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Original Article

Alteration of the Inflammatory and Anti-Inflammatory Cytokine Profiles of Peripheral Blood Mononuclear Cell in Crohn's Disease Patients after Following up

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Abstract

Background: Crohn's disease (CD) has a chronic course, which its recurrence varies widely among different patients. In this study we prospectively analyzed blood samples of 19 CD patients. Alteration in transcription of inflammatory and anti-inflammatory cytokines was analyzed compared with household members after three month follow up.

Methods: CD patients were diagnosed based on clinical symptoms, endoscopic and histopathologic characteristics. Nineteen CD patients and their households were evaluated from Jun 2019 to Feb 2021 at Tehran university hospitals. CD activity score, biological, clinical and demographic data of the patients were recorded at two time point intervals. Bacteriological tests were done using aerobic and anaerobic blood cultures. To investigate transcriptional alterations, peripheral blood mononuclear cells (PBMCs) were isolated using Ficol centrifugation method and relative quantitative real-time PCR was done to determine the expression level of IFN-γ, TNF-α, IL10, and FOXP3 cytokines.

Results: Our results showed a correlation between fecal calprotectin level (709.8 ± 554.6), C-reactive protein concentration (18.1 ± 15.9), and erythrocyte sedimentation rate (30.4 ± 17.9) with disease activity (Flare/remission). IL10 and Foxp3 anti-inflammatory gene's expression were significantly (P = 0.003 for IL10 and P = 0.008 Foxp3) higher during the flare and remission in patients with active disease respectively. Bacteriological examination showed infection with *Streptococcus* spp. and *Clostridium* spp. in two CD patients during flares, which was correlated with upregulation and down-regulation of IL10, TNF-α, IFN-γ and FOXP3 proteins, respectively.

Conclusion: Occurrence of bacteremia, and higher amount of CAP, CRP and ESR are correlated with higher level of transcription for inflammatory cytokines, which could effectively reflect the disease activity. Raise in FoxP3 transcription proposed change in Treg sub-population in PBMC or its activity during the CD remission phase.

Keywords: Crohn's disease; Inflammation; Cytokines; Regulatory T cell; Blood culture

Introduction

Crohn's disease (CD) is an inflammatory disorder of the gastrointestinal tract characterized by remission and relapses with symptoms of bloody diarrhea, abdominal pain and bleeding (1, 2). The causes of CD are not clear yet, but it is linked to immunological, genetic and environmental factors (3). While it is generally accepted that CD is resulted from an altered immune response to the gut microbiota, our knowledge about this contribution is poor (4).

Current data show divergent cytokine patterns among the CD and healthy controls and also between different phases of the disease (5). The imbalance of T helper (Th)1/Th2 subsets and also induction of Th17 cytokines has been implicated in the pathogenesis of CD (6). Microbes and their metabolites can induce high levels of antibody and T cell response in the intestinal tract leading to the secretion of cytokines, such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ, which is correlated with the disease development and exacerbation (7).

Although dysregulation of immune responses in CD has been studied extensively over recent years, data about involvement of microbes in this interplay remains controversial (4). Microbial translocation and shedding of their metabolites into blood is associated with chronic inflammation (8). Interaction of these microbes/metabolites with circulating monocytes and macrophages in the bloodstream could prime them to differentiate into tissue-specific macrophages and dendritic cells.

In healthy people, it is well known that the intestinal mucosal macrophages are involved in tolerance to commensal microbiota that exerts through their anti-inflammatory and anergic state; however, in CD patients it seems that transfer of bacterial components, such as peptidoglycan and endotoxins, from the intestine to the bloodstream can disturb natural maturation cycle of progenitor monocytes leading to the generation immature macrophages (9, 10).

Although understanding exact mechanisms involving the CD immunopathogenesis remain to be elucidated, current study was done to show transcriptional alteration in peripheral blood mononuclear cell (PBMC) during different phases of the disease. Accordingly, transcriptional profile of inflammatory and anti-inflammatory markers (IFN-γ, TNF-α, IL10, and FOXP3 cytokines) was measured in peripheral blood monocytes and macrophages during the flare and remission phases in CD patients in compare to their households. Disease activity index, biological markers of inflammation, and bacteremia were assessed concurrently to show possible links for blood-intestine interaction in these patients.

