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Fecal Lactoferrin, Fecal Calprotectin, Transforming growth factor-b1, and CRP in Evaluation of Disease Activity in Egyptian patients With Ulcerative colitis

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Abstract

Aims: to analyze the usefulness of fecal lactoferrin, fecal calprotectin, transforming growth factor-B1,Endoscopic Activity Index, Clinical Activity Index, C- reactive protein, and blood leucocytes in monitoring disease activity in Egyptian patients with ulcerative colitis. Methods: One hundred and twenty patients with ulcerative colitis were enrolled and scored according to the endoscopic part of the Rachmilewitz Index. **Patients and controls:** Patients and controls provided fecal and blood samples for measuring Lactoferrin, calprotectin, TGF-B1, CRP, and leucocytes. Results: The values in ulcerative colitis patients (n = 120) compared to controls (n = 30): Fecal lactoferrin: *703.6±657.6* versus*7.01±3.9 µg/g*, calprotectin: *867.4±561.2* versus *36.3±15.3* µg /g, TGF-B1: *386.9±246.7 versus 5.9±1.8* pg /ml, CRP:*19.4±14.7* versus *3.2±0.9* mg/L, blood leucocytes: *11.6±3.8* versus *6.8±1.9* g/L (for all P< 0.001). Endoscopic disease activity correlated significantly with lactoferrin (Spearman's rank correlation coefficient r =0.949), calprotectin (r = 0.923), TGF-B1 (r = 0.918), Clinical Activity Index (r = 0. 761), CRP (r = 0.851), and blood leucocytes (r = 0.681). Fecal lactoferrin, calprotectin and TGF-B1 levels were significantly lower in ulcerative colitis patients with inactive disease (endoscopic score 0 -3, Lactoferrin 48.7±24.9 µg/g, calprotectin 81.5 ± 48.8 µg / g, TGF-B1 42.5 ± 36.5 pg/mL , P < 0.001 for both lactoferrin and calprotectin, but P <0.059 for TGF-B1), compared to patients with mild (score 4 – 6, lactoferrin 465.7 ± 217.4 µg/g, calprotectin 420.4 ±244.8 µg/g, TGF-B1 269.6 ± 80.3 pg/mL, P < 0.001), moderate (score 7 – 9, lactoferrin 678.7 ± 258.6 µg/g, calprotectin 1074.9 ± 303.5 µg/g, TGF-B1 391.5 ± 56.8 pg/mL, P < 0.001), and high disease (score 10 – 12 , lactoferrin 1624.5 ± 327.2 µg/g, calprotectin 1466.5.2 ± 31.5 µg/g, TGF-B1 690.9 ± 160.1 Pg/ mL, P < 0.001). The overall accuracy for detection of histopathological active disease was 96.9 % for fecal lactoferrin, 96.6 for fecal calprotectin, 94.5 % for TGF-B1, 90 % for Endoscopic Activity Index, 87 % for Clinical Activity Index, and 65 % for both blood leucocytes and CRP. **Conclusion**: Fecal lactoferrin, fecal calprotectin and TGF-B1 correlated significantly with endoscopic disease activity, clinical activity index, CRP, and blood leucocytes. Furthermore, lactoferrin and calprotectin were suitable markers that can differentiate endoscopically and histopathologically inactive from active disease. Also TGF-B1 was used as a useful marker to distinguish mild from moderate and high active disease. Thus, these three biomarkers may be used for monitoring ulcerative colitis activity.

Keywords: Fecal Lactoferrin, Fecal Calprotectin, Transforming Growth Factor-B1, Ulcerative Colitis, Disease Activity, Biomarkers, Rachmilewitz Activity Index.

Introduction

Ulcerative colitis [UC] is a chronic relapsing disease. The determination of inflammatory activity is crucial for the assessment of clinical decision making and for the tailoring of therapy (1). To define remission in ulcerative colitis, a standard based on clinical symptoms and/or endoscopy is proposed. A variety of disease activity indices are available for UC and several different symptom-based activity scores, composite scores, and patient evaluation scoring systems have been used and published (1,2,3,4). Two widely used scores are the CAI by Rachmilewitz (5) and the Mayo UC Disease Activity Index by Sutherland (6). Both contain clinical and endoscopic items.

Both contain clinical and endoscopic items. The Rachmilewitz Score has the advantage that the clinical as well as the endoscopic part can be used separately. Furthermore, it is easy to calculate and increasingly used in clinical practice (7). Colonoscopy and biopsy are useful in the assessment of intestinal mucosal inflammation of patients with ulcerative colitis, but these examinations can be a heavy burden to the patient (8). Several standard markers as erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), acute phase protein e.g., albumin, and platlets are used to aid in diagnosing and monitoring the disease. However, these markers lack specificity for gastrointestinal tract inflammation (9).

Fecal markers may be more specific for assessing intestinal disease activity. Specifically, Lactoferrin and calprotectin have been detected in stools in direct proportion to neutrophil migration through the gastrointestinal tract (10).

Lactoferrin (LF) is a long and widely used fecal biomarker of intestinal inflammation. It is an iron-biding glycoprotein with a molecular mass of about 80 kDa present in secondary (specific) granules especially in mature neutrophilic granulocytes [11,12-14]. Although it provides an excellent quantifiable marker of neutrophilic inflammation, several exocrine cells also secrete lower amounts of this protein that are often present in lower concentrations in many fluids such as normal human milk, tears, synovial fluid, and serum. Its presence in breast milk has raised concerns about the validity of low levels of lactoferrin measured in the stools of exclusively or even partially breastfed children. LF is stable in fecal samples at room temperature for up to 5 days allowing samples to be sent to the laboratory [15]. During intestinal inflammation neutrophils infiltrate the mucosa and markedly increase LF levels that can be readily measured in feces or gut lavage fluid [16,17]. Studies evaluating lactoferrin in the diagnosis of IBD show that it exhibited similar performance to fecal calprotectin and correlated better than C-reactive protein with mucosal inflammation by endoscopy [18-20].

Calprotectin is a calcium- and zinc- binding S 100 family of proteins that inhibits metalloproteinase, has antifungal activity and induces apoptosis in cell culture [20, 21,22]. It makes up about 5% of the total protein content in the neutrophil and about 60% of the cytosolic proteins (23). It is an important granulocyte cytosolic protein that is closely related to fecal excretion of 111-indium labeled leucocytes, deemed to be the gold standard for measuring intestinal inflammation (24). This kind of protein can resist metabolic degradation caused by intestinal bacteria and is relatively stable in stools for up to one week at room temperature. It can differentiate between patients with organic or non-organic intestinal disease and can be useful in detecting colorectal cancers and inflammatory disorders, and also can be useful in predicting a relapse of inflammatory bowel disease (25-27). When the level of these markers is low, the presence of active inflammation in the colon is unlikely (28, 29, 30).

