, clinical and histopathological activity:
A) The patients with UC were divided into two groups, based on the endoscopic, clinical and total Mayo score, respectively. A fecal level of IL-17 was determined by ELISA;
B) The patients with UC were divided into two groups according to several histological characteristics group I – scores 0–I; group II – scores II–III.
A statistical significance was tested by the Mann–Whitney Rank Sum test.

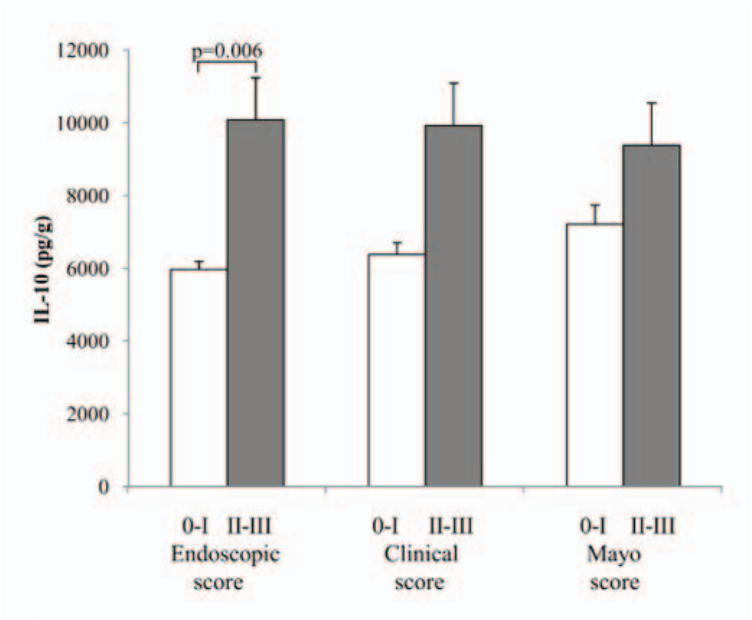

Fig. 4 – The increased concentration of IL-10 in feces of patients with the higher Mayo score. The patients with ulcerative colitis were divided into two groups, based on the endoscopic, clinical and total Mayo score group I – scores 0–I; group II – scores II–III. A fecal level of IL-10 was determined by ELISA. A statistical significance was tested by the Mann–Whitney Rank Sum test.