Materials and Methods

Study design and patients

To compare alterations in the extent of the transcription of common inflammatory and non-inflammatory genes in PBMCs, confirmed Iranian 18-40 years old cases of CD patients based on clinical symptoms, endoscopic and histopathologic characteristics were recruited in the present study from Jun 2019 to Feb 2021 and followed during three months between flare up and remission stages at Tehran university hospitals. Healthy households of the CD patients were considered as control group (11). The disease severity was estimated according to the CD Activity Index (CDAI) defined by Best et al. (12). Based on the CDAI scores, patients were divided in to groups including inactive CD (CDAI < 170 points) and active CD (CDAI > 170 points) (13). Laboratory tests (WBC (white blood cell), CAP (calprotectin), CRP (C reactive protein), ESR (erythrocyte sedimentation rate), Alb (albumin), Hem (hemoglobin) and Hct (hematocrit)) and pathological information were recorded for all patients. Individuals with history of systemic antibiotic administration at least during last three months, hospitalization in last

four weeks, pregnant patients, or those who exposed to corticosteroids or anti-inflammatory drugs were excluded.

All patients and healthy controls signed an informed consent form before their inclusion in the investigation. This study was approved by the Ethics Committee at Tehran University of Medical Sciences (IR.TUMS.SPH.REC.1398.060).

Sampling and microbiological analyses

Blood sampling was done two times from all the participants during a three months period. A volume of 20 mL heparinized blood samples was collected, transferred to laboratory immediately after phlebotomy, and divided into two RNase/DNase free tubes to perform blood culture, PBMCs isolation and RNA extraction. Each thioglycolate and tryptic soy broth (TSB) mediums were inoculated by 5 mL of the blood samples and the bottles were placed in the 37 °C incubator under anaerobic and aerobic conditions, respectively. Growth of bacteria in both of the inoculated bottles were followed up to 14 days. Bacterial characterization was done by Gram-staining (directly on blood smears and grown colonies on blood agar and Columbia agar media), and biochemical tests (14).

Isolation of PBMCs

PBMCs were isolated from heparinized peripheral blood by Ficol (Lymphodex Inno -Train, Germany) gradient centrifugation (15). In brief, heparinized blood was diluted with an equal volume of phosphate-buffered saline (PBS), pH 7.4 and 7 mL of diluted blood was layered over 3 mL of the Ficol and centrifuged at 400 × g for 30 min at room temperature. The PBMCs was carefully removed by pipetting and washed with PBS by centrifugation at 250 × g for 5 min. The cells were resuspended in PBS and counted for cell concentration.

RNA extraction and cDNA synthesis

Total RNA was extracted from fresh PBMCs according to the RNX-Plus protocol (SinaClon, Tehran, Iran). The quantity and quality of extracted RNA was assessed using nano drop (Nano Drop™ One Microvolume UV-Vis Spectrophotometers) and gel electrophoresis, respectively. The adjusted concentration of RNA samples treated with DNase I (Thermo Fisher Scientific, Waltham, Massachusetts, United States) was used for cDNA synthesis using thermo scientific revert aid first strand cDNA synthesis kit (Thermo Fisher Scientific, USA) in a reaction primed by a random hexamer according to the manufacture's instruction.

Relative quantitative real-time PCR

To do relative quantitative real-time PCR, reaction mixture containing 3 µL of template cDNA, 1 µL (10 pM) of forward and reverse primers, 10 ul of SYBR green master mix (Amplicon, Brighton, UK), and 10 µL of sterile distilled water was provided. The StepOnePlusTM real-time PCR System (Applied Biosystems, Foster City, CA, USA) was used to analyze relative difference in transcription of IFN-γ, TNF-α, IL10 and FOXP3 genes (Table 1). Efficacy of the primers and melting curve analyses were done to ensure specific amplification. All the experiments were done in duplicate for all the samples tested. Data analysis was carried out using the $2^{-\Delta\Delta CT}$ method to evaluate expression level of target genes by normalization to the *ActB* housekeeping gene. RNA extract of Caco-2 intestinal epithelial cell line (Pasteur Institute of Iran, Iran) was used for optimization of the real time PCR conditions.