Transforming growth factor-b1 (TGF-B1) belongs to a family of multifunctional polypeptides produced by Lymphoid and non-lymphoid cells. It has five different isomers. TGF-B1 has confirmed its effects on cell Proliferation, immunosuppression, and wound healing (29). It plays a great role as an inducer of fibrosis and myofibroblast generation and in a biological process called Epithelial-to-Mesenchymal Transition (EMT) in colonic diseases (30).

EMT is a well established biological phenomenon important in normal tissues and organ development and in the pathogenesis of diseases (such as chronic inflammation-related fibrosis, colorectal carcinogenesis, cancer invasion, and in mucosal healing. The inhibition of EMT seems to minimize chronic inflammation related wall fibrosis in the colon (30). In inflammatory bowel disease, TGF-B1 produced and secreted from the cells in the lamina propria and the epithelium in the colon, it controls proliferation and takes part in healing and fibrosis (31).

C-reactive protein (CRP) is a pentameric protein composed of five monomers and is one of the most important acute phase human proteins. Under normal conditions, CRP is produced by hepatocytes in small amounts (1 mg/L). However, following an acute phase stimulus such as inflammation, the production of CRP increases rapidly in the hepatocytes under the influence of interleukin (IL)-6, the tumor necrosis factor (TNF-a), and IL-1b (1,7). Mahmoud et al. (10) identified significant correlations between CRP and the clinical, endoscopic, and histological activity of the inflammatory bowel diseases (IBDs). A high CRP level is mainly determined by the precipitating cause such as the activity of the disease, and its half-life is constant for approximately 18 h. Despite these characteristics, there is heterogeneity in the CRP response between Crohn's disease (CD) and ulcerative colitis (UC). CD presents a strong CRP response, whereas the CRP response in UC is modest to absent (51).

Patients and Methods

One hundred and twenty patients known to have active ulcerative colitis who presented to the outpatient clinics and were inpatients of the Gastroenterology Unit of Internal Medicine Department of El Hussien University hospital (documented clinically, endoscopically, and histologically) were enrolled in the study, including 6 patients with proctitis, 78 patients with left sided colitis, 19 patients with extensive colitis, and 17 patients with pancolitis. They were 70 females (58.3%) and 50 males (41.7%). Their mean ages were 37.5(±11.63) years. Thirty healthy controls were members of staff of the hospital (12 males and 18 females) with a mean age of 39.3(± 2.3) years were included with no confirmed abnormality in the upper or lower digestive tract. The Ethical Research and Review Committee of the Hospital approved the study protocol, and informed consent was obtained from the participants.

Determination of fecal lactoferrin concentration: The stool samples were collected and placed in plastic containers, frozen and stored at –72°C until analysis. Fecal concentration of lactoferrin was determined with an IBD-SCANTM quantitative immunoenzymatic test (catalogue no. 30351l TECHLAB, USA). The test uses antibodies to human lactoferrin. The samples were diluted at 1:100, 1:400, 1:1000 and 1:4000 and further handled according to the manufacturer's instructions. Absorbance of the samples was determined with an ElizaMatTM 3000 reader (DRG MedTek, Poland). Fecal calprotectin was quantitated using an Enzyme Linked Immunoassay (ELISA) test (Calprest, Eurospital Trieste, Italy). The results of the test samples were calculated by the standard curve and were expressed as micrograms per milliliter. According to the manufacturer, the lactoferrin cut-off point as positive was 13 µg/ g feces.

Determination of fecal calprotectin concentration: A single stool sample was collected from each patient in screw capped plastic containers that avoids toilet, water artifact and simplifies laboratory sampling at the beginning of the study. The stool samples were frozen (-20) until calprotectin determination. Fecal calprotectin was quantitated using an Enzyme Linked Immunoassay (ELISA) test (Calprest, Eurospital Trieste, Italy). The results of the test samples were calculated by the standard curve and were expressed as micrograms per milliliter. According to the manufacturer, the calprotectin cut-off point as positive was 50 µg/ g feces.

Blood samples for measurement of a full blood count, CRP, and transforming growth factor-b1 were delivered by the patients within 3 days prior to endoscopy.

Transforming growth factor-B1 was measured in venous blood obtained after an overnight fasting. Results were expressed as the mean of the results of the sample. Five milliliter blood was taken (without using a tourniquet) on EDTA. The blood samples were immediately placed on ice. The plasma was spun for 30 min. in 1000 Xg and later 10 min. at 10,000 Xg to remove platlets (which contain a large amount of TGF) to obtain the platelet poor plasma (PPP). The activation of TGF-B1 was obtained by acidification of PPP with 2.5 N acetic acid/ 10 urea and later Ph was adjusted up to 7.2 – 7.6 using 2.7 N NaOH/ 1 MHEPES. The analysis was done by ELISA following h Human TGFB1 Immunoassay Protocol (Quantikine, R & D Systems) method. According to the manufacturer, the TGF-B1 cut-off for counting as positive was 7.0 pg/L.

Blood leucocytes (normal range 4-11 g/l), hemoglobin (normal range for women 12- 16 g/dl, for men 13-18 g/dl), a sedimentation rate (normal range for men and women up to 50 years up to 20mm/h and 15 mm/h; normal range for persons older than 50 years up to 30 mm/h and up to 20 mm/h), as well as CRP that was measured using a latex immunoturbidimetric test {CRPLX, Tina-quant, Roche/Hitachi} (upper limit of normal < 6mg/ L) were determined as routine laboratory values.

Inclusion criteria were disease duration more than 3 months, complete colonoscopy including at least 6 colonic biopsies from UC- affected colon and rectum, informed consent, age from 18 – 65 years, fecal samples delivered from 3 to 1 day before colonoscopy and bowel preparation was not started until the fecal specimens were collected.

Exclusion criteria were incomplete colonoscopy, history on non-steroidal anti-inflammatory drugs and/or antibiotics during the three months preceding enrolment, colorectal cancer, crohn's disease, urinary incontinence (fear of contamination of fecal samples), inability to collect fecal samples, pregnancy, history of colorectal surgery and alcohol abuse.

