

Trefoil Factor 3 is Highly Predictive of Complete Mucosal Healing Independently and in Combination with C-Reactive Protein in Patients with Ulcerative Colitis

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ABSTRACT

Aim: In the current study we aimed to evaluate the role of trefoil factor 3 (TFF3) as a marker for complete mucosal healing (MH) in patients with ulcerative colitis (UC).

Methods: We enrolled 116 consecutive UC patients. Trefoil factor 3 levels were measured by ELISA and were compared to the clinical activity, assessed by Lichtiger Index, fecal calprotectin (FCP) and C-reactive protein (CRP) levels. Colonoscopy was performed in all the patients and the findings were graded according to Mayo endoscopic score (EMS) and UC endoscopic index of severity (UCEIS).

Results: Trefoil factor 3 levels were significantly correlated with Lichtiger Index ($r=0.736$, $p<0.001$), EMS ($r=0.811$, $p<0.001$), UCEIS ($r=0.820$, $p<0.001$), FCP ($r=0.696$, $p<0.001$) and CRP ($r=0.405$, $p<0.001$). The TFF3 cut-off level of 6.74 ng/ml indicated complete MH (EMS=0; UCEIS=0) with a sensitivity and specificity of 0.879 and 0.869, respectively (area under the curve, AUC 0.927; 95% confidence interval, 0.877–0.976). The DeLong's test revealed no significant difference between the AUC of TFF3+CRP and the AUC of FCP ($Z=1.717$, $p=0.086$), AUC of TFF3+FCP ($Z=1.908$, $p=0.056$), and AUC of TFF3+CRP+FCP ($Z=1.915$, $p=0.056$). However, the AUC of TFF3+CRP showed significant difference compared with the AUC of TFF3 ($Z=2.210$, $p=0.027$) and the AUC of CRP ($Z=3.145$, $p=0.002$) for predicting complete MH.

Conclusions: Trefoil factor 3 levels correlated significantly with clinical activity, endoscopic indices, CRP and FCP in our patients. TFF3 is a highly predictive marker of complete MH independently and in combination with CRP in patients with UC.

Key words: inflammatory bowel diseases – biomarkers – fecal calprotectin – trefoil factor 3.

Abbreviations: CRP: C-Reactive protein; FCP: fecal calprotectin; IBD: inflammatory bowel disease; MH: mucosal healing; TFF3: trefoil factor 3; UC: ulcerative colitis; UCEIS: ulcerative colitis endoscopic index of severity.

INTRODUCTION

Ulcerative colitis (UC) is a lifelong chronic inflammatory bowel disease (IBD) characterized by periods of remission and relapse [1]. It is currently defined by a continuous mucosal inflammation of the rectum and a variable extent of the colon, without granulomas on mucosal biopsies [1].

In the past, clinical remission was the main therapeutic goal for patients with UC and the disease management aimed to control the symptoms, such as rectal

bleeding and increased frequency of bowel movements [2]. However, there is mounting evidence that achieving clinical remission without mucosal healing (MH) is not associated with reduced rates of hospitalization or colectomy over the years [3]. Hence, MH has become the treatment target in UC patients, leading to improvement of short- and long-term outcomes [4-6].

The use of repeated endoscopy is the most accurate way to evaluate MH, however this is invasive, time-consuming, and expensive [7]. Therefore, identification of noninvasive markers to accurately detect mucosal inflammation is needed. To date, the best studied inflammatory markers are C-reactive protein (CRP) and fecal calprotectin (FCP). Despite the reported correlation between endoscopic activity and CRP [8], data are still insufficient to warrant its broad use in UC. There are many promising results for FCP [9, 10], but more studies are needed to clarify adequate surveillance strategies and cut-off

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levels before its broad implementation in clinical practice. Consequently, there is still need for new markers to identify intestinal inflammation in IBD.

