RESEARCH ARTICLE

Usefulness of selected laboratory markers in ulcerative colitis

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ABSTRACT. ***Introduction:*** **This study aimed to compare the accuracy of selected laboratory markers in assessing disease activity in patients with ulcerative colitis (UC). The analysis included serum IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α, IFN-γ, hsCRP, peripheral regulatory T cells, as well as fecal calprotectin and lactoferrin.** ***Patients and methods:*** **A group of 45 adults with UC was enrolled in the study. Disease activity was assessed using the Mayo endoscopic index, while for clinical activity scoring, the Clinical Activity Index (CAI) was used. Concentrations of markers investigated were estimated by means of flow cytometry and enzyme-linked immunosorbent assays: the results were correlated with both indices.** ***Results:*** **The study demonstrated that both fecal markers, i.e. calprotectin (r = 0.880, *P<0.001*) and lactoferrin (r = 0.799, *P<0.001*) correlated closely with the Mayo endoscopic score, and might be used to evaluate the severity of UC in the clinical setting. The correlation of these markers with CAI was also significant, with r = 0.831 for calprotectin (*P<0.001*) and r = 0.672 for lactoferrin (*P<0.05*). As for the other markers investigated, only IL-6 (r = 0.598, *P<0.001*), IL-17A (r = 0.587, *P<0.005*), and TNF-α (r = 0.701, *P<0.001*) correlated closely with the Mayo endoscopic index. The correlation of the markers with CAI was also significant, though weaker, with r = 0.525 for IL-6 (*P<0.001*), r = 0.587 for IL-17A (*P<0.05*), and r = 0.624 for TNF-α (*P<0.001*).** ***Discussion:*** **Despite the fact, that UC is generally considered to be an IL-13-driven, Th2-like type of disease, markers of inflammation such as serum interleukin (IL)-6, IL-17, TNF-α, fecal calprotectin and lactoferrin might be useful in assessing disease activity.**

Key words: ulcerative colitis, laboratory markers, calprotectin, lactoferrin, IL-6, IL-17, TNF-α, Mayo index, CAI

Ulcerative colitis (UC) is a chronic, relapsing, immunologically-mediated disease characterized by continuous colonic mucosal inflammation that extends proximally from the rectum. It typically presents in the second and third decade of life with bloody diarrhea and abdominal cramps. The clinical manifestation of UC is however heterogeneous, with different clinical courses. In addition, the behavior of UC can change over time. Assessment of disease activity in UC patients can be based on clinical disease activity indices, endoscopic indices, serum markers, fecal markers and miscellaneous tests [1-3]. Clinical indices however, provide an indirect measurement of disease activity only, and may not accurately reflect the inflammatory activity found by endoscopic and histological examination. Endoscopy, in turn, is accurate, but it is invasive and expensive. This has led to the search for reliable and inexpensive biological markers that could be used to assess disease activity in the clinical practice setting. The most widely held hypothesis for the pathogenesis of UC is that overly aggressive, acquired (T cell) immune responses to a subset of commensal enteric bacteria develop in genetically susceptible hosts, and environmental factors precipitate the onset or reactivation of the disease [4]. Despite the fact that UC is generally considered to be an IL-13-driven, Th2-like type of disease [5], various markers of inflammation, including serum interleukin (IL)-6, IL-17, TNF-α, C-reactive protein (CRP), fecal calprotectin and lactoferrin, seem to be the most obvious candidates due to the inflammatory nature of the disease.

For instance, IL-6 is a potent, pleiotropic cytokine known to regulate T cell differentiation, activation and resistance to apoptosis, and thereby controling the balance between proinflammatory and regulatory T cell subsets [6]. It is produced by various cell types: however, its primary sources are monocytes and macrophages at sites of inflammation during acute inflammation, as well as T cells in chronic inflammation [7]. This cytokine is considered to be an accurate marker of ongoing inflammation [8]. Elevated concentrations of serum IL-6 have been found in patients with active UC: however, the correlation between cytokine concentration and disease severity remains controversial [9, 10].

Current evidence indicates that the Th17 response may also play a role in the pathogenesis of the disease [11, 12]. This newly identified subset of Th cells, distinct from Th1/Th2, produces various proinflammatory cytokines, including IL-17A (often referred to as IL-17), IL-17F, IL-21, IL-22 and TNF-β, and plays an important role in immunity against extracellular pathogens. Although the evidence implies the possible involvement of Th17 cells in the pathogenesis of UC [13, 14], as in other autoimmune and inflammatory diseases [15], a protective role for IL-17

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in intestinal inflammation has also been proposed [16]. In spite of the unclear role of Th17 cells in the pathogenesis of UC, increased levels of IL-17 have been observed in the serum of patients with active UC [14, 17], in addition to increased concentrations of circulating Th17 cells and a decreased ratio of Treg/Th17 cells [18, 19].

TNF-α, in turn, has been implicated in the pathogenesis of various inflammatory conditions, and its importance has been highlighted by the efficacy of anti-TNF antibodies or administration of soluble TNF receptors (TNFRs) in controlling disease activity [20], including UC [21]. It is produced predominantly by activated macrophages and T lymphocytes [22], and mediates multiple proinflammatory signals that play central roles in the pathogenesis of IBD, including neutrophil recruitment to the local site of inflammation, activation of both coagulation and fibrinolysis, as well as the induction of granuloma formation [23]. Elevated levels of TNF-α have been found in the serum, stool and intestinal tissue of UC patients [24-26].