All patients underwent total colonoscopy for determining the severity of disease. Indications for colonoscopy were clinically active disease, assessment of endoscopic activity after medical treatment and dysplasia surveillance for longstanding disease. The aim of colonoscopic examination was to confirm diagnosis, estimate disease extent, and obtain colonoscopic biopsy specimens using Pentax videoscope Ec-3840 L. Biopsies were immediately fixed in 10% neutral buffered formalin. Formalin-fixed paraffin embedded samples were prepared for histopathology and stained by hematoxylin and eosin for histological grading. The degree of inflammation was graded on a four point scale; normal (no significant inflammation), mild (elevated number of mucosal leucocytes but intact epithelium), moderate (aggregates of leucocytes with crypt abscesses and erosions but no ulceration of the epithelium), and severe (significant ulceration of the epithelium by mononuclear cell infiltrate). Histological grading was performed by a pathologist without knowledge of endoscopic or laboratory features.

Disease activity was determined through using Colitis Activity Index (CAI) by Rachmilewitz, which includes a combination of clinical and endoscopic parts (Table1). The Clinical Activity Index (CAI) ranges from 0-29 points, namely weekly calculation of bowel frequency, blood in stools, well-being, abdominal pain, fever, extra intestinal symptoms, erythrocyte sedimentation rate, and hemoglobin level. The Endoscopic Activity Index (EAI) ranges from 0 – 12 points.

Table 1. *Rachmilewitz Index for Ulcerative Colitis.*

Clinical Activity Index	Score
1. Number of stools weekly:	
< 18	0
18-35	1
36-60	2
>60	3
2. Blood in stools:	
None	0
Little	2
A lot	4
3. Investigator's global assessment of symptomatic satate:	
Good	
Average	0
Poor	1
Very poor	2
4. Abdominal pain/cramps:	
None	0
Mild	1
Moderate	2
Severe	3
5. Temperature:	
37 – 38	0
>38	3
6. Extraintestinal manifestations:	
Iritis	3
Erythema nodosum	3
Arthritis	3
7. Laboratory findings:	
Sedimentation rate > 50 mm in 1st h.	1
Sedimentation rate > 100 mm in 1st h.	2
Hemoglobin < 100 g/L	4
Endoscopic Activity Index	
1. Granulation scattering reflected light:	
No	0
Yes	2
2. Vascular pattern:	
Normal	0
Faded/disturbed	1
Completely absent	2
3. Vulnerability of mucosa:	
None	0
Slightly increased (Contact bleeding)	2
Greatly increased (Spontaneous bleeding)	4
4. Mucosal damage (mucin, fibrin, exudates, erosions. Ulcers):	
None	
Slight	0
Pronouced	2

The Clinical Index ranges from 0 – 29 points, the Endoscopic Activity Index from 0 – 12 points.

Statistical Analysis

Statistical analysis was performed using the statistical package SPSS version 16. The data were expressed as mean ± SD. They were compared by t- student test for comparison between two groups and ANOVA f- test when more than two groups were compared. The association between Endoscopic disease activity, Clinical activity, fecal calprotectin, TGF-B1, CRP, and blood leucocytes was assessed by f-test. Also, Pearson's r correlation and chi – square test were used.

Results

One hundred and twenty patients with ulcerative colitis were included in the study. The mean age was 39.5 ± 11.6 years, and 53.8% were women. The mean duration of disease to the current colonoscopy was 4.5 ±3.4 years (range, 1 – 15 years), none had history of surgery. Disease location in ulcerative colitis patients was as follows: proctitis (4.6 %), left sided colitis (60 %), extensive colitis (14.6 %), and 13.1 % of patients had pancolitis.

Mean levels of fecal Lactoferrin, calprotectin and TGF-B1 were *703.6±657.6* (*0.55-2080 µg/g*), *867.4±561.2* (range from 9 – 1500 µg/g), *386.9±246.7* (range from 6 – 800 pg/mL) respectively.

All patients presented with variable grades of diarrhea (mean number of motions per week was *37.9±23.1*, bloody stool based on weekly average was positive in 97 patients (A little in 49.5% and A lot in 33%). Abdominal cramps were detected in 102 patients (85 %). Extra intestinal manifestations were detected in 55 % (Arthritis in 33.3 %, Erythema nodosum in 16.6 %, and Iritis in 15.1 %).

The clinical and laboratory characteristics of our patients in comparison to controls were shown in table 2.

Table 2: Clinical and laboratory characteristics of Ulcerative Colitis patients (n = 120) and controls (n = 30).

	Ulcerative colitis patients		*Controls*		*t-test*	*P value*
	Range	*Mean ± SD*	*Range*	*Mean ± SD*		
No. of motion per week	*5-80*	*37.9±23.1*	*9-16*	*12.9±2.5*	*11.9*	*0.001*
Temp.	*37-39*	*37.6±0.5*	*36.5-37.2*	*36.9±0.2*	*13.4*	*0.001*
ESR	*5-105*	*38.8±25.4*	*3-30*	*11.5±7.4*	*11.7*	*0.001*
CRP	*1-52*	*19.4±14.7*	*2-5*	*3.2±0.9*	*12.1*	*0.001*
Blood Leukocytes	*4.5-22*	*11.6±3.8*	*4.5-11*	*6.8±1.9*	*13.04*	*0.001*
Hemoglobin	*7-13*	*10.9±1.4*	*13-17*	*15.3±1.2*	*-23.9*	*0.001*
Platlet count	*190-650*	*395.8±156.0*	*180-410*	*280.2±75.9*	*7.5*	*0.003*
Fecal Lactoferrin	*0.55-2080*	*703.6±657.6*	*0.26-13*	*7.01±3.9*	*11.6*	*0.001*
Fecal Calprotectin	*9-1500*	*867.4±561.2*	*10-55*	*36.3±15.3*	*16.3*	*0.001*
TGF-B1	*6-800*	*386.9±246.7*	*1-7*	*5.9±1.8*	*16.9*	*0.001*

In our patients, the severity of disease was determined according to the Rachmilewitz Index for ulcerative colitis. The Rachmilewitz Clinical Activity Index was divided into 4 subgroups, 22 patients in remission (18.3 %), 38 patients were mild (31.7%), 37 patients were moderate (30.8%) and 23 (19.2 %) patients were severe. The Endoscopic Activity Index was divided into 4 subgroups: 22 patients in remission (18.3 %), 25 patients were mild (20.8 %), 39 patients were moderate (32.6 %), and 34 patients were severe (28.3 %). The controls were healthy persons from the clinical and laboratory staff willing to provide blood and fecal samples, 60 % were females, mean age 41.1 ± 13.3 years (range from 22 – 62).