Trefoil factors (TFFs) include a family of three mucin-associated peptides secreted by goblet cells in the intestinal mucosa [11]. They have a key role in maintaining mucosal barrier integrity [12] and are up-regulated at the site of mucosal damage [13, 14]. It has recently been suggested that serum levels of TFF3 can predict disease activity [15] and reflect MH in UC patients with minimal disease activity [16]. However, its potential to predict complete MH independently or in combination with other biomarkers has never been addressed.

In the current study, we aimed to evaluate the diagnostic role of TFF3 as a noninvasive marker for complete mucosal healing in patients with UC and to compare its levels with those of FCP and CRP.

METHODS

This was a prospective observational study. We prospectively recruited patients referred to the IBD outpatient clinic of “Tsaritsa Yoanna” University Hospital in Sofia between October 2015 and February 2018. As inclusion criteria we used: age 18–85 years, known UC diagnosed according to the ECCO Guidelines [1] and completion of a written informed consent. The exclusion criteria were: colorectal cancer or colon polyps, indeterminate colitis, history of colorectal surgery, pregnancy, history of active non-steroidal anti-inflammatory drugs (NSAIDs) intake (2 tablets/week), oral steroids or steroid enemas intake in the last 3 months, infectious colitis and primary immunodeficiency.

We defined clinical remission in UC as a Lichtiger Clinical Activity Index of 3 points or less [17] and endoscopic remission as Ulcerative Colitis Endoscopic Index of Severity (UCEIS) of 0 [18]. As a complete MH we defined UCEIS of 0 and EMS of 0.

All enrolled patients underwent a full medical assessment including a detailed medical history and physical examination. All therapies received by the patients before enrollment were recorded. All patients underwent colonoscopy within two weeks from the visit. Serum and fecal samples were collected within 1–2 days before colonoscopy. Serum levels of TFF3, CRP and FCP levels were assessed in all the patients.

Enzyme immunoassay

Serum levels of TFF3 were measured by ELISA (Quantikine ELISA, Human TFF3 Immunoassay, R&D Systems, Inc., USA). We strictly followed the manufacturer's instructions.

Fecal calprotectin

Calprotectin was analyzed in stool samples by means of point-of-care desk-top Quantum Blue Reader® (POC Reader) method. It is a lateral flow technology based on ELISA techniques. We performed the test according to the manufacturer's instructions (Quantum Blue® Calprotectin, Bühlmann Laboratories AG, Switzerland) [19]. The POC device uses internal standards within a range of 30–300 µg/g and a sensitivity of <10 µg/g, thus, guarantying consistency in results. When we received results >300 µg/g, we performed additional 1:10 dilution with extraction buffer according to

the manufacturer's instructions, allowing us to receive FCP levels up to 3000 µg/g. FCP values above the upper limit of the measurement ranges were registered as 3000 µg/g and FCP values below the lower limit were accordingly registered as 30 µg/g.

Statistical analysis

The statistical analysis was performed using SPSS for Windows, Version 25.0. (SPSS Inc., Chicago, USA) and MedCalc Statistical Software, Version 16.4.3 (MedCalc Software bvba, Ostend, Belgium; 2016). Descriptive statistic for tabular and graphical presentation of results was used. The inter-rater agreement of the endoscopic indices was analyzed using Cohen's weighted Kappa coefficient. Correlation analysis was performed using Spearman's and Pearson's correlation coefficient. The comparison between the coefficients of correlations was conducted using Steiger's Z test, known also as Meng's test [20]. The receiver operating characteristic (ROC) curves for the TFF3, CRP and FCP levels were assessed to predict the complete MH. The comparison between the area under the ROC curve was analyzed using DeLong's test [21]. A p value of <0.05 was considered statistically significant.

Ethics approval

The current study was approved by the Ethics Committee of “Tsaritsa Yoanna” University Hospital in Sofia, Bulgaria. Before initiating this study, written informed consent was obtained from all patients. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008) as reflected in a priori approval by the institution's human research committee.