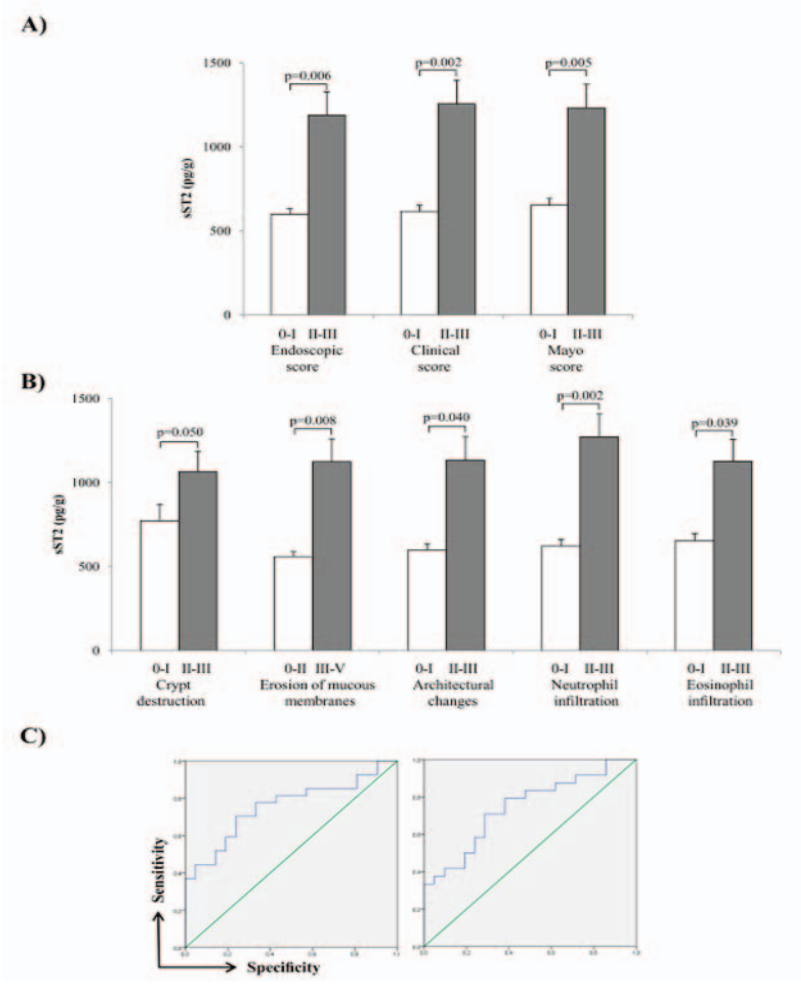

Fig. 5 – Differences in fecal sST2 in the patients according to the change in the endoscopic, clinical and histological scores:
A) The patients with ulcerative colitis were divided into two groups, based on the endoscopic, clinical and total Mayo score, respectively. A fecal level of sST2 was determined by ELISA;
B) The patients with UC were divided into two groups according to several histological characteristics, respectively. A statistical significance was tested by the Mann-Whitney Rank Sum test;
C) Reciever operating characteristic (ROC) curves illustrate the specificity and sensitivity of fecal sST2 comparing the clinical score and the total Mayo score.

Clinical activity, endoscopic and histo-pathologic characteristics of ulcerative colitis are associated with higher fecal sST2

We noticed a higher fecal level of sST2 in the patients with advanced endoscopic, clinical and total Mayo score ($p < 0.05$), Figure 5A. Further, sST2 was significantly increased in the patients with a strong crypt destruction, erosion of the mucous membranes, architectural changes, neutrophil infiltration and eosinophil infiltration ($p < 0.05$), Figure 5B. The analysis of receiver operating characteristic (ROC) curves of fecal sST2 for various stages and parameters of UC found that the sST2 level in feces could predict the disease severity. The analysis showed that sST2 can be a valuable marker for

distinguishing the clinical score (sensitivity 77.8%, specificity 66.7%) and the total Mayo score (sensitivity 79.2%, specificity 61.9%). The optimal cut- off value estimated for sST2 that allows the discrimination of stages of UC progression was 624.0 pg/g.

Fecal sST2 and IL-17 concentrations significantly correlated with disease severity

The relationship between the fecal sST2/IL-17 and the clinico-pathological parameters of patients with UC were summarized in Table 2. The analysis revealed positive correlation between the fecal sST2/IL-17 and parameters and markers of disease severity. There is the moderate positive correlation between fecal sST2/IL-17 and the endoscopic score (r = 0.390; p = 0.005/ r = 0.330; p = 0.014), clinical score (r = 0.441; p = 0.002/ r = 0.330; p = 0.016), total Mayo score (r = 0.442; p = 0.004 r = 0.356; p = 0.013), crypt destruction (r = 0.267; p = 0.049 r = 0.269; p = 0.047), erosion of the mucous membranes (r = 0.376; p = 0.006 r = 0.407; p = 0.003), architectural changes (r = 0.300; p = 0.038 r = 0.409; p = 0.003), neutrophil infiltration (r = 0.480; p = 0.001 r = 0.411; p = 0.002) and eosinophil infiltration (r = 0.304; p = 0.038 r = 0.305; p = 0.028). Finally, we found the strong positive correlation between fecal sST2 and IL-17 (r = 0.771; p = 0.001).

Table 2
Correlation between the fecal level of sST2 and IL-17 and the parameters of disease severity in the patients with ulcerative colitis

Variables	Spearman`s rho	*p* value	Spearman`s rho	*p* value
	sST2		IL-17	
Endoscopic score	0.390	0.005	0.330	0.014
Clinical score	0.441	0.002	0.330	0.016
Mayo score	0.422	0.004	0.356	0.013
Crypt destruction	0.267	0.049	0.269	0.047
Erosion of the mucous membranes	0.376	0.006	0.407	0.003
Architectural changes	0.300	0.038	0.409	0.003
Neutrophil infiltration	0.480	0.001	0.411	0.002
Eosinophil infiltration	0.304	0.038	0.305	0.028
IL-17	0.771	0.001		

Note: A statistical significance was tested by the Spearman correlation coefficient.

Discussion

Recently, studies have pointed on a significance of measuring biomarkers in feces [23–27]. These kinds of tests have a significant promise. In this way, proteins and molecules produced by intestinal mucosa are measured, which reflects the intestinal inflammation [27]. Recent studies have considered the potential as well as the diagnostic accuracy of different non-invasive fecal markers in the detection of IBD and as indicators of the therapy response [23–27].

Our results revealed that the levels of all measured biomarkers in feces reflected the disease activity. A domination of type 1 response, manifested by a significantly higher fecal level of TNF-α, was detected in the patients with the advanced endoscopic score, higher clinical and total Mayo score and intense cript destruction, erosion of the mucous membranes, architectural changes, neutrophil and eosinophil infiltration, respectively. This indicates that TNF-α correlates with the severity of UC. TNF-α is already established as master cytokine implicated in the pathogenesis of various inflammatory conditions, particularly IBD [28, 29]. Its importance has been confirmed in many studies using anti-TNF antibodies in controlling the disease activity [30, 31]. The main sources of TNF-α are the activated macrophages and Th1 cells [32, 33]. An elevated level of TNF-α in the sera, stool or intestinal tissue were detected in the UC patients [34, 35], while the systemic values of TNF-α correlate with the clinical activity of UC [36].