The relationship between endoscopic Activity Index with Fecal Lactoferrin, Fecal Calprotectin, TGF-B1, CRP, and blood leukocytes are shown in table 3.

Table 3: Correlation of the Endoscopic Activity Index subgroups with fecal lactoferrin, fecal calprotectin, TGF-B1, the Clinical Activity Index, CRP, and Blood leucocytes.

Endoscopic Activity Index	No	Fecal Lactoferrin		Fecal Calprotectin		TGF-B1		CRP		Blood Leukocytes		Clinical Activity Index	
		Range	Mean± SD	Range	Mean± SD	Range	Mean± SD	Range	Mean ±SD	Range	Mean±SD	Range	Mean±SD
Remission (0-3)	22	0.55-80	48.7±24.9	9-140	81.5±48.8	6-100	42.5±36.5	3.6-12	5.8±2.2	4.5-9	6.4±1.4	0-17	6.9±4.7
		P<0.001		P<0.001		NS		NS		NS		P<0.001	
Mild (4-6)	25	13-750	465.7±217.4	15-1045	420.4±244.8	90-320	269.6±80.3	4-16	8.7±3.4	5-12	10.2±2.1	4-23	15.5±4.8
		P<0.001		P<0.001		P=0.005		NS		NS		P=0.002	
Moderate (7-9)	39	250-1000	678.7±258.6	400-1450	1074.9±303.5	300-450	391.5±56.8	1-34	17.9±9.3	10-14	11.9±0.8	4-23	16.1±4.9
		P<0.001		P<0.001		P<0.001		P<0.001		p<0.001		P<0.001	
Severe (10-12)	34	1000-2080	1624.5±327.2	1390-1500	1466.5±31.5	420-800	690.9±160.1	16-52	37.8±10.4	12.5-22	15.7±2.9	7-27	21.9±5.3

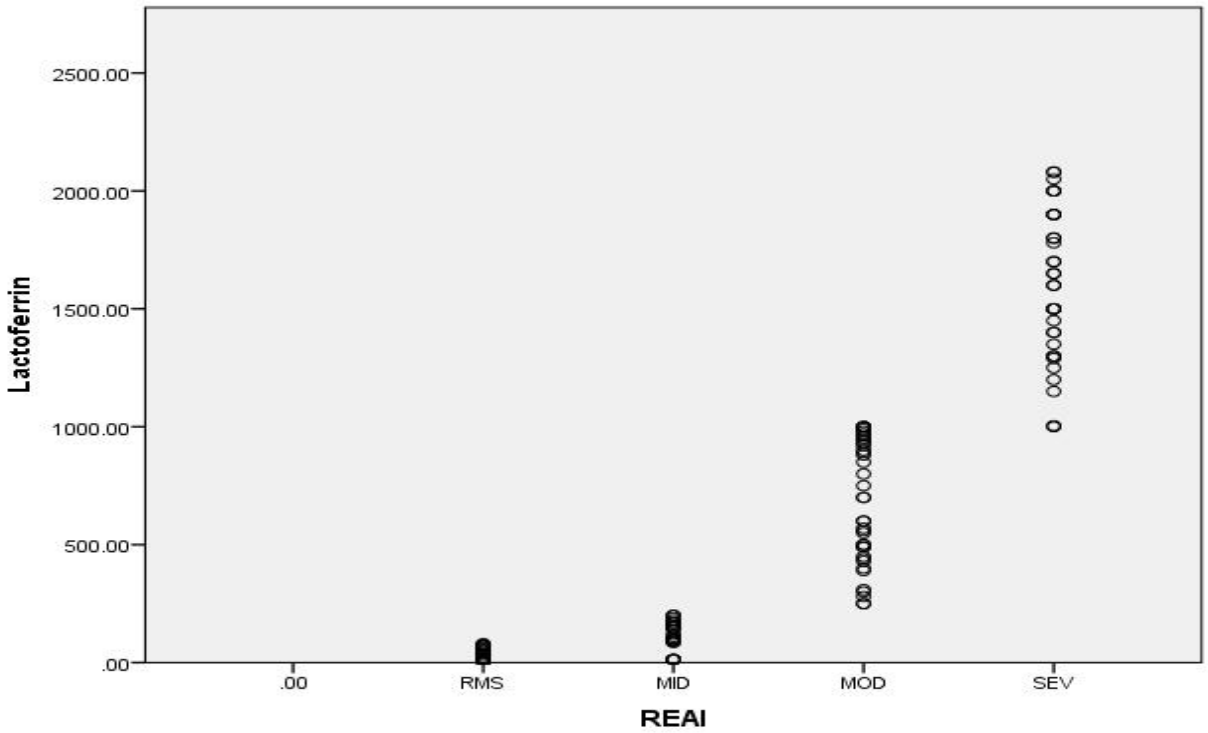

Figure 1.Scatter plot diagram illustrating the correlation of the Rachmilewitz Endoscopic Activity Index with fecal lactoferrin (Spearman's correlation coefficient = 0.949).