RESULTS

The current study enrolled 116 UC patients, 62 (53.4%) were men; the median age at diagnosis of UC was of 39.4 ± 9 (19–65) years. The proportion of proctitis, left-sided colitis and extensive colitis in the cohort was 15.5%, 43.1%, and 41.4%, respectively. Fifty-six (48.3%) of the patients were with quiescent disease and 60 (51.7%) with active UC. An overview of the demographic patient characteristics at baseline is provided in Table I.

The mean FCP level was 926.28 µg/g (30–3000; SD 1101.72), the mean CRP level was 2.11 mg/dl (0.02–28.62; SD 4.69) and the mean TFF3 level was 8.03 ng/ml (4.6–13.54; SD 2.76), whereas the median EMS, UCEIS and Lichtiger Index values were 1.25 (0–3; SD 1.31), 2.13 (0–7; SD 2.25), and 4.72 (0–17; SD 4.75), respectively (Table II). Fifty patients (43%) were with complete MH and 66 (57%) with active UC. The mean levels of the three biomarkers, clinical and endoscopic indices are provided in Table II.

Correlation of the TFF3 level with FCP and CRP levels

Trefoil factor 3 levels exhibited significant correlation with FCP levels (Pearson correlation coefficient $r=0.696$, $p<0.001$) and CRP levels ($r=0.405$, $p<0.001$) (Figs. 1D, 1E). Moreover, FCP levels correlated significantly with CRP levels ($r=0.451$, $p<0.001$) (Fig. 1F). When comparing the degree of correlation between the TFF3 levels and other biomarkers, FCP showed

Table I. Characteristics of Ulcerative Colitis Patients Enrolled in the Study

Characteristics	Number	Percentage (%)
No. patients	116	-
Gender		
Male	62	53.4
Female	54	46.6
Age, mean \pm SD, range (years)	39.4 \pm 9 (19-65)	-
Duration of the disease, mean \pm SD (years)	4.1 \pm 2.8	-
Smoking habit		
Smokers	32	27.6
Never smoked	70	60.3
Ex-smokers	14	12.1
Disease location		
Proctitis – E1	18	15.5
Left-sided colitis – E2	50	43.1
Extensive colitis – E3	48	41.4
Therapy		
None	7	6.0
Topical 5-ASA	17	14.7
Systemic 5-ASA	58	50.0
Azathioprine	34	29.3
TNF-alpha inhibitor	23	19.8

a better correlation with the TFF3 levels, as compared to CRP (Steiger's Z test $Z = 3.209$, $p < 0.01$).

Correlation of the TFF3 level with the disease activity index and endoscopic indices

Trefoil factor 3 levels exhibited significant correlation with Lichtiger Index (Spearman correlation coefficient $r = 0.736$, $p < 0.001$), EMS ($r = 0.811$, $p < 0.001$) and UCEIS ($r = 0.820$, $p < 0.001$) (Figs. 1A, 1B and 1C). Furthermore, FCP levels correlated significantly with EMS ($r = 0.839$, $p < 0.001$) and UCEIS ($r = 0.872$, $p < 0.001$). C-reactive protein levels also had significant correlation with EMS ($r = 0.610$, $p < 0.001$) and UCEIS ($r = 0.665$, $p < 0.001$). Trefoil factor 3 levels had a significantly better correlation with UCEIS compared to CRP ($z = -2.911$, $p < 0.01$) but a similar correlation with UCEIS compared to FCP levels ($Z = 1.707$, $p < 0.01$). UCEIS showed very strong

correlation with EMS ($r = 0.934$, $p < 0.001$). The kappa coefficient of inter-rater agreement for MES and UCEIS were 0.80 and 0.76, respectively.