Current publications indicate that the type 17 immune response also play an important role in the biology of UC [37, 38]. The type 17 response is mediated mainly by IL-17, produced by the Th-17 cells and $CD8^+$T cells, during chronic inflammation [39]. Our data revealed a significantly increased fecal level of IL-17 in the patients with more severe stage of UC (the advanced endoscopic and clinical score and histological grade). We also obtained the positive correlation between fecal IL-17 and disease severity (the advanced endoscopic score, higher clinical and total Mayo score). Moreover, the fecal level of IL-17 is in a positive correlation with histologically advanced and more severe disease (intense cript destruction, erosion of the mucous membranes, architectural changes, neutrophil and eosinophil infiltration, respectively). Although many studies support the pro-inflammatory role of IL-17 in the genesis and progression of UC [38, 40], some demonstrated a protective role of IL-17 in IBD [41]. IL-17 can mediate a protective function in an experimental model of UC [42]. In humans, recent publications showed that the IL-17 levels were increased in the UC patients, and correlated with the disease severity [37, 38, 40, 43].

Interleukin 10 is one of the most important anti-inflammatory cytokines known to suppress an inflammation in mucosa [44]. It is produced mainly by the macrophages, dendritic cells, Th2 and Treg cells [45]. We found the significant increment of IL-10 in feces of patients with the advanced endoscopic score, and this trend was detected in the patients

with the higher clinical and total Mayo score (did not reach statistical significance). Based on our results, we believe that the relatively enhanced fecal level of IL-10 in the patients with severe UC is a way to compensate for an intense inflammation and the enhanced type 1 and type 17 immune response.

Interleukin 33 presents the alarmin and cytokine that plays a role in the polarization of immune response toward type 2 [46]. Soluble ST2 is a decoy receptor for IL-33 [24]. The increased systemic values of sST2 were detected in the patients with the chronic inflammatory diseases [47, 48] and correlate with the disease severity [49]. The serum sST2 is proposed as a potential biomarker of the UC activity [49]. Our goal was to determinate whether fecal sST2 correlated with the severity of UC. We demonstrated, for the first time, a significantly increased fecal level of sST2 in the patients with more severe stage of UC (higher endoscopic, clinical and total Mayo score, respectively). Moreover, fecal sST2 was significantly increased in the patients with the intense cript destruction, erosion of the mucous membranes, architectural changes, neutrophil and eosinophil infiltration, respectively. Also, it was demonstrated that fecal sST2 correlated closely with the endoscopic, clinical and total Mayo score as well as with the histopathological parameters of affected tissue, and might be used to evaluate the severity of UC. This observation is in line with previous reports indicating a direct association between the systemic sST2 and endoscopic score of UC as well as the histopathological stage [49]. To our knowledge, this is the first study revealing direct association between fecal sST2 and the degree of endoscopic, clinical and histopathological activity of UC. Further in this study, we envisaged the possible role of fecal sST2 as a biomarker in preceding the disease severity and progression. The analysis of the ROC curves of sST2 and the disease parameters revealed that sST2 could predict the advanced endoscopic and clinical score, at good sensitivity and specificity. According to our findings, fecal sST2 could be a valuable marker for the UC severity and activity. The best cut-off point for the sST2 concentration was obtained at a threshold of 624.0 pg/g, predicting the active disease.

Another novel finding in this study was a strong positive correlation between IL-17 and sST2 in feces of the UC patients. Both biomarkers correlated closely with the parameters of disease severity. Little is known about the IL-17/sST2 relation. Recent studies of inflammatory diseases, demonstrate that IL-33 suppresses the type 17 immune response and subsequent production of IL-17 [50, 51]. Taken together, the enhanced local values of sST2 can neutralize and reduce concentration of present IL-33 and thus suppress the IL-33 inhibition of IL-17.

Conclusion

The increased local values of sST2, reflected trough higher fecal concentration, in the UC patients with the higher endoscopic, clinical and total Mayo score, as well as the histopathological parameters of affected tissue may be considered as a sign of the disease progression and, consequently, of a poor prognosis for the patients. An increment of sST2 may facilitate the IL-17 production, via the IL-33 reduction, implicating immunomodulatory role of sST2 in enhancing the ongoing proinflammatory processes. Furthermore, the fecal values of sST2 can be used as a valuable marker for the UC severity. These observations support the possible role of fecal sST2 as an activity marker of UC and its potential use as a therapeutic target.

Declaration of interest

The authors declare that they have no conflict of interests.

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