Other biological markers of inflammation that might be useful in assessing the UC activity include serum C-reactive protein (CRP) and neutrophil-derived proteins such as calprotectin and lactoferrin. CRP is one of the most important proteins that are up-regulated during acute-phase stimulus in humans. Under normal conditions, the baseline concentration of CRP in the plasma is around 0.8 mg/L, and is, in part, genetically regulated [27, 28]. However, in the presence of an acute-phase stimulus such as IL-6, TNF-α and IL-1β, CRP production is up-regulated within hours, and may reach concentrations that are 500- to 1000-fold higher than under basal conditions [29]. The short half-life of CRP (19 h) also ensures that the concentrations quickly decrease once the acute-phase stimulus disappears, making CRP a valuable marker of inflammation [30]. Medical treatment does not seem to have a direct effect on CRP production in hepatocytes, and changes in the CRP response during treatment are caused by the effect of the therapy on the underlying disease. CRP has been widely used as a parameter of inflammatory activity in a variety of infectious and inflammatory diseases. It has also been found to correlate with clinical parameters of disease activity in CD [30, 31]: however its correlation with disease activity in UC is unclear [31].

Calprotectin is, in turn, an abundant, calcium-binding protein of the S100 family that is derived predominantly from neutrophils and, to a lesser extent, from monocytes and macrophages [32]. It has bacteriostatic and fungistatic properties, and its markedly elevated concentrations have been found in both plasma and stool in infectious and inflammatory conditions, including UC [33]. Similarly to calprotectin, it has been observed that fecal lactoferrin levels quickly rise after an influx of neutrophils into intestinal lumen during inflammation. In active IBD, the concentration of lactoferrin can rise several hundred times [34]. Both fecal markers, calprotectin and lactoferrin, are sensitive to inflammation: however, they are not disease-specific.

This study aimed to compare the accuracy of selected biological markers in assessing disease severity and activity in patients with UC. In particular, it was aimed at examining how reliably these marker measurements reflect endoscopic severity in UC, and at determining cutoff levels for endoscopically-visible inflammation. The analysis included biological markers such as serum IL-2, IL-6, IL-17, TNF-α, IFN-γ, high-sensitive CRP (hsCRP), peripheral $CD4^+CD25^+FOXP3^+$ regulatory T cells, as well as fecal calprotectin and lactoferrin.

PATIENTS AND METHODS

The project was approved by the Ethics Committee at Poznan University of Medical Sciences. A group of 45 consecutive adults with UC was enrolled to the study. All patients were Caucasian. The disease had been diagnosed and confirmed by endoscopic and radiological means. Demographic and clinical characteristics of the patients were retrieved from medical notes and patient interviews. The endoscopic Mayo scoring system was used to grade mucosal inflammation at colonoscopy. This index is commonly used in both clinical trials and clinical practice [35]. The score can range from 0 to 3, with 0 indicating inactive disease, 1 mildly active disease (erythema, decreased vascular pattern, friability), 2 moderately active disease (marked erythema, absent vascular pattern, friability, erosions), and 3 severely active disease (spontaneous bleeding, ulceration). The Mayo score was calculated within seven days of the blood and stool sampling. Clinical activity was assessed using the Clinical Activity Index (CAI) [36]. The index combines objective measurements, such as number of stools in one week, temperature, and extraintestinal manifestations (EIM) with subjective findings, including blood in stools, abdominal pain/cramps, and global assessment of symptomatic state. The total index score ranges from 0 to 25. Degrees of clinical activity were defined as follows: inactive disease (0-4), mild activity (5-10), moderate activity (11-17), and high activity (18-25). Only patients treated with conventional therapies, including 5-aminosalicylates, prednisolone, budesonide, azathioprine, mercaptopurine, methotrexate and antibiotics, were recruited into the study.

Serum samples were cytometrically tested for concentrations of IL-2, IL-6, IL-17, TNF-α, and IFN-γ using the Human Th1/Th2/Th17 Cytokine Cytometric Bead Array Kit (BD Biosciences Pharmingen, San Diego, CA, USA). Serum samples were also used to evaluate, in a local laboratory, the hsCRP concentrations. Elevated hsCRP was defined as >3.0 mg/L. The cytometric analysis was performed to evaluate the levels of $CD4^+CD25^+FOXP3^+$ regulatory T cells (BioLegend, San Diego, CA, USA) in the peripheral blood of investigated subjects. Fecal concentrations of calprotectin were measured using CalproLab™ ELISA kit (Calpro AS, Lysaker, Norway), with the normal range of calprotectin defined as <100 μg/g of stool. Fecal concentrations of lactoferrin were measured using IBD-SCAN (Techlab, Blacksburg, VA, USA), with the normal range of lactoferrin defined as <7.25 μg/g of stool. All laboratory tests were performed according to instructions provided by manufacturers. Descriptive variables are presented as means and medians with range, and categorical variables as frequencies with percentages. Frequencies were compared with the χ^2 test, and means between two groups with the Mann-Whitney test. The two-tailed Spearman's rank-order correlation coefficient served for analyzing any correlation between variables. All statistical analyses were performed with the Statistical Package for the Social Sciences version 15.0 (SPSS, Chicago, IL, USA). Values were considered significant when $P \leq 0.05$.

Table 1
Clinical and demographic characteristics of patients with ulcerative colitis (UC)