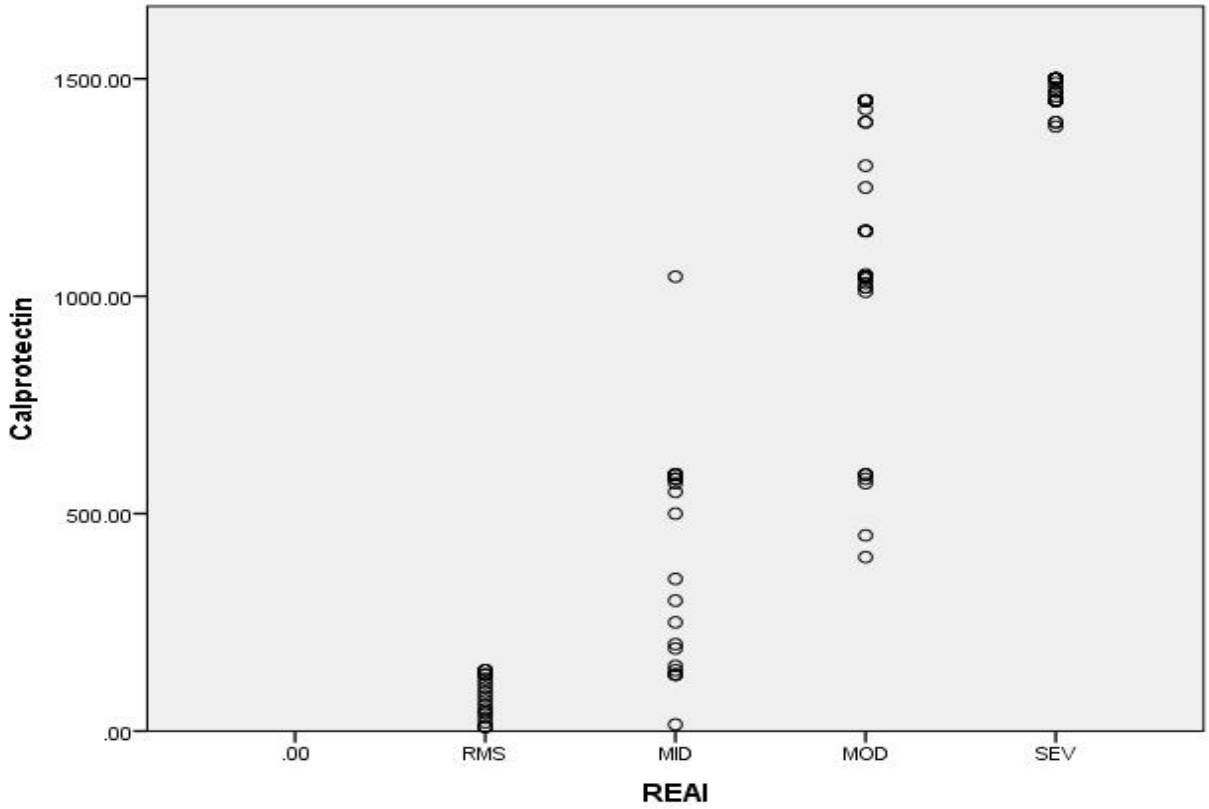

Figure 2.Scatter plot diagram illustrating the correlation of the Rachmilewitz Endoscopic Activity Index with fecal calprotectin (Spearman's correlation coefficient = 0.923).

The relationship between the different subgroups of Endoscopic Activity Index with their corresponding Clinical Activity Index (CAE), fecal Lactoferrin, fecal Calprotectin, TGF-B1, CRP, and blood leucocytes (values given as mean ± SD, range, ANOVA F- test) is shown in Table 4.

Table 4: Relationship between Endoscopic Activity Index subgroups with ESR, Platelet count, Blood leukocytes, CRP, TGF-B1, Fecal lactoferrin, fecal calprotectin, and Clinical Activity Index.

Endoscopic Activity Index		Remission	Mild	Moderate	Severe	F	p. value
ESR	Range	5-15	5-30	20-49	45-105	0.420	0.741
	Mean±SD	10.9±2.5	21.7±5.8	8.3±0.8	71.8±19.4		
Platelet count	Range	190-220	200-460	210-520	440-650	1.512	0.246
	Mean±SD	200.0±8.3	298.6±81.9	401.9±81.5	587.0±69.3		
Blood leukocytes	Range	4-9	5-12	10-14	12.5-22	3.825	0.001
	Mean±SD	6.4±1.4	10.2±2.1	11.9±0.8	17.7±2.9		
CRP	Range	3.6-12	4-16	1-34	16-52	4.206	0.001
	Mean±SD	5.8±2.2	8.7±3.4	17.9±9.3	37.8±10.4		
TGF-B1	Range	6-100	90-320	300-450	420-800	4.896	0.001
	Mean±SD	42.5±63.6	269.9±80.3	391.5±56.8	690.9±160.1		
Fecal lactoferrin	Range	0.55-80	13-750	250-1000	1000-2080	8.204	0.001
	Mean±SD	48.1±24.9	456.7±217.4	678.7±258.6	1624.6±327.2		
Fecal calprotectin	Range	90-140	15-1045	400-1450	1390-1500	7.543	0.001
	Mean±SD	81.5±48.8	420.4±244.8	1074.9±303.5	1466.5±31.5		
Clinical Activity Index	Range	0-13	4-17	4-23	7-27	11.098	0.001
	Mean±SD	6.7±4.4	15.5±4.8	16.0-4.9	21.9±5.3		

When comparing the ability to discriminate between the various subgroups of the EAI, the mean fecal Lactoferrin in patients with remission was 48.7 (± 24.9), mean fecal Lactoferrin in patients with mild activity was 465.7 (±217.4), while the mean fecal Lactoferrin value among patients with moderate activity was 678.7 (±258.6) and the mean fecal Lactoferrin value in patients with high activity was 1624.5 (± 327.2). These results revealed a good significant relationship between fecal Lactoferrin values and the different subgroups of EAI as determined by Rachmilewitz criteria ($P < 0.001$).

Fecal Calprotectin in this study can discriminate the various subgroups of the EAI, the mean fecal Calprotectin in patients with inactive disease was 81.5 (± 48.4), mean fecal Calprotectin in patients with mild activity was 420.4 (± 244.8), while the mean fecal Calprotectin value among patients with moderate activity was 1074.9 (± 303.5) and the mean fecal Calprotectin value in patients with severe activity was 1466.5 (± 31.5). These results showed a significant relationship between fecal Calprotectin values and the different subgroups of EAI as determined by Rachmilewitz criteria ($P < 0.001$).

TGF-B1 in this study can't discriminate inactive endoscopic activity from mild activity index ($P < 0.059$), but can discriminate mild activity from moderate activity index ($P < 0.005$), and moderate activity index from high endoscopic activity index ($P < 0.001$).

The CRP and blood leucocytes in this study can't discriminate inactive endoscopic activity index from mild endoscopic activity index, can't discriminate mild activity index from moderate activity index, but can discriminate between moderate activity index from the severe one ($p < 0.001$) as shown in table 4.

The relationship between the different clinical activity index subgroups with their corresponding fecal Lactoferrin, fecal Calprotectin, TGF-B1, and endoscopic activity revealed that, the mean fecal Lactoferrin, fecal Calprotectin, TGF-B1, endoscopic activity index, sedimentation rate, blood leukocytes and CRP differed significantly between inactive from mild clinical activity index, mild from moderate clinical activity index and moderate from high clinical activity index (for all $P < 0.001$)as shown in table 5.

Table 5: Relationship between Clinical Activity Index with ESR, blood leukocytes, CRP, TGF-B1, Fecal lactoferrin, fecal calprotectin, and Endoscopic Activity Index.