Statistical Analysis

Statistical analyses were performed using SPSS version 26 and GraphPad Prism7 software. Correlation between the relative expression values of genes in CD patients and controls was analyzed by Spearman correlation nonparametric test. Gene expression differences during phase 1 and phase 2 of active and inactive patients was analyzed using Wilcoxon test and Kruskal-Wallis test was use to analyze clinical and laboratory findings among the active and inactive groups. A *P*-value ≤0.05 was considered statistically significant. ClustVis tool was used for principal component analysis and heatmap drawing to show correlation between the disease stages, transcription levels, clinical and laboratory findings.

Table 1: The sequences of primers used in relative quantitative real-time PCR for IFN-γ, TNF-α, IL10, FOXP3, and ActB genes

Gene		*Oligonucleotide sequence (5' to 3')*	*Tm (°C)*	*Amplicon size (bp)*
IFN-γ	F	GACTATGCGATGAGCGTGAT	58.4	109
	R	CCGTCGTAGTTAGGGATGAA	58.4	
TNF-α	F	TGGCGCGATCACGTCAACA	59.5	91
	R	TGCACCGCATCCTCCAATC	59.5	
IL10	F	GGAATATTGCACAATGGGCGC	61.2	112
	R	CACAAGGGAACGCCTATCTC	60.5	
FOXP3	F	GAAACAGCACATTCCCAGAGTTC	62.9	100
	R	ATGGCCCAGCGGATGAG	57.3	
actB	F	ATGTGGCCGAGGACTTTGATT	59.5	111
	R	AGTGGGGTGGCTTTTAGGATG	61.2	

TM; Annealing temperature, bp; Base pair, F; Forward, R; Reverse

Results

Activity index and laboratory/clinical findings

In this study, out of 38 recruited patients with IBD, 19 patients (18-40 years old) were selected based on the inclusion and exclusion criteria. Seven (36.8%) and 12 (63.2%) patients were men and women, respectively. In the control group, 10 men (52.6%) and nine women (47.4%) were included (18-65 years old). Based on the activity index scores, all patients were divided into two groups, including eight inactive (CDAI < 170) and 11 active (CDAI > 170) CD patients (Table 2). Erythema, colon involvement, mucosal exudates and ulcer were the most common features found in 18, 17, 16 and 13 patients, respectively. Furthermore, abscess (three patients), granulomatous colitis (two patients), colitis (two patients) and hyperplasia (one patients) were as the other pathological features detected. The BMI and CAP values, and laboratory test results including WBC, CRP, ESR, Alb, Hem and Hct, are shown in Table 2. Differences of all these tests between inactive and active patients were statistically significant (*P* values are shown in Table 2) except for BMI, WBC and Alb.

Table 2: The mean CDAI scores, BMI, CAP values and laboratory tests of 19 Crohn's disease patients.

Tests	*Disease stage (No. of patients)*		*P value*
	Inactive (8) (mean ± SD)	Active (11) (mean ± SD)	
CDAI	158.1 ± 10.5	243.5 ± 37.9	0.00
BMI	23.7 ± 4.6	22.1 ± 4.6	0.34
CRP	6.0 ± 6.8	18.1 ± 15.9	0.05
CAP	132.3 ± 148.5	709.8 ± 554.6	0.01
ESR	9.9 ± 9.4	30.4 ± 17.9	0.01
Hem	13.8 ± 1.1	11.7 ± 1.5	0.01
Hct	41.5 ± 3.7	35.4 ± 4.5	0.01
WBC	8028.8 ± 2165.5	7218.2 ± 1637.6	0.36
Alb	3.7 ± 0.4	3.7 ± 0.9	0.46