Characteristic	UC (n = 45)
Gender *n (%)*	
Female	30 (66.7%)
Age at diagnosis	
Mean (yrs ± SD)	40.4 ± 5.6
Median (range) yrs	39.1 (31.2-56.3)
Early onset (<40 yrs) *n (%)*	23 (51.1%)
Duration of disease	
Mean (yrs ± SD)	2.7 ± 0.2
Median (range) yrs	2.3 (0.0-5.6)
Family history of IBD *n (%)*	13 (28.9%)
Hospitalization *n (%)*	
Once	19 (42.2%)
At least twice	8 (17.8%)
Colectomy *n (%)*	6 (13.4%)
5-ASA	42 (93.3%)
Glucocorticoids	19 (42.2%)
Immunosuppressants	10 (22.2%)
Smoking behavior at diagnosis *n (%)*	
Smoker	10 (22.2%)
Former smoker	19 (42.2%)
Non-smoker	16 (35.6%)
Mayo endoscopic score n (%)	
Inactive disease (0)	5 (11.1%)
Mildly active disease (1)	4 (8.9%)
Moderately active disease (2)	21 (46.7%)
Severely active disease (3)	15 (33.3%)
Clinical activity index (CAI) n (%)	
Inactive disease (0-4)	5 (11.1%)
Mildly active disease (5-10)	6 (13.4%)
Moderately active disease (11-17)	15 (33.3%)
Severely active disease (18-25)	19 (42.2%)
UC location *n (%)*	
Proctitis (E1)	11 (24.4%)
Left-sided (E2)	15 (33.3%)
Pancolitis (E3)	19 (42.2%)
EIM *n (%)*	4 (8.9%)

Abbreviations: 5-ASA – 5-aminosalicylates; CAI – clinical activity index; EIM – extraintestinal manifestations; IBD – inflammatory bowel diseases; SD – standard deviation; UC – ulcerative colitis; yrs – years.

Table 2
Clinical disease activity and laboratory markers of patients with ulcerative colitis.

Parameter	UC (n = 45)
Mayo endoscopic score	
median (range)	2 (0-3)
mean (95% CI)	2 (1.7-2.3)
CAI	
median (range)	16 (0-20)
mean (95% CI)	13 (11.6-15.1)
hsCRP [mg/L]	
median (range)	4.4 (0.0-16.3)
mean (95% CI)	5.2 (4.2-6.2)
Calprotectin [μg/g]	
median (range)	699.0 (30-2013)
mean (95% CI)	781.1 (610.5-951.8)
Lactoferrin [μg/g]	
median (range)	536.0 (11-2289)
mean (95% CI)	647.6 (469.9-825.3)
IL-2 [pg/mL]	
median (range)	6.0 (0.0-12.1)
mean (95% CI)	5.8 (4.6-7.0)
IL-4 [pg/mL]	
median (range)	0.0 (0.0-7.8)
mean (95% CI)	1.1 (0.4-1.8)
IL-6 [pg/mL]	
median (range)	15.0 (0.0-33.1)
mean (95% CI)	19.6 (0.0-52.8)
IL-10 [pg/mL]	
median (range)	7.4 (0.0-35.8)
mean (95% CI)	8.2 (5.8-10.6)
IL-17 [pg/mL]	
median (range)	17.5 (0.0-62.6)
mean (95% CI)	19.2 (15.5-22.9)
TNF-α [pg/mL]	
median (range)	25.0 (0.0-58.7)
mean (95% CI)	24.4 (20.2-28.6)
IFN-γ [pg/mL]	
median (range)	9.7 (0.0-20.8)
mean (95% CI)	10.0 (8.2-11.9)
Treg [%Th]	
median (range)	1.3 (0.1-4.8)
mean (95% CI)	1.7 (1.4-2.0)

Abbreviations: CDI – clinical activity index; IFN – interferon; IL – interleukin; TNF – tumor necrosis factor; Th – T helper cell; Treg – regulatory T cell; 95% CI – 95% confidence interval.

RESULTS

Clinical and demographic characteristics

Clinical and demographic characteristics of the UC patients participating in the study are presented in *table 1*. The group included 45 adults, 30 females (66.7%) and 15 males (33.3%), with a mean age of 40.4 ± 5.6 (median: 39.1) years at diagnosis. Mean CAI was 13 (95%CI: 11.6-15.1), while the median score was 16 (range: 0-20). Based on CAI, 5 (11.1%) patients had inactive, 6 (13.4%) mild, 15 (33.3%) moderate, and 19 (42.2%) severe disease. According to the Mayo endoscopic index, 5 (11.1%) individuals had inactive, 4 (8.9%) mild, 21 (46.7%) moderate, and 15 (33.3%) severe disease. The distribution of the extent of UC was 24.4% proctitis, 33.3% left-sided, and 42.2% pancolitis.

Table 2 presents the Mayo endoscopic and CAI scores, as well as hsCRP and cytokine serum concentrations, peripheral Treg cell concentrations and fecal marker concentrations of the patients investigated. Thirty (66.7%) patients had elevated serum levels of hsCRP (normal range<3.0 mg/L). The mean serum concentration of hsCRP was 5.2 (95% CI: 4.2-6.2) mg/L, and the median serum concentration was 4.4 (range: 0-16.3) mg/L. In this cohort, 46 (86.7%) patients had elevated fecal levels of calprotectin (normal range:<100 μg/g). The mean fecal concentration of calprotectin was 781.1 (95% CI: 610.5-951.8) μg/g, while the median fecal concentration was 699.0 (range: 30-2013) μg/g. Increased fecal levels of

lactoferrin (≥7.25 μg/g of stool) were found in all patients investigated, with a mean fecal concentration of 647.6 (95% CI: 469.9-825.3) μg/g and a median fecal concentration of 536.0 (range: 11-2289) μg/g.

As for the cytokines, the serum concentrations were as follows: IL-2: mean concentration (95% CI) of 5.8 (4.6-7.0) pg/mL and median (range) of 6.0 (0.0-12.1) pg/mL; IL-4: mean concentration (95% CI) of 1.1 (0.4-1.8) pg/mL and median (range) of 0.0 (0.0-7.8) pg/mL; IL-6: mean concentration (95% CI) of 19.6 (0.0-52.8) pg/mL and median (range) of 15.0 (0.0-33.1) pg/mL; IL-10: mean concentration (95% CI) of 8.2 (0.0-10.6) pg/mL and median (range) of 7.4 (0.0-35.8) pg/mL; IL-17: mean concentration (95% CI) of 19.2 (15.5-22.9) pg/mL and median (range) of 17.5 (0.0-62.6) pg/mL; TNF-α: mean concentration (95% CI) of 24.4 (20.2-28.6) pg/mL and median (range) of 25.0 (0.0-58.7) pg/mL; and IFN-γ: mean (95% CI) concentration of 10.0 (8.2-11.9) pg/mL and median (range) of 9.7 (0.0-20.8) pg/mL. The mean (95% CI) level of peripheral $CD4^+CD25^+FOXP3^+$ regulatory T cells in the cohort was 1.7 (1.4-2.0%), while the median (range) level was 1.3 (0.1-4.8%).