Clinical Activity Index		Remission	Mild	Moderate	Severe	F	p. value
ESR	Range	5-15	10-46	10-55	12-105	3.575	0.001
	Mean±SD	11.4±2.9	27.4±9.6	38.7±11.4	79.2±20.2		
Blood leukocytes	Range	4.5-9	4-13	4.7-15	4.8-22	3.467	0.001
	Mean±SD	6.6±1.3	10.6±2.1	12.3±1.5	16.4±3.7		
CRP	Range	3-12	1-16	6-36	6-52	3.989	0.001
	Mean±SD	5.8±2.2	9.9±3.9	22.5±10.03	41.04±10.6		
TGF-B1	Range	6-90	7-430	7-780	6-800	4.885	0.001
	Mean±SD	46.1±33.2	306.1±99.5	404.7±108.5	386.9±246.7		
Fecal lactoferrin	Range	0.55-150	13-1000	30-2000	4.9-2080	8.327	0.001
	Mean±SD	39.9±37.2	324.7±251.8	834.6±504.8	1637.3±419.9		
Fecal calprotectin	Range	9-140	10-1150	20-1500	30-1500	8.221	0.001
	Mean±SD	89.7±43.5	627.8±383.3	1155.4±393.1	1416.3±296.6		
Endoscopic Activity Index	Range	0-7	1-12	3-12	10-12	8.057	0.001
	Mean±SD	2.7±2.1	7.2±2.6	10.2-1.9	12.9±0.4		

The Endoscopic Activity Index (EAI) correlated significantly with the levels of fecal Lactoferrin, (Spearman's rank correlation coefficient r = 0.949), fecal Calprotectin (r = 0.923) TGFB1 (r =0.918), the Clinical Activity Index (r= 0.761), CRP (r= 0.851), and blood leukocytes (r= 0.681). For all items $P < 0.001$ was found (Table 6) and figures (1, 2).

Table 6: Correlation between Endoscopic Activity Index with Fecal Lactoferrin, Fecal Calprotectin, TGF-B1, and CRP

Endoscopic Activity Index	r	P value	Significance
Fecal Lactoferrin	0.949	0.001	HS
Fecal Calprotectin	0.923	0.001	HS
TGF-B1	0.918	0.001	HS
CRP	0.851	0.001	HS
Clinical Activity Index	0.761	0.001	HS
Blood Leukocytes	0.681	0.001	HS

From the histopathologic aspect, 20 patients (16.7 %) were normal, 35 were mild (29.2 %), 40 were moderate (33.3 %) and 12 patients were severe (20.8 %). There were a significant correlation between the results of histopathology with fecal Lactoferrin (r = 0.847), fecal Calprotectin (r= 0.845), TGF-B1 (r = 0.770), Endoscopic Activity Index (r = 0.678), Clinical Activity Index (r = 0.695), CRP (r = 0.798), and blood leukocytes (0.589). For all the P value was < 0.001 as shown in Table 7.

Table 7: Correlation of Histopathology with Fecal calprotectin, TGF-B1, Endoscopic Activity Index, Clinical Activity Index, CRP, and blood leukocytes.

Histopathology	Spearman's Rank Correlation Coefficient (r)	No.	Significance
Fecal Lactoferrin	0.847	120	0.001
Fecal Calprotectin	0.845	120	0.001
TGF-B1	0.770	120	0.001
Endoscopic Activity Index	0.678	120	0.001
Clinical Activity Index	0.695	120	0.001
CRP	0.798	120	0.001
Blood leukocytes	0.589	120	0.001

The correspondence between the results of histopathological examination and the classification based on the parameter cut-offs was analyzed for each parameter and was expressed as the percentage of the samples that were correspondingly identified (specificity and sensitivity). The specificity was highest for fecal Lactoferrin and fecal Calprotectin and lowest for Clinical Activity Index. The specificity rates for fecal Lactoferrin, fecal Calprotectin, TGF-B1, Endoscopic Activity Index, Clinical Activity Index, CRP, and blood leukocytes were 85 %, 83 %, 66 %, 64 %, 50 %, 60 % and 63 % respectively. The sensitivity for both fecal Lactoferrin and fecal Calprotectin was relatively high, but was relatively low for blood leucocytes.

The sensitivity rates for fecal Lactoferrin, fecal Calprotectin, TGF-B1, Endoscopic Activity Index, Clinical Activity Index, CRP, and blood leucocytes were 97.8 %, 97.5 %, 96.5 %, 93 %, 89%, 64.5 % and 66% respectively as shown in Table 8.

Table 8: Sensitivity, Specificity. Positive predictive value (PPV), Negative predictive value (NPV), and the Overall Accuracy of Fecal Lactoferrin, Fecal calprotectin, TGF-B1, Clinical Activity Index, Endoscopic Activity Index, CRP, and blood leucocytes in predicting Histopathologically Active Disease.

Marker	Cut-off	Sensitivity	Specificity	PPV	NPV	Accuracy
Fecal Lactoferrin	≥13 µg/g	97.8 %	85 %	98.9%	72 %	96.9 %
Fecal Calprotectin	≥50 µg/g	97.5 %	83 %	98.6 %	71 %	96.6 %
TGF-B1	≥ 7pg/mL	96.5 %	66%	97.6 %	57 %	94.5 %
Endoscopic Activity Index	≥ 3	93 %	64 %	96 %	33 %	90 %
Clinical Activity Index	≥ 5	89 %	50 %	96 %	25 %	87 %
Blood leukocytes	≥ 8 gm/L	64.5 %	60	76.9 %	45 %	65 %
CRP	≥ 6mg/L	66 %	63	78.8 %	48 %	65 %

Discussion

The clinical course of ulcerative colitis is quite variable and characterized by episodes of relapse and remission. One of the most prominent histological features observed in ulcerative colitis is infiltration of the neutrophils into the inflammed mucosa at an early stage of inflammation. The neutrophils are major sources of inflammatory cytokines, chemokines, proteases, and reactive oxygen derivatives, as well as a full complement of factors needed to exacerbate mucosal inflammation and tissue injury. Active inflammation in ulcerative colitis is associated with an acute phase reaction and migration of leucocytes to the gut.

Thus various proteins can be measured in serum and feces (11).