Values are mean ± standard deviations (SD). $P < 0.05$ means statistically significant differences. CDAI; Crohn's Disease Activity Index, BMI; Body Mass Index, CRP; C Reactive Protein, CAP; Calprotectin, ESR; Erythrocyte Sedimentation Rate, Hem; Hemoglobin, Hct; Hematocrit, WBC; White Blood Cell, Alb; Albumin

Bacterial isolates

Totally, two blood samples (2/19) of CD patients were positive for general cultivable aerobic and anaerobic bacteria. *Clostridium* spp. was isolated from a female patient with the clinical findings including, ulcer, erythema, colon involvement and mucosal exudates. *Streptococcus* spp. was isolated from blood of a male with ulcer, colitis, colon involvement and mucosal exudates. Both the patients were in flare period with CDAI of 297 and 265 scores, respectively. There were no positive cultures among the control group.

Alteration in transcription of inflammatory and non-inflammatory genes

Alteration in expression levels of IFN-γ, TNF-α, IL10, and FOXP3 genes in PBMC of CD patients compared with related samples from their households as reference, was measured during three months period (Fig. 1).

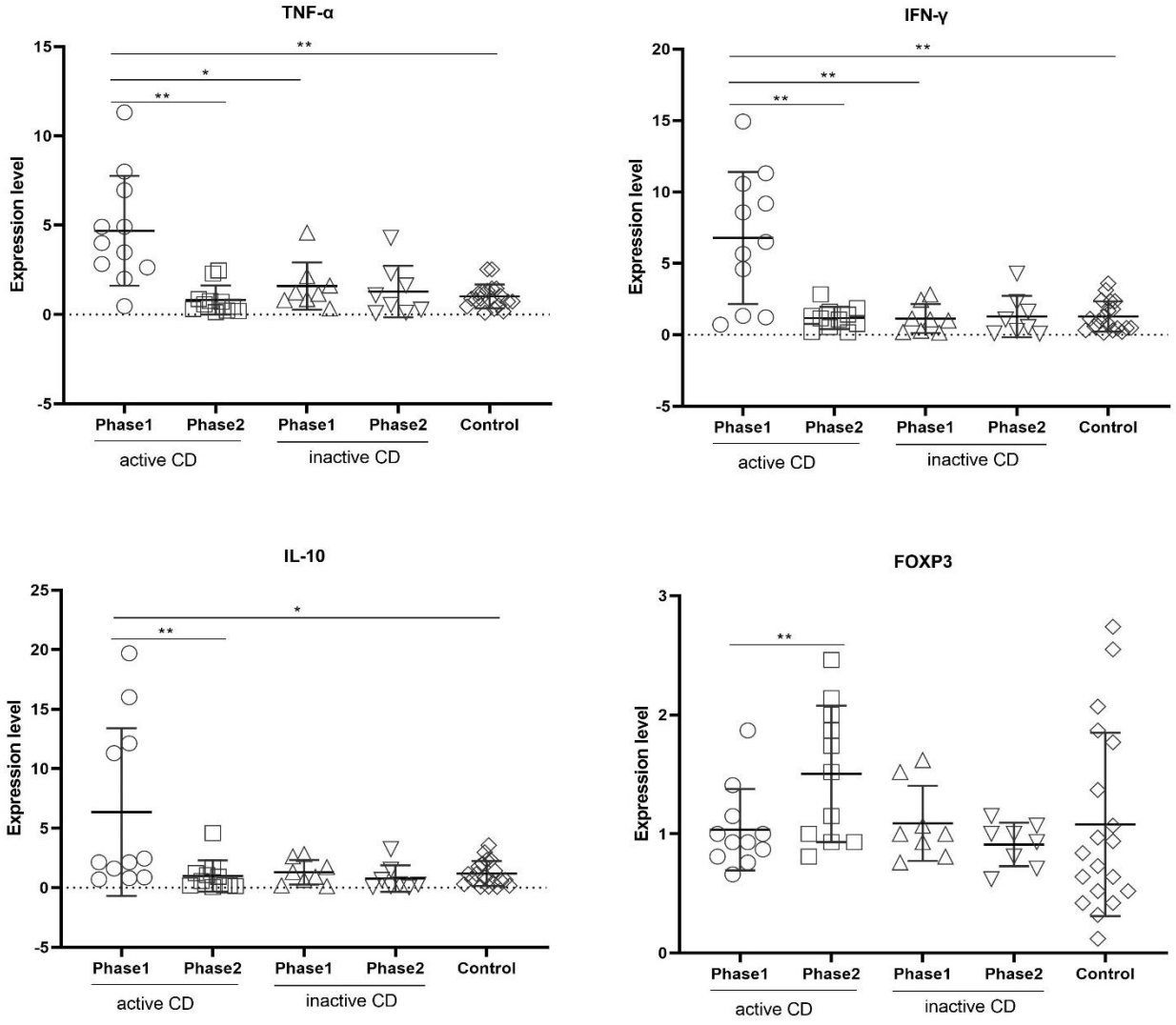

Fig. 1: Expression level of IL-10, TNF-α, IFN-ϒ and FOXP3 genes in Crohn's disease patients and healthy controls. *; *P* value < 0.05, **; *P* value < 0.01