Analysis of the ROC curves

ROC curve analysis indicated a TFF3 cut-off level of 6.74 ng/ml (area under the curve [AUC], 0.927; 95% CI, 0.863 - 0.967) for predicting complete MH defined as both UCEIS and EMS values of 0. The sensitivity and specificity of the cut-off value of 6.74 ng/ml for complete MH were 87.9% and 86.9 %, respectively (Fig. 2C). ROC analysis revealed CRP cut-off level of 0.43 mg/dl (AUC, 0.865; 95% CI, 0.789 - 0.921) for predicting complete MH with sensitivity of 78.8% and specificity of 76% (Fig. 2B). ROC curve analysis showed a FCP cut-off level of 99 μ g/g (AUC, 0.988; 95% CI, 0.947 - 0.999) for predicting complete MH. The sensitivity and specificity of this cut-off value for complete MH were 97% and 98 %, respectively (Fig. 2A).

After combining the biomarkers among them, the AUC of TFF3+CRP was 0.960 (95% CI, 0.907-0.988), the AUC of TFF3+FCP was 0.990 (95% CI, 0.951-1.000), and the AUC of TFF3+CRP+FCPP was also 0.990 (95% CI, 0.951-1.000) (Figs. 3 and 4).

When comparing the AUC of TFF3 with the AUC of CRP and the AUC of FCP for predicting complete MH, the DeLong's test showed lack of significant difference for the former ($Z = 1.543$, $p = 0.0123$) and significant difference for the latter ($Z = 2.380$, $p = 0.017$) (Fig. 2D). There was a significant difference between the AUC of TFF3 and the AUC of TFF3+CRP ($Z = 2.210$, $p = 0.027$), the AUC of TFF3+FCP ($Z = 2.525$, $p = 0.012$), and AUC of TFF3+CRP+FCPP ($Z = 2.482$, $p = 0.013$).

DeLong's test revealed no significant difference between the AUC of TFF3+CRP and the AUC of FCP ($Z = 1.717$, $p = 0.086$), AUC of TFF3+FCP ($Z = 1.908$, $p = 0.056$), and AUC of TFF3+CRP+FCPP ($Z = 1.915$, $p = 0.056$). However, the AUC of TFF3+CRP showed significant difference with the AUC of TFF3 ($Z = 2.210$, $p = 0.027$) and the AUC of CRP ($Z = 3.145$, $p = 0.002$) for predicting complete MH. The DeLong's test did not find significant difference between the AUC of FCP alone and the AUC of any combination of biomarkers.

DISCUSSION

It is increasingly believed that achieving MH may improve quality of life, prevent relapses, minimize hospitalizations, and prevent complications such as colorectal cancer, and

Table II. Mean levels of Lichtiger Index, Mayo endoscopic score (EMS), Ulcerative Colitis endoscopic index of severity (UCEIS), fecal calprotectin (FCP), C-reactive protein (CRP) and trefoil factor 3 (TFF3) in all patients with UC, UC patients with mucosal healing (MH) and active UC patients. All data are presented in mean levels, minimal and maximal values and standard deviation.

	All UC patients	UC patients with MH	Active UC
Number	116	50	66
Lichtiger Index	4.72 (0-17; SD 4.75)	0.64 (0-2; SD 0.77)	7.82 (0-17; SD 5.86)
EMS	1.25 (0-3; SD 1.31)	0	2.20 (0-3; SD 0.96)
UCEIS	2.13 (0-7; SD 2.25)	0	3.74 (1-7; SD 1.68)
FCP (μ g/g)	926.28 (30-3000; SD 1101.72)	50.66 (30-156; SD 26.19)	1589.62 (51-3000; SD 1053.76)
CRP (mg/dl)	2.11 (0.02-28.62; SD 4.69)	0.29 (0.02-1.30; SD 0.35)	3.48 (0.04-28.62; SD 5.86)
TFF3 (ng/ml)	8.03 (4.6-13.54; SD 2.76)	5.79 (4.60-10.93; SD 1.07)	9.73 (4.69-13.54; SD 2.41)