Several laboratory markers have evolved in the diagnosis and follow-up of ulcerative colitis patients. These include blood leucocyte counts, ESR, and C- reactive protein. Fecal biomarkers have emerged as important tools to assess intestinal inflammation, whether due to inflammatory infections, such as shigellosis or *C. difficile* colitis, or to inflammatory bowel disease (IBD), be it ulcerative colitis or Crohn's disease (11,13, 32,3). As relatively specific biomarkers of neutrophilic inflammation in the intestinal mucosal (34), these tests have the advantages of being noninvasive, rapid, simple and relatively inexpensive (16). Several clinical studies have shown the usefulness of fecal biomarkers of inflammation in the diagnosis or in the tracking of disease activity; these include the stool measurement of sensitive biomarkers that include such neutrophil-granular proteins as lactoferrin (LF) and calprotectin (FC) (10).

Lactoferrin (LF) is a component of the innate immune system, with antimicrobial activity as a bactericide and fungicide, as well as being a major constituent of neutrophil granules that is released during apoptosis (35). During intestinal inflammation polymorphonuclear neutrophils infiltrate the mucosa, increasing LF concentration in feces proportional to neutrophil translocation to the GI tract (36). Studies investigating whether fecal lactoferrin can be used as a noninvasive marker to distinguish IBD from non inflammatory conditions, especially irritable bowel syndrome (IBS), have yielded variable results (9,10, 37)

Out of numerous neutrophil derived proteins present in stools, calprotectin a calcium- and zinc-binding protein that inhibits metalloproteinase is probably the most promising. It is the major protein found in monocytes, macrophages and constitutes 50-60% of neutrophil cytosolic proteins (38,39). several studies have showed high fecal calprotectin levels are directly correlated with the quantification of the neutrophilic infiltrate in the gut mucosa as an indicator of infectious and inflammatory conditions (14, 40).

Transforming growth factor-B1 plays an important role in the pathophysiology of inflammatory bowel disease (IBD). In inflammatory bowel disease (IBD), TFG-B1 is produced and secreted from the cells in the lamina propria and the epithelium of the small bowel and colon. TGF-B1 controls proliferation and differentiation of intact epithelial cells, and plays a role in wound healing and increase in fibrosis during inflammation. It enhances the production of extracellular matrix by intestinal cells and fibroblast-mediated contraction of the collagen matrix, and also regulates the function of leucocytes and epithelial cells as well as their products (29,30). TGF-B1 may be used as a sensitive marker of ulcerative colitis activity and also can be used as a marker in differentiating inactive from active ulcerative colitis (31, 41).

In this study, we try to evaluate any relationship that might exist between the mucosal neutrophil infiltration represented by Lactoferrin, calprotectin, TGF-B1, CRP, sedimentation rate, and the ulcerative colitis disease activity represented by Rachmilewitz criteria (8).

From this study, fecal Lactoferrin, calprotectin and serum TGF-B1 correlate very closely with endoscopic disease activity, they were the only three markers that could discriminate inactive from mild, moderate, and severe active disease.

Our results showed that fecal Lactoferrin, fecal calprotectin and transforming growth factor-b1 concentrations were significantly higher in patients than controls ($P < 0.001$). Also, fecal Lactoferrin and fecal calprotectin in this study were significantly differentiate inactive from mild, moderate, and high active disease (21.22, 42),but the serum TGF-B1 can't differentiate inactive from mild activity disease, but significantly differentiate mild, moderate and high active disease (29, 41). Our results correlate with Mahmoud et al. 2015, who founded that fecal lactoferrin and fecal calprotectin concentrations were significantly higher in patients with active ulcerative colitis than in patients with inactive ulcerative colitis and had a better correlation with disease activity index than the CRP, ESR concentrations (10).

Alian et al. 2009, Iman et al. 2009, Hassan et al. 2013, Langhorest et al.2016, founded that active inflammatory bowel disease patients had statistically significant elevation in fecal lactoferrin and fecal calprotectin than patients with inactive disease (7, 9, 24, and 42). Langhorest et al stated that the assessment of activity in UC can be performed on different levels such as clinical activity, biochemical activity by measuring blood or fecal biomarkers, endoscopy, and histology. Clinical remission in ulcerative colitis does not necessarily imply biochemical, endoscopic, or histological remission. Non-invasive fecal biomarkers like fecal lactoferrin and calprotectin, are highly sensitive to mucosal level and have the potential to significantly add to our understanding of active inflammation in every day patient care (42). Xiang et al concluded that fecal lactoferrin appears to have good diagnostic precision in distinguishing IBD from IBS both in adults and children with fecal lactoferrin being a more sensitive assay in patients with active than inactive IBD (37). Iman et al stated that fecal calprotectin was significantly elevated in inflammatory bowel disease cases in comparison to controls and is a good marker in differentiating Egyptian patients with ulcerative colitis from healthy controls (9).

The fecal lactoferrin level has been shown to be a stable and accurate biomarker for the leucocyte degranulation seen in cases of intestinal inflammation. In particular, the fecal lactoferrin level has been proved to be a useful tool for diagnosing IBD (37) and differentiating between active and inactive disease (42, 43). Furthermore, the fecal lactoferrin level has been found to correlate well with the endoscopic severity of colonic IBD (16). Sagawa et al. stated that fecal lactoferrin level is a useful biomarker of the mucosal findings in ulcerative colitis. Although endoscopy is the gold standard, the faecal lactoferrin level can be used as a biomarker in differentiating patients with inactive and active ulcerative colitis from healthy controls (43). During intestinal inflammation neutrophils infiltrate the mucosa and markedly increase lactoferrin levels that can be readily measured in feces or gut lavage fluid (15, 16). Studies evaluating lactoferrin in the diagnosis of IBD show that it exhibited similar performance to fecal calprotectin and correlated better than C-reactive protein with mucosal inflammation by endoscopy (17, 18, and 19). Joishy *et al.* (15) also found that fecal lactoferrin correlated with disease activity indices and erythrocyte sedimentation rate in pediatric patients with IBD. Mara et al stated that lactoferrin (a secondary granule marker) and calprotectin (a neutrophil cytoplasmic marker) can provide simple, noninvasive assessments of intestinal inflammation that correlate closely with each other, do not require protein normalization and can differentiate patients with ulcerative colitis from healthy controls (44). Alian et al. found that fecal calprotectin concentrations were significantly elevated in ulcerative colitis patients than controls and can discriminate inactive from mild, moderate, and high active disease (7).

As regard to serum transforming growth factor-B1 (TGF-B1) in our results, it might be considered as a sensitive marker of ulcerative colitis activity and there was a significant elevation of TGF-B1 concentrations in ulcerative colitis patients than controls. It can also be used for evaluation of inflammatory activity in ulcerative colitis and can discriminate mild from moderate, and high disease activity.