Accordingly, overexpression of IL10, TNF-α and IFN-γ genes was orderly detected in eight, 10 and 11 patients with active disease during phase 1, which declined significantly three months post medication (*P* values= 0.01, 0.00 and 0.003) respectively (Table 3 and Fig. 1). No significant change in the transcription of IL10, TNF-α, IFN-γ and FOXP3 genes was detected in patients with inactive CD during phase 1 and patients with active and inactive CD during the second phase of sampling. Foxp3 showed expression level equal to the control group during the first and second phases of the follow up except for remission phase of active CD patients ($P < 0.01$). A correlation was detected between expression level of IL10/TNF-α and IFN-γ that was statistically significant ($P < 0.01$, Fig. 2).

Table 3: Gene expression level of 19 Crohn's disease patients during flare/remission

Tests		***Disease stage (No. of patients)***		***P value***
		Inactive (8) (mean ± SD)	Active (11) (mean ± SD)	
IL10	Phase 1	1.31 ± 1.03	6.36 ± 7.03	0.003
	Phase 2	0.78 ± 1.1	0.99 ± 1.32	0.2
TNF-α	Phase 1	1.59 ± 1.33	4.69 ± 3.08	0.004
	Phase 2	0.95 ± 0.82	0.81 ± 0.81	0.5
IFN-ϒ	Phase 1	1.14 ± 1.01	6.78 ± 4.62	0.003
	Phase 2	1.28 ± 1.45	1.18 ± 0.78	0.8
Foxp3	Phase 1	1.09 ± 0.32	1.04 ± 0.34	0.1
	Phase 2	0.91 ± 0.18	1.51 ± 0.57	0.008

Values are means ± standard deviations (SD). Data represent units obtained with the RT-qPCR normalized to *actB*. $P < 0.05$ means statistically significant differences