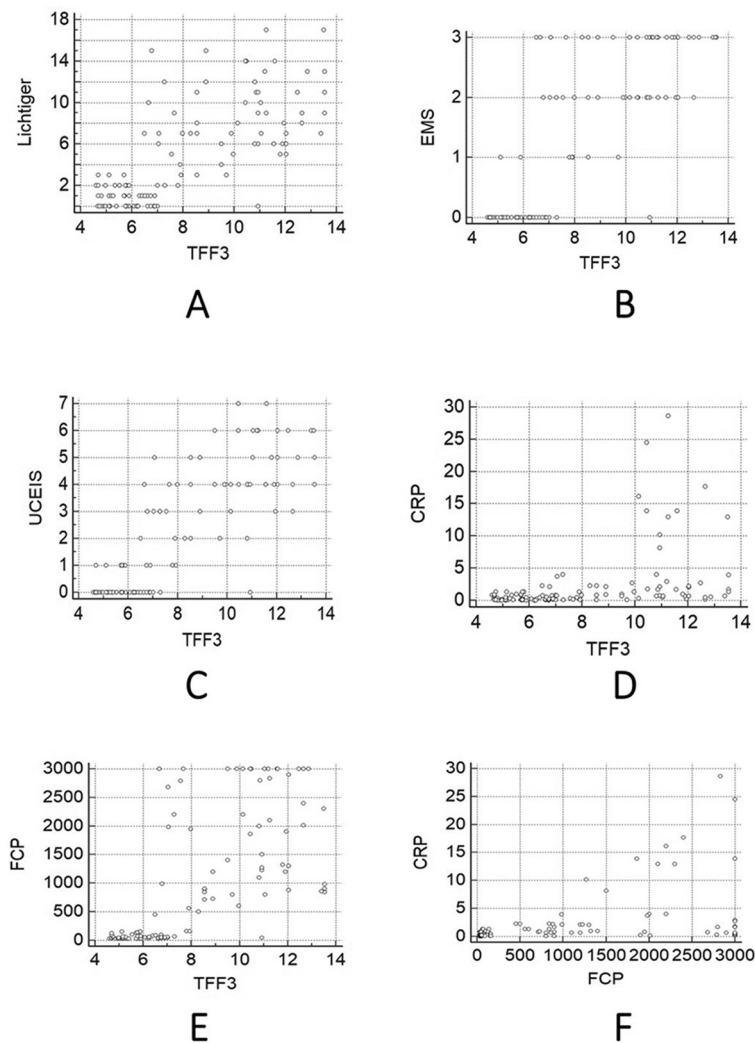


Fig. 1. Correlation between trefoil factor levels and A) Lichtiger Clinical Activity Index; B) Mayo endoscopic score (EMS); C) Ulcerative Colitis Endoscopic Index of Severity (UCEIS); D) C-reactive protein (CRP); E) Fecal calprotectin (FCP); and correlation between FCP and CRP (F).

need for surgery in UC patients [22]. Furthermore, MH is currently viewed as the best predictor of disease prognosis [2]. However, it is not still clear whether the “gold standard” for MH assessment, i.e. endoscopy with biopsies, could be replaced by any non-invasive biomarker, more convenient to use in everyday clinical practice. As a factor that plays a pivotal role in maintaining the integrity of intestinal mucosa, TFF3 could be considered as a potential candidate.

According to our best knowledge, the current study is the first that aimed to evaluate the potential of TFF3 to predict complete MH in UC patients independently or in combination with other biomarkers. Overall, we found that TFF3 levels were highly predictive of complete MH in UC. Moreover, the combination of TFF3 and CRP was able to predict complete MH even better than TFF3 alone and with no significant difference when compared to FCP.