The values for CAI and concentrations of investigated markers in the different endoscopic activity groups are presented in *table 3*. Some previous reports have suggested the possible differences in levels of biological markers, particularly for fecal parameters and CRP, dependent on the location of the UC [37], therefore the analysis of concentrations of markers investigated according to disease location was also performed. No statistically significant differences were found however among the given subgroups (*P>0.05*), although the level of serum hsCRP seemed be related to the extent of the disease, as suggested by other authors (*table 4*) [31].

Correlation analysis

In our cohort of UC patients, the Mayo endoscopic scores correlated significantly with CAI (Spearman's rank order correlation coefficient (r) = 0.815, *P<0.001*), serum concentrations of IL-6 (r = 0.598, *P<0.001*), IL-17 (r = 0.587, *P<0.005*), TNF-α (r = 0.701, *P<0.001*), and fecal concentrations of calprotectin (r = 0.880, *P<0.001*) and lactoferrin (r = 0.799, *P<0.001*) (*figure 1*). In contrast, there was no significant correlations between the Mayo endoscopic scores and other markers investigated, such as serum concentrations of hsCRP (r = 0.273, *P>0.05*), IL-2 (r = 0.213, *P>0.05*), IL-10 (r = 0.144, *P>0.05*), IFN-γ (r = 0.251, *P>0.05*) or Treg cells (r = -0.172, *P>0.05*). The correlations between CDAI and parameters investigated were similar, as follows: serum concentrations of IL-6 (r = 0.525, *P<0.001*), IL-17 (r = 0.587, *P<0.05*), TNF-α (r = 0.624, *P<0.001*) and fecal concentrations of calprotectin (r = 0.831, *P<0.001*) and lactoferrin (r = 0.672, *P<0.05*). Correlation analysis is shown in *table 5*.

ROC curve analysis

ROC curve analysis revealed a cutoff IL-6 level of 9.6 pg/mL in predicting endoscopically-active disease (Mayo endoscopic score ≥1), with a sensitivity of 95%, a specificity of 80%, a positive predictive value (PPV) of 95%, and a negative predictive value (NPV) of 50% (AUC (95% CI) = 0.930 (0.844-1.000), *P<0.001*). Similarly, a serum IL-17 concentration of 6.6 pg/mL gave a sensitivity of 92%, a specificity of 60%, a PPV of 95%, and an NPV of 50.0% (AUC (95% CI) = 0.928 (0.840-1.000), *P<0.001*). A serum TNF-α concentration of 7.6 pg/mL gave a sensitivity of 95%, a specificity of 80%, a PPV of 97%, and an NPV of 80% (AUC (95% CI) = 0.975 (0.931-1.000), *P<0.001*)). As for the fecal markers, a calprotectin concentration of 79.5 μg/g of stool gave a sensitivity of 97%, a specificity of 80%, a PPV of 91%, and an NPV of 80% (AUC (95% CI) = 0.995 (0.979-1.00), *P<0.001*), while a lactoferrin concentration of 76.5 μg/g of stool gave a sensitivity of 97%, a specificity of 80%, a PPV of 95%, and an NPV of 60% in predicting endoscopically-active disease (AUC (95% CI) = 0.968 (0.915 – 1.00), *P<0.001*).

DISCUSSION

This study aimed to evaluate theusefulness of various fecal and serum markers in assessing disease severity and activity in patients with UC. It has been demonstrated that both fecal markers, i.e. calprotectin (r = 0.880, *P<0.001*) and lactoferrin (r = 0.799, *P<0.001*), correlate closely with the Mayo endoscopic score, and might be used to evaluate the severity of UC in the clinical setting. This observation is in line with previous reports indicating both fecal neutrophil-derived proteins as useful surrogate markers of mucosal disease activity in IBD [38-40]. The correlation of these markers with CAI was comparable, with r = 0.831 for calprotectin (*P<0.001*) and r = 0.672 for lactoferrin (*P<0.05*). ROC curve analysis showed that the best cutoff point for calprotectin concentration was obtained at a threshold of 79.5 μg/g, predicting endoscopically active disease (Mayo endoscopic score ≥1) with 97% sensitivity and 80% specificity (PPV = 91%, NPV = 80%). The choice of the fecal calprotectin cutoff point is still under discussion, since some researchers suggest a threshold of 50 μg/g [41, 42], while others 100 μg/g or higher [38, 39]. A recently conducted meta-analysis reported a pooled sensitivity of fecal calprotectin testing to be 93% (95% CI: 0.85-0.97), and a pooled specificity of 96% (95% CI: 0.79-0.99) [43]. As for lactoferrin, the cutoff value of 76.5 μg/g gave a sensitivity of 97%, a specificity of 80%, a PPV of 95%, and an NPV of 60% in predicting endoscopically-active disease. Another meta-analysis reported the pooled sensitivity of fecal lactoferrin to be 80% (95% CI: 78%-83%), and the pooled specificity to be 82% (95% CI: 79%-84%) [44]. To date, information on the comparative performance of fecal calprotectin and lactoferrin tests in IBD is rather sparse, but most authors suggest the superiority of the former [45, 46], which was also observed in this study.