Indeed, Irena et al. and Kilic et al. noted that in ulcerative colitis, the mean level of TGF-B1 in active disease was higher than in remission and can be used as a marker for differential diagnosis of these stages (41, 45). In a few studies TGF-B1 was measured in bowel tissue by an immunohistochemical method. Kanazawa et al. studied the expression of TGF-B1 in paraffin-embedded samples from bowel tissue and the concentration in blood, basic fibroblast growth factor (b-FGF), endothelin-1 (ET-1), and vascular endothelial growth factor (VEGF). They examined 11 patients with ulcerative colitis, 11 patients with crohn's disease, and 10 healthy controls. Expression of TGF-B1 in the endothelial cells was not found in either the ulcerative colitis or the crohn's disease group. They noted moderate or weak expression of TGF-B2 and TGF-B3 in the inflammatory cells in 5 cases of active ulcerative colitis and in 4 cases of active crohn's disease (46). Some studies were conducted in pediatric patients (65 children suffering from crohn's disease and 23 patients from ulcerative colitis. They found that TGF-B1 was significantly higher in patients with crohn's disease in remission than in active disease (47, 48, and 49). In another study, Wedrychowicz et al. assessed the influence of exclusive enteral nutrition on serum concentration of TGF-B1 and vascular endothelial growth factor (VEGF) in 24 patients with crohn's disease and 15 patients with ulcerative colitis; they found increased serum TGF-B1 in ulcerative colitis patients versus the crohn's disease group and controls (50).

In this study, there was a good correlation between the Rachmilewitz Clinical Activity Index (CAI) and the endoscopic activity index (EAI) and the results correlate with the results done by Alian and his collageous (7). Other studies (1, 50), graded ulcerative colitis patients according to the Rachmilewitz Clinical Activity Index only and the Endoscopic Activity Index did not follow this score.

Focusing on the evaluation of the relationship that might exist between the mucosal neutrophil infiltration represented by fecal lactoferrin, fecal calprotectin, TGF-B1, CRP, blood leukocytes, and the Rachmilewitz activity indices, the present study revealed that fecal lactoferrin and fecal calprotectin correlated significantly with the TGF-B1, blood leucocytes, CRP, ESR, Rachmilewitz Activity Indices, but not correlated with the platelet count and sedimentation rate. Similar findings were found in various studies. Langhorst et al and Alian et al. found a good correlation between the concentrations of fecal lactoferrin, fecal calprotectin, Rachmilewitz Activity Indices, CRP, and blood leukocytes in ulcerative colitis patients (7, 42). Also, Xiang et al and Mahmoud et al. founded a good correlation between fecal calprotectin, ESR, CRP, and ulcerative colitis activity index in ulcerative colitis patients (8, 10). Kilic et al. found a significant correlation between TGF-B1 levels and CRP, whereas no significant correlation was established between the other parameters (blood leucocytes, ESR, fibrinogen level, and platelet count) (45).On the other hand, Irena et al. found a good correlation between TGF-B1 and the concentrations of CRP and platelet count and can be used for evaluation of inflammatory activity in ulcerative colitis and to a lesser extent can also be used for evaluation of inflammatory activity in crohn's disease (41).

In our results, ulcerative colitis has a weak CRP response. Our explanation is that, in ulcerative colitis the inflammation is confined to the mucosa, and also polymorphisms in the CRP gene are responsible for interindividual differences in CRP production in humans (51). Thus, CRP doesn't seem to be an adequate biomarker for the assessment of endoscopic disease activity in ulcerative colitis.

Fecal lactoferrin and fecal calprotectin predict the severity of colorectal inflammation with increased concentrations strongly associated with advanced histological grades of colorectal inflammation (19, 22, 26. 33, 37). In our study, there was a significant correlation between fecal lactoferrin, fecal calprotectin concentrations and the results of histopathological examinations ($P < 0.001$). Similar results were found in a study done by Xiang et al and Hassan et al. they stated that fecal lactoferrin and fecal calprotectin concentrations correlated more closely to histologic than to macroscopic colonic inflammation. This suggests that fecal lactoferrin and fecal calprotectin

concentrations may show early inflammation that is not detectable macroscopically during colonoscopy (24, 37).

As regard to histopathology that is considered as the gold standard test for diagnosis of ulcerative colitis, the test performance (given by sensitivity / specificity / positive predictive value / negative predictive value and accuracy in percent) of fecal lactoferrin, fecal calprotectin, TGF-B1, Endoscopic Activity Index, Clinical Activity Index, CRP, and blood leucocytes in predicting the positivity and negativity of ulcerative colitis, the fecal lactoferrin with a cut-off ≥ 13 µg/g had the best overall accuracy (96.9 %) followed by fecal calprotectin with a cut-off ≥ 50 µg / g (96.6 %), followed by TGF-B1 with a cut-off ≥ 7pg/ mL (94.5 %), then Endoscopic activity index (90 %), Clinical Activity Index (87 %), and lastly CRP and blood leucocytes (for both 65 %) for detection of active disease. These results agreed with more results done by other researchers (7, 8, 10, 29, 37, and 41).

From the present study, we concluded that: Fecal lactoferrin and fecal calprotectin were the only two markers that could reliably discriminate inactive from active ulcerative colitis and have the potential to replace endoscopy in disease monitoring and are considered as an objective advances to grading the mucosal disease activity in patients with ulcerative colitis. The usefulness of these fecal biomarkers as screening tests may be helpful in the selection of cases for endoscopic examination. The advantages of both fecal lactoferrin and fecal calprotectin are the simplicity, non-invasiveness, and relatively low cost. Although inferior to Lactoferrin and calprotectin measurements, the Rachmilewitz Clinical Activity Index had good correlation with endoscopic disease activity. Transforming growth factor-B1 can be used in the early diagnosis of ulcerative colitis exacerbation. It can be used for evaluation of inflammation activity in ulcerative colitis and correlated with elevated concentrations of fecal lactoferrin, fecal calprotectin, Endoscopic Activity Index, blood leucocytes, CRP, Clinical Activity Index, and histopathology. Thus, TGF-B1 can be used as a marker for differentiating mild active from moderate and high active ulcerative colitis patients. Further studies are needed for determining the value of fecal lactoferrin, fecal calprotectin and TGF-B1 in other organic diseases, their guidance for choosing the best modality of treatment and their use as biomarkers of remission and success of management. Nevertheless, more studies with larger patient groups are necessary.

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