Although there are several cellular and animal studies that demonstrated the key role of TFF3 in the integrity maintenance of intestinal mucosa, we are lacking clinical data about its potential in IBD management. Vestergaard et al. [23] did not find any significant fluctuations of TFF3 in three patients with UC who underwent treatment with prednisolone with clinical improvement. Grønbaek et al. [24] found that serum TFF3 levels correlated with disease activity

indices in patients with UC and they noted a trend towards reduction in TFF3 levels with clinical improvement after therapy with steroids. In a recent study, Srivastava et al. [16] showed that serum TFF3 could point out patients with MH in a group of UC patients in clinical remission or with mild activity with reasonable sensitivity and specificity. However, the MH was defined as Baron score 0 or 1, and the latter did not represent a complete MH. In one of our recent studies we showed that the mean levels of TFF3 in active UC were significantly higher than those identified in patients with quiescent UC, which were similar to those of the healthy controls [15]. In the current study we demonstrated a strong correlation of TFF3 with clinical activity, endoscopic indices and FCP, showing its potential as a marker for disease activity and complete MH in UC patients. Unfortunately, TFF3 is not a useful marker for disease activity in patients with Crohn's disease [15, 25].

Many non-invasive markers have been investigated over years, but still an ideal marker to detect and monitor MH in UC is needed. Nowadays, FCP is the most studied and the most sensitive one. Based on our ROC curve analysis we could state that FCP significantly predicts complete MH in UC patients. In spite of the fact that CRP and TFF3 in combination with FCP enhanced the predictability of complete MH, combination

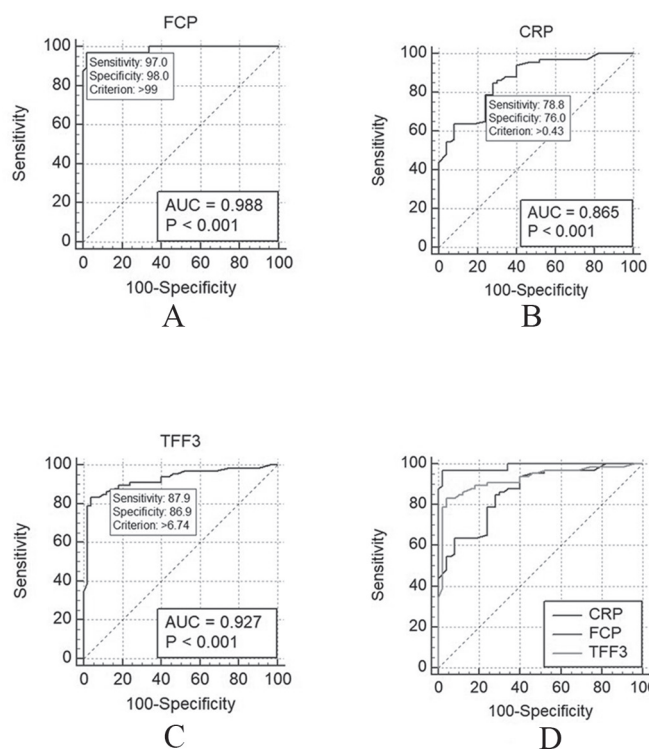


Fig. 2. ROC curve analysis showing the AUC of the A) FCP levels; B) CRP levels and C) TFF3 for predicting complete mucosal healing defined as both UCEIS and EMS values of 0. D) Comparison of the ROC curves of CRP, FCP and TFF3. TFF3 trefoil factor 3, CRP C-reactive protein, FCP fecal calprotectin, ROC receiver operating characteristics, AUC area under curve, EMS Mayo endoscopic score, UCEIS ulcerative colitis endoscopic index of severity.

of other markers to FCP did not affect significantly the predictability of complete MH.

Although FCP is proved to be the best marker of disease activity in UC, some concerns have been raised. When groups of UC patients with different levels of disease activity are compared, considerable overlaps in FCP levels between the groups were reported [26]. This makes it difficult to interpret the results for the individual patient. A significant day-to-day variation in FCP in patients with UC has also been described [27-29]. Furthermore, there is a lack of validated cut-off level to predict MH in UC patients. A recent meta-analysis by Rokkas et al. [30] shows best specificity of 78.2% for cut-off levels of FCP greater than 100 µg/g, which is in line with our results.

Interestingly, the combination of TFF3 and CRP has comparable predictability of complete MH to FCP, which gives an opportunity to UC patients to avoid stool sampling and to be followed-up just by blood tests. This could be extremely useful for patients reluctant to handle fecal material.