As for other markers investigated only IL-6 (r = 0.598, *P<0.001*), IL-17 (r = 0.587, *P<0.001*), and TNF-α (r = 0.701, *P<0.001*) correlated closely with the Mayo endoscopic score. The correlation beween these markers and CAI was also significant, although weaker, with r = 0.525 for IL-6 (*P<0.001*), r = 0.494 for IL-17 (*P<0.001*), and r = 0.624 for TNF-α (*P<0.001*). IL-6 is a potent, pleiotropic cytokine known to regulate T cell differentiation, activation and resistance to apoptosis, thereby controlling the balance between proinflammatory and regulatory T cell subsets [6]. It is produced by various cell types, however, its primary sources are monocytes and macrophages at sites of inflammation during acute

Table 3
CAI and selected biological markers according to the Mayo endoscopic index.

Mayo endoscopic index	**CAI**	**hsCRP [mg/L]**	**Calprotectin [μg/g]**	**Lactoferrin [μg/g]**	**IL-2 [pg/mL]**	**IL-4 [pg/mL]**	**IL-6 [pg/mL]**	**IL-10 [pg/mL]**	**IL-17 [pg/mL]**	**TNF-α [pg/mL]**	**IFN-γ [pg/mL]**	**Treg [%Th]**
Inactive disease (0)												
n = 5												
mean (95%CI)	2.2 (0.0-4.4)	2.6 (0.0-5.7)	51.2 (24.6-7.8)	54.0 (17.0-91.0)	3.6 (0.0-7.9)	1.0 (0.0-3.8)	8.4 (0.3-16.5)	6.3 (0.0-13.7)	5.8 (0.0-13.3)	3.7 (0.0-7.6)	5.3 (1.1-9.7)	2.1 (0.0-4.5)
median (range)	2.0 (0-4)	3.1 (0.0-5.6)	43.0 (30.0-82.0)	61.0 (13.0-86.0)	5.2 (0.0-7.7)	0.0 (0.0-5.0)	9.0 (0.0-17.6)	9.3 (0.0-12.4)	5.0 (0.0-13.2)	5.0 (0.0-8.6)	5.2 (0.0-9.3)	1.1 (0.4-4.8)
Mildly active (1)												
n = 4												
mean (95%CI)	8.0 (3.0-13.0)	2.5 (0.0-7.4)	201.7 (39.6-363.9)	119.0 (0.0-257.7)	5.0 (0.0-10.6)	2.0 (0.0-8.2)	16.4 (2.1-30.8)	5.6 (0.0-16.8)	13.7 (3.2-24.1)	12.1 (0.0-25.2)	6.3 (0.0-18.8)	2.1 (1.1-3.1)
median (range)	7.5 (5-12)	2.1 (0.0-6.1)	208.5 (77.0-313.0)	134.5 (11.0-196.0)	6.2 (0.0-7.7)	0.0 (8.1)	15.8 (6.2-28.1)	3.7 (0.0-14.8)	14.4 (5.0-21.0)	15.3 (0.0-18.0)	4.6 (0.0-16.2)	2.2 (1.2-2.7)
Moderately active (2)												
n = 21												
mean (95%CI)	13.5 (11.7-15.3)	6.0 (4.5-7.7)	621.1 (508.9-733.3)	476.3 (353.7-598.8)	5.8 (3.8-7.9)	0.8 (0.0-1.7)	20.5 (17.1-23.9)	7.9 (4.1-11.8)	17.0 (14.2-17.5)	24.0 (19.7-28.3)	11.3 (8.4-14.2)	1.7 (1.3-2.2)
median (range)	12.0 (5-20)	5.3 (2.0-16.3)	630 (218.0-1236.0)	533.0 (78.0-919.0)	7.3 (0.0-12.1)	0.0 (0.0-3.9)	20.0 (6.1-33.0)	5.9 (0.0-35.8)	17.5 (5.0-27.3)	25.4 (6.6-42.1)	12.4 (0.0-19.3)	1.3 (0.1-43.7)
Severely active (3)												
n = 15												
mean	18.4	5.3	1403.0	1226.3	6.7	1.4	31.1	9.8	28.2	35.2	10.8	1.4
(95%CI)	(17.9-18.9)	(3.6-6.7)	(1164.1-1641.9)	(882.5-1570.0)	(4.8-8.6)	(0.1-2.9)	(24.7-37.5)	(5.1-14.4)	(19.7-36.6)	(28.5-41.8)	(7.8-13.3)	(0.9-1.8)
median (range)	18.0 (17-20)	5.0 (2.1-12.9)	1461.0 (834.0-2013.0)	1003.0 (262.0-2289.0)	6.0 (0.0-12.1)	0.0 (0.0-6.0)	28.4 (17.9-52.8)	8.0 (0.0-43.7)	25.3 (8.2-62.6)	30.0 (19.7-58.7)	10.5 (0.0-19.8)	1.2 (0.6-3.7)

Abbreviations: CAI – clinical activity index; IFN – interferon; IL – interleukin; TNF – tumor necrosis factor; Th – T helper cell; Treg – regulatory T cell; 95% CI – 95% confidence interval.

Table 4
Concentrations of biological markers investigated according to disease location.