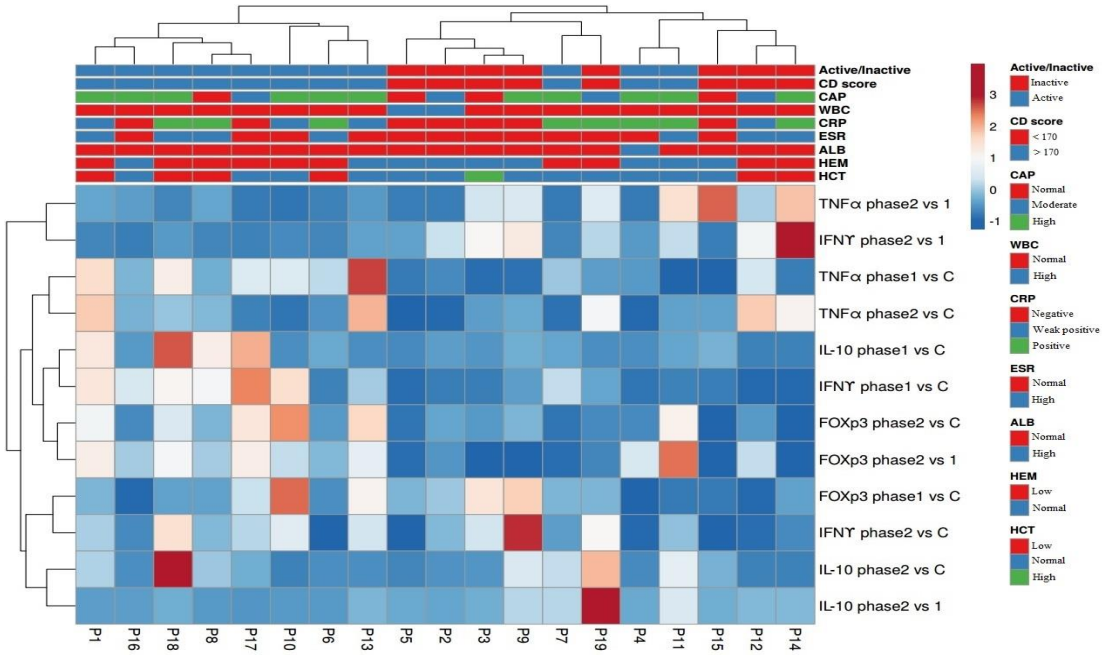

Fig. 2: Heatmap illustration of correlation between the gene expression levels with disease stages, clinical and laboratory findings among the 19 Crohn's disease patients. Columns were labeled with P1-P19 showing 19 patients. 12 bottom rows show the expression level of all IL10, TNF-α, IFN-γ and FOXP3 genes during phase 1 and 2 when compared to the controls and each other. Nine top rows demonstrate the clinical and laboratory findings. CD scores; Crohn's Disease Activity Index, CAP; Calprotectin, WBC; White Blood Cell, CRP; C Reactive Protein, ESR; Erythrocyte Sedimentation Rate, Alb; Albumin, Hem; Hemoglobin, Hct; Hematocrit

Correlation of transcription level of all genes with the disease stages, clinical and laboratory findings are shown in Figure 2. Eight out of 11 active patients were classified in a same cluster and the remained three active patients were placed in two different clusters among inactive patients based on the lower expression levels of the studied genes. The alteration detected in gene expression level was positively correlated with disease stage, CAP, CRP, ESR levels; however, a negative correlation was detected in relation to Hem and Hct values.

Discussion

This study demonstrates that increased CAP, CRP and ESR better correlates with the Crohn's disease activity than blood leukocytes (WBC) and albumin. In accordance to our results, discrimination of disease severity using fecal calprotectin have already shown in several studies (16, 17). However, CRP and ESR were also significantly increased in active patients, but the role of CRP was not very noticeable to reflect disease stage as reported by Langhorst et al. (17). In addition, hemoglobin and hematocrit were negatively correlated with disease severity in our patients. This could explained by the bloody diarrhea and bleeding that were known as symptoms characterize the Crohn's disease (2). Dysbiosis of the intestinal microbiota is considered as an important risk factor in CD onset through a dysregulated mucosal immune response (18). Different aerobic and anaerobic bacterial species are reported in association to the CD occurrence in IBD patients (19, 20). In current study, *Clostridium* and *Streptococci* genera were the only two bacterial isolates characterized in blood culture of two patients in active phase of the disease. These patients showed increased expression of IFN-γ, TNF-α and IL10 genes in the early stage (> 2 folds) and this expression was greater than culture negative patients except in two of them for IFN-γ and IL10 genes and one patient for TNF-α. However, values of other markers such as CRP, ESR and CAP were not significantly higher than those with negative blood culture. Although the transcription of IFN-γ and IL10 remained high during the remission in patient with *Clostridium* spp. infection, reduction of TNF-α transcription was seen after medication during the remission. As for the *Streptococcus* spp. positive patient, the only gene that remained overexpressed during remission was *TNF-a* (> 2 folds). As *Clostridium* spp. and *Streptococcus* spp., it previously demonstrated that Gram-positive bacteria could able to trigger monocytes and macrophages, major producers of inflammatory cytokines like TNF-α (21). Incidence of bacterial species in blood samples of CD patients were also reported previously. *Mycobacterium avium paratuberculosis* (MAP) was reported from blood of 47% (9/19) CD patients by Chamberlin et al. (22). They concluded that MAP infection in CD patients may be causative or secondary and this is consistent with what is known about the genetic risk factors of CD (susceptibility of immunodeficient people to MAP infection and CD). In a study, *Clostridium difficile* infection and bacteremia with multidrug-sensitive *Escherichia coli* was reported from CD patients (23). First bacteremia case due to *C. difficile* in a CD patient was reported (24). They reviewed the literature for previously reported cases and concluded that *C. difficile* bacteremia is associated with a significant mortality rate and need aggressive antibiotic therapy (24). Thus, in accordance to previous investigations, our findings support the role of bacterial infection in CD patients.