CONCLUSIONS

In the present study we found that serum human TFF3 is significantly correlated with clinical activity, endoscopic indices and FCP in UC patients. Moreover, TFF3 is a highly predictive biomarker of complete MH independently and in combination with CRP in patients with UC. Future studies on different patient populations are required to further explore

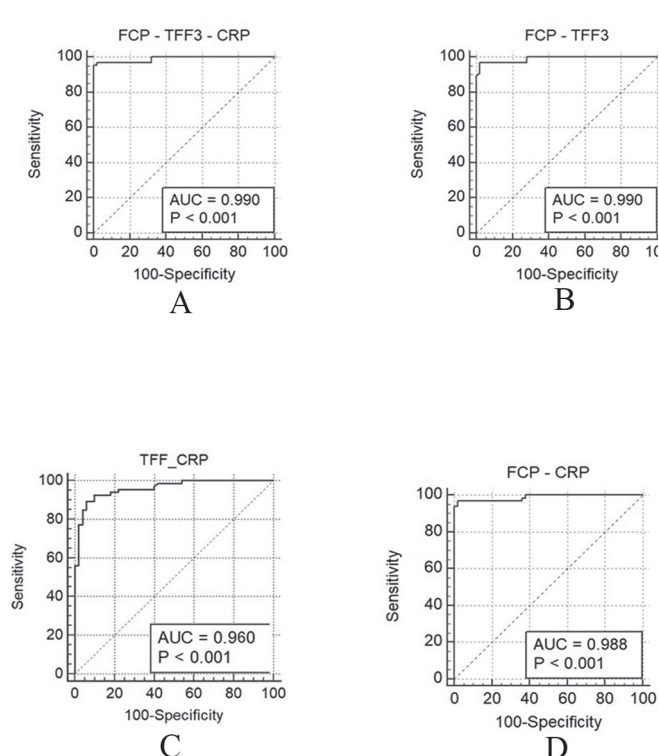


Fig. 3. ROC curve analysis showing the AUC of the A) FCP levels in combination with TFF3 and CRP levels (FCP-TFF3-CRP); B) FCP levels in combination with TFF3 levels (FCP-TFF3), C) TFF3 levels in combination with CRP levels (TFF3_CRP) and D) FCP levels in combination with CRP levels (FCP-CRP) for predicting complete mucosal healing defined as both UCEIS and EMS values of 0. (For abbreviations see Fig. 2).

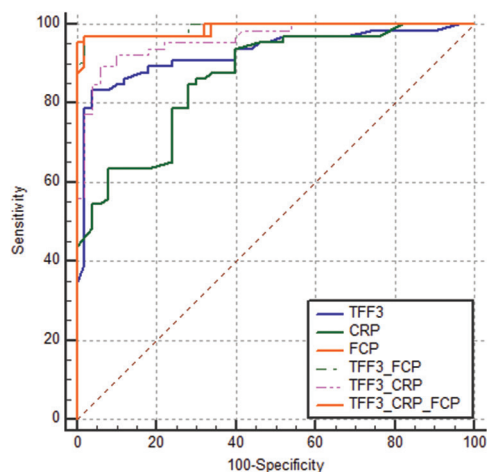


Fig. 4. Comparison of the ROC curves of TFF3, CRP, FCP, combination of TFF3 and FCP levels (TFF3_FCP), combination of TFF3 and CRP levels (TFF3_CRP) and combination of the three markers (TFF3_CRP_FCP) for predicting complete mucosal healing defined as both UCEIS and EMS values of 0. (For abbreviations see Fig. 2).

the predictability of TFF3 and its combination with CRP for evaluating MH in UC patients.

Conflicts of interest: None to declare.

Authors' contributions: R.N and V.N designed the study, enrolled and followed up the patients; performed statistical analyses; collected and analyzed the data and wrote the manuscript. T.V. performed the research and participated in study design. V.G. and L.T. managed the patients, collected and analyzed data, and supervised the project. All authors approved the final version of the manuscript

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