Disease location	hsCRP [mg/L]	Calprotectin [μg/g]	Lactoferrin [μg/g]	IL-2 [pg/mL]	IL-4 [pg/mL]	IL-6 [pg/mL]	IL-10 [pg/mL]	IL-17 [pg/mL]	TNF-α [pg/mL]	IFN-γ [pg/mL]	Treg [%Th]
Proctitis											
n = 11 (24.4%)											
mean (95%CI)	4.5 (2.7-6.4)	616.5 (346.7-886.4)	489.3 (237.0-741.6)	7.4 (5.3-9.5)	0.6 (0.0-1.8)	19.5 (12.2-26.7)	7.3 (3.1-11.6)	16.6 (12.6-18.0)	24.0 (17.9-30.1)	11.6 (7.2-15.9)	1.9 (1.2-2.6)
median (range)	5.8 (2.0-12.9)	630.0 (37.0-1990)	313.0 (183.0-1879.0)	7.9 (0.0-10.7)	0.0 (0.0-6.0)	18.8 (0.0-52.8)	7.4 (0.0-21.0)	18.0 (5.0-27.3)	27.4 (0.0-47.1)	12.8 (0.0-19.3)	1.3 (0.8-3.7)
Left-sided											
n = 15 (33.3%)											
mean (95%CI)	5.1 (3.5-6.8)	834.0 (441.5-1226.5)	886.7 (498.2-1275.3)	5.9 (3.6-8.3)	1.6 (0.5-3.2)	27.6 (21.6-33.7)	6.6 (3.7-9.5)	26.4 (16.1-36.7)	19.8 (12.6-27.1)	9.1 (5.5-12.7)	1.8 (1.2-2.4)
median (range)	4.1 (0.0-16.3)	755.0 (169-1892)	740.0 (183.0-1879.0)	6.0 (0.0-12.1)	0.0 (0.0-7.8)	28.0 (14.8-43.6)	5.8 (0.0-14.8)	22.6 (11.3-62.6)	18.0 (0.0-44.1)	8.4 (0.0-18.6)	1.6 (0.6-4.8)
Pancolitis											
n = 19 (42.2%)											
mean (95%CI)	6.2 (3.9-8.5)	880.5 (583.5-1177.5)	634.1 (310.2-958.0)	4.8 (2.8-6.6)	1.1 (0.4-2.1)	21.5 (16.4-26.7)	9.8 (4.8-14.8)	17.1 (11.1-23.0)	31.4 (20.9-41.8)	9.9 (7.1-12.7)	1.4 (0.9-1.9)
median (range)	5.8 (2.0-12.9)	740.0 (183.0-1879.0)	355.0 (13.0-2289.0)	5.8 (0.0-11.9)	0.0 (0.0-5.7)	19.5 (5.2-46.2)	8.3 (0.0-35.8)	16.5 (0.0-52.9)	28.7 (13.2-58.7)	9.3 (0.0-19.8)	1.2 (0.1-3.7)

Abbreviations: IFN – interferon; IL – interleukin; TNF – tumor necrosis factor; Th – T helper cell; Treg – regulatory T cell; 95% CI – 95% confidence interval.