Our results showed a significantly higher expression level of TNF-α and *IFN-γ* in CD patients in comparison to the healthy controls. This finding was consistent with previous studies and indicated production of a higher levels of proinflammatory cytokines from PBMC among patients with CD, demonstrated a close association with the disease activity (25). Elevated expression of TNF-α and IFN-γ genes was observed in 91% (10/11) and 73% (8/11) of the patients with active CD during the flare phase, respectively (≥ 2 folds), respectively, while 20% (2/10) and 12.5% (1/8) of the patients showed overexpression of these two genes during the remission phase. In a study,

higher serum level of TNF-α was reported in 100% of CD patients with the active stage. They demonstrated increased level of TNF-α production by PBMCs in the presence of lipopolysaccharide (LPS), as a marker of endotoxemia, among IBD patients in comparison to healthy ones (26). A significantly higher serum level of IFN-γ in patients with active Crohn's disease than those in inactive form or healthy controls was demonstrated in Japan (27). These authors concluded that the elevated IFN-γ in sera of Crohn's disease patients may be related to the immune reaction that could be originated from the inflammation in the intestine (27). Therefore, imbalance of immunity and production of proinflammatory cytokines by PBMCs (outside of the inflammation site) may have a pathological meaning in IBD patients and correlate with disease activity. In the case of anti-inflammatory cytokines, significantly higher transcription of IL10 was recorded in the flare phase of active CD patients than in the remission phase. This difference was mainly related to 4 patients that showed >10 folds increase in expression level of IL10 gene during the flares. In fact, normal and higher levels of IL10 were detected in targeting CD patients as demonstrated previously (28, 29). This inconsistency could be result of variations in age, severity of disease and commensal flora tolerance (30). Impaired IL10 production has been reported in other studies on severe cases of CD patients (31). In a study by Lindsay J et al., increased numbers of IL10 producing PBMCs were shown during inflammation in patients with CD (32). Although there were no noticeable changes in Foxp3 transcription of our subjects when inactive CD patients compared to the control group, statistically significant differences in *Foxp3* gene expression between phase 1 and 2 of active patients were recorded, where three out of 11 active CD patients showed more than 2 folds expression during remission. The increasing level of *FoxP3* transcription could be due to the change in the amount of Treg sub-population in PBMCs or its activity during the remission phase (33). Similar to our study, Wang et al. showed elevated *Foxp3* mRNA and the protein in the inflamed mucosa of patients with active CD compared with the healthy controls (34). The lack of intestinal specimens was one of the limitations of our investigation and further studies are needed to better correlate these findings from the blood samples with the intestinal specimens of CD patients.

Conclusion

CAP, CRP and ESR results were positively correlated with disease activity and negative correlation was found for Hem/Hct values. Significant differences in gene expression of IFN-γ, TNF-α and IL10 were detected during phase 1 active CD and these differences were significant when active patients were compared to inactive patients. IL10 transcription showed normal and elevated levels in active patients which can indicate the simultaneous regulatory T cell activation and tolerance responses. Active patients showed increased anti-inflammatory response (Foxp3) during follow-up period. Increased inflammatory and anti-inflammatory responses were demonstrated in patients with bacteremia. Further studies are needed to show possible role of bacteremia as a noticeable complication in exacerbation of CD.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declared no conflict of interest.

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