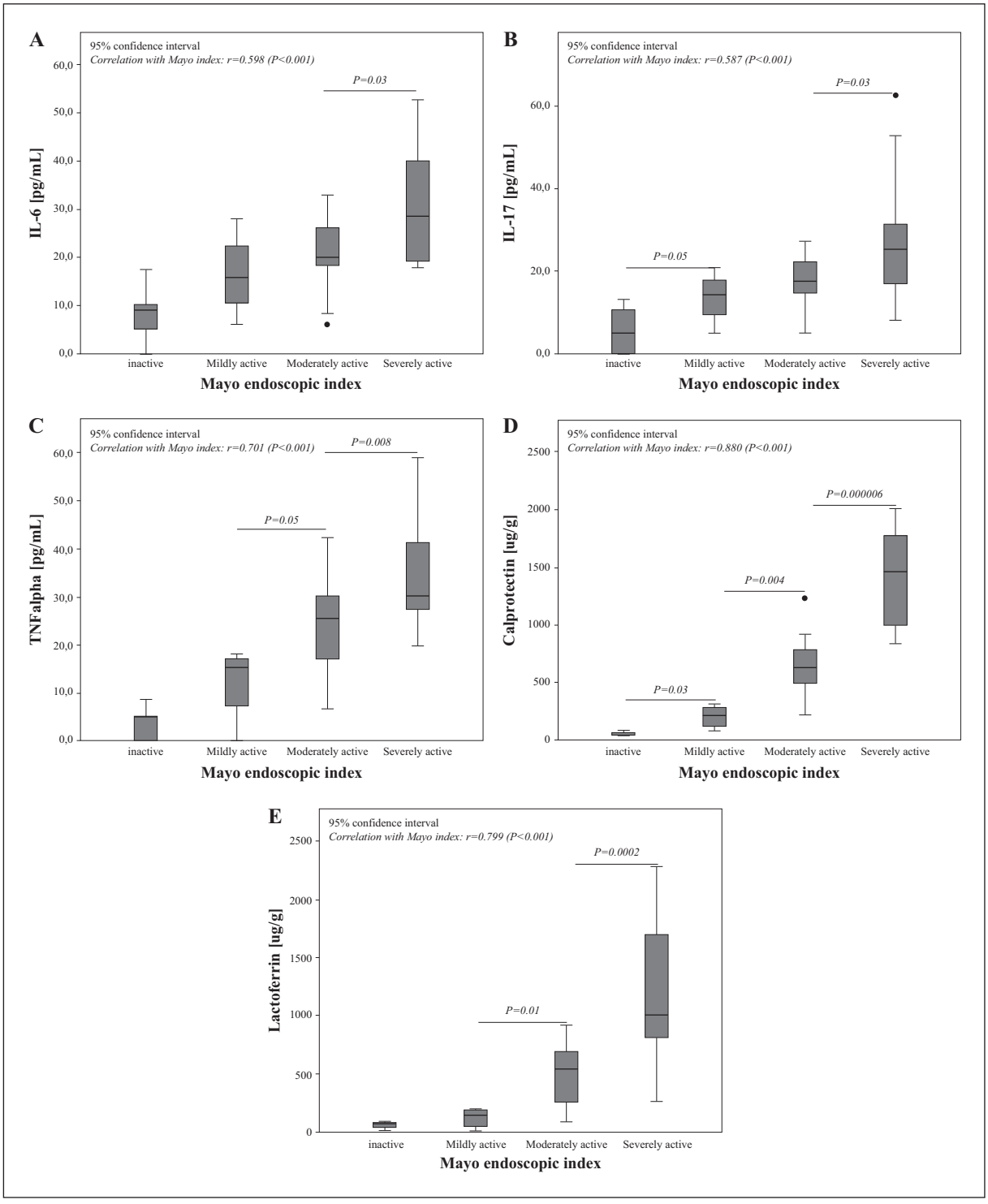

Figure 1

A) Box plot of mean serum IL-6 concentrations according to Mayo endoscopic groups. **B)** Box plot of mean serum IL-17A concentrations according to Mayo endoscopic groups. **C)** Box plot of mean serum TNF-α concentrations according to Mayo endoscopic groups. **D)** Box plot of mean fecal calprotectin concentrations according to Mayo endoscopic groups. **E)** Box plot of mean fecal lactoferrin concentrations according to Mayo endoscopic groups. Abbreviations: *r – Spearman's rank order correlation coefficient; P – p-value*.

inflammation, as well as T cells in chronic inflammation [47-49]. At the beginning of acute inflammation, it plays a key role, being the main inducer of acute phase reactants such as CRP, fibrinogen and serum amyloid A protein. When its activity as a proinflammatory cytokine persists, acute inflammation turns into chronic inflammation that includes an immune response. In particular, IL-6 has a detrimental role that favors mononuclear cell accumulation at sites of injury, mainly through MCP-1 production, angioproliferation, and anti-apoptotic function on T cells. Originally called B-cell-stimulating factor (BSF)-2 or hepatocyte-stimulating factor, IL-6 was shown to induce proliferation of B cells [50] and to induce the acute phase response in the liver [51]. Elevated IL-6 serum levels have been detected in acute and chronic inflammation, and this cytokine is considered to be an accurate marker of ongoing inflammation [8]. Altered IL-6 production has been found in various inflammatory states including IBD,

Table 5
Correlation between the Mayo endoscopic index and the clinical activity index (CAI) with biological markers investigated in patients with ulcerative colitis.

Parameter	r	r
CAI	0.815**	0.815**
hsCRP	0.273	0.299
Calprotectin	0.880**	0.831**
Lactoferrin	0.799**	0.672**
IL-2	0.213	0.202
IL-4	0.069	0.114
IL-6	0.598**	0.525**
IL-10	0.144	0.164
IL-17	0.587**	0.494**
TNF-α	0.701**	0.624**
IFN-γ	0.251	0.281
Treg	-0.172	-0.134

Abbreviations and symbols: CAI – clinical activity index; IFN – interferon; IL – interleukin; r – Spearman's rank order correlation coefficient; P – P-value; TNF – tumor necrosis factor; Treg – regulatory T cell; * $P \leq 0.05$; ** $P \leq 0.001$.

rheumatoid arthritis, psoriasis, multiple sclerosis, mesangial glomerulonephritis, asthma, fever, sepsis etc [52]. Elevated concentrations of serum IL-6 have been found in patients with active UC, however, the correlation between the cytokine concentration and diseases severity remains controversial [9, 10, 53, 54]. It has also been demonstrated that IL-6 might be a useful marker for predicting relapse of disease in both UC and CD patients [52]. Although UC has been traditionally considered to be a Th2 disease, with IL-13 as the effector cytokine, accumulating evidence suggests an important role for IL-6 in the pathogenesis of the disease [55, 56]. For instance, IL-6-dependent activation of STAT3 has been found in lamina propria T cells from UC and CD patients [56]. Furthermore, IL-6 trans signaling has been shown to protect T cells of IBD patients from apoptosis [57]. Additional proof of a functional role for IL-6 in IBD comes from various preclinical models of the disease. IL-6 has been shown to be upregulated in various mouse models of colitis [56, 58]. Anti-IL-6R antibody treatment was able to reduce T cell-driven intestinal inflammation in the widely used transfer colitis model [59]. Our study showed that serum IL-6 concentrations closely correlate to the severity of the disease. ROC curve analysis in this study revealed a cutoff IL-6 serum level of 9.6 pg/mL in predicting endoscopically-active disease (Mayo endoscopic score ≥ 1), with a sensitivity of 95%, a specificity of 80%, a PPV of 95%, and an NPV of 50%.

IL-6 has been shown to suppress the differentiation of inducible regulatory $Foxp3^+$ T cells, and thereby regulates the balance between pro-inflammatory and immunosuppressive T cells [60]. IL-6 signaling in T cells is also of critical importance for the differentiation of Th17 cells, which are characterized by the expression of the transcription factor RAR-related orphan receptor γt (RORγt) and the secretion of large amounts of IL-17A [61], another cytokine found increased in UC. It is the primary effector of Th17 cells, but it is also produced by other cell types, including $CD8^+$ T cells, γδ T cells, neutrophils, and possibly mast cells [62]. IL-17 plays an important role in the protective immunity against intracellular pathogens such as bacteria and fungi [63]. Genetic studies and correlative expression data in disease tissues have pointed to the role of IL-17 and Th17 cells in the pathogenesis of various autoimmune disorders [62, 64]. The pathological involvement of IL-17 also seems to be proved by the therapeutic effect achieved in clinical trials with anti-IL-17A monoclonal antibody in plaque psoriasis [65], psoriatic arthritis [66], ankylosing spondylitis [67], and rheumatoid arthritis [68]. Mounting evidence seems to support the view that Th17 cells are also somehow involved in the pathogenesis of IBD. A high expression of IL-17 mRNA was found in intestinal mucosa from IBD patients [13, 69]. Several mouse studies have identified IL-23 as a major driver of intestinal inflammation via inflammatory mediators including IL-17 and IL-6 [12, 58, 70]. A genome-wide association study (GWAS) has indicated that IL-23R and other genes involved in Th17 differentiation are associated with susceptibility to UC [71, 72]. It is not clear how IL-23R polymorphism might predispose to UC, however, the identification of both disease-protective and risk-associated variants of the gene suggests that IL-23 signaling may play a crucial role in maintaining immune homeostasis in the intestine. Although these observations suggest the importance of the IL-23/IL-17 axis in UC, the exact role of IL-17 in its pathogenesis remains unclear, since its protective role in intestinal inflammation has also been proposed based on T cell-dependent and T cell-independent models of colitis [73, 74]. This view is supported by the poor therapeutic effect, accompanied by a higher rate of adverse events compared to placebo group, obtained in a recent phase II clinical trial of anti-IL-17 therapy in active CD patients [24]. It should be remembered that Th17 cells constitute a heterogonous subpopulation, differing in properties and functions, depending on the context, e.g. cytokine milieu at sites of inflammation [75]. Despite the unclear role of IL-17 in UC it seems that serum concentrations closely correlate to the severity of the disease [14]. ROC curve analysis in this study revealed a cutoff IL-17 serum level of 6.6 pg/mL in predicting endoscopically-active disease (Mayo endoscopic index ≥ 1), with a sensitivity of 92%, a specificity of 60%, a PPV of 95%, and an NPV of 50%.

TNF-α, a third cytokine found in this study to correlate closely with the severity of the UC, has a strong proinflammatory effect on a broad range of cells. TNF-α has been implicated in the pathogenesis of various inflammatory conditions, and its importance has been highlighted by the efficacy of anti-TNF antibodies or administration of soluble TNF receptors (TNFRs) in controlling disease activity [20]. It is produced predominantly by activated macrophages and T lymphocytes, and, to a lesser extent, by mast cells, B cells, natural killer (NK) cells, neutrophils, endothelial cells, smooth and cardiac muscle cells, fibroblasts and osteoclasts [22]. TNF-α mediates multiple proinflammatory signals that play a central role in the pathogenesis of IBD, including neutrophil recruitment to the local site of inflammation, activation of both coagulation and fibrinolysis, as well as the induction of granuloma formation [23]. TNF-α has been found in the serum, stool and intestinal tissue of IBD patients [24-26]. In CD tissues, TNF-α-positive cells have been found deeper in

the lamina propria and in the submucosa, while TNF-α immunoreactivity in UC is mostly located in subepithelial macrophages [76]. TNF-α exerts its proinflammatory effect via nuclear factor kappa beta (NF-κβ), a pivotal transcription factor that increases the expression of various cytokines, enzymes, and adhesion molecules [77]. Increased TNF-α production and NF-κβ nuclear translocation have been noted in lamina propria mononuclear cells derived from both UC and CD patients [78]. It is known that TNF-α is capable of upregulating other proinflammatory cytokines such as IL-6 and IL-1β, thus amplifying the inflammatory cascade [79]. A TNF-α-induced increase in intestinal epithelial tight junction permeability has been also suggested as an important proinflammatory mechanism contributing to intestinal inflammation in IBD [80]. Although the role of TNF-α in disease pathogenesis is more evident in CD [81], it has been observed that both tissue and serum levels of TNF-α correlate with the severity of UC [26, 82]. The same has also been demonstrated for the levels of serum soluble TNF receptor I and II [83]. It is worth mentioning however, that the role of TNF-α blocking agents in UC is less clear than in the case of CD, and recent studies have yielded conflicting results. A systemic review concluded that in patients with moderate to severe UC whose disease is refractory to conventional treatment with corticosteroids and/or immunosuppressive agents, infliximab is effective in inducing clinical remission, including clinical response, promoting mucosal healing and reducing the need for colectomy, at least in the short term [84]. ROC curve analysis in this study revealed a cutoff TNF-α serum level of 7.6 pg/mL in predicting endoscopically-active disease (Mayo endoscopic index $\geq$1), with a sensitivity of 95%, a specificity of 80%, a PPV of 97%, and an NPV of 80%.

The study has some limitations. A relatively small number of patients in the study might have contributed to unexpected and/or non-significant results. Particularly, a larger population of patients in remission could have allowed the calculation of cutoff values more precisely. The study proved however, that both fecal markers, calprotectin and lactoferrin, closely correlate with the Mayo endoscopic score and CAI. It also showed that IL-17, despite its vague role in the pathogenesis of UC, might also be a useful marker in assessing the activity and severity of the disease. However, further research is needed to determine whether serum IL-17 can serve as screening and monitoring tests to reduce the number of people undergoing invasive endoscopy. The same applies to other proinflammatory cytokines investigated, such as IL-6 and TNF-α. It is worth remembering that in addition to high costs, endoscopy is a time-consuming and invasive procedure. Complications of endoscopy, related to the invasiveness of the procedure or to anesthesia are rare, but present. For instance, several retrospective studies have reported the incidence of a small perforation after colonoscopy to be in the range of 0.032% (1 in 3115 patients) to 0.9% (1 in 111) [43].

In conclusion, the majority of systemic markers currently used seem to have low sensitivity and specificity for intestinal inflammation and do not correlate well with symptoms and disease activity indexes. Fecal markers, such as calprotectin and lactoferrin, have the theoretical advantage of having higher specificity for the diagnosis of gastrointestinal diseases since their concentrations are not elevated in extradigestive processes. They also seem to correlate better with lesions in the colonic mucosa. Laboratory tests based on serum markers, on the other hand, are faster and easier to perform in the clinical setting. Despite all the disadvantages however, it seems that measuring fecal or serum marker concentrations might be a useful screening tool for identifying patients who are most likely to need endoscopy for suspected UC. Biological markers might also help to monitor patients undergoing treatment [85, 86] or to predict relapse [44, 87], particularly in the context of the introduction of biological treatments in IBD.

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