

## Interleukin 23 P 19 Expression in Patients with Ulcerative Colitis and Its Relation to Disease Severity

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### Abstract

**Background and study aims:** The purpose of this study is to determine whether mucosal expression of IL 23 p 19 has role in the pathogenesis of ulcerative colitis and its relation to disease severity.

**Materials and methods:** This study was carried out on 50 patients with ulcerative colitis and 10 normal individuals as control. They were divided into: Group I: 27 patients with mild to moderate disease. Group II: 23 patients with severe disease. Group III: 10 normal individuals. All patients and control were subjected to histopathological study, IL-23p19 immunohistochemical staining, IL-23R expression by flow cytometry and serum IL-23 by ELISA.

**Results:** There is significant increased in IL-23p19 gene and IL-23R in ulcerative colitis patients compared with control. Significant positive correlation was detected between increased expression of IL-23 p19 gene, IL-23R, high serum IL-23 and severity of the disease.

**Conclusions:** increased expression of IL-23 p19 is associated with ulcerative colitis and may indicate a role in its pathogenesis and that targeted therapy directed against IL-23 p19 may be effective in the treatment. Also increased expression of IL-23p19 gene and IL-23R with high serum IL-23 correlate positively with disease severity.

**Keywords:** Interleukin 23 p 19 gene; IL-23 R expression; Ulcerative colitis

### Introduction

Inflammatory bowel disease (IBD) consists of two distinct diseases, Crohn's disease (CD) and ulcerative colitis (UC). Both diseases are thought to arise due to combination of genetic variations and alteration in bacterial flora which can subsequently drive a dysregulated immune response that results in chronic intestinal inflammation [1,2].

Interleukin 23 (IL-23) is a member of a small family of proinflammatory cytokine, consisting of a p19 subunit and a common p40 subunit [3]. The receptor for interleukin-23 (IL-23 R) consists of the IL-12R beta 1 subunit and a novel component termed IL-23R [4], which is expressed predominantly on T lymphocytes, and natural killer (NK) cells and to a smaller extent on monocytes, macrophages and dendritic cells (DCs) [5]. IL-23 plays a crucial role in the pathogenesis of a number of immune-mediated inflammatory diseases by recruitment of several inflammatory cells [6,7]. IL-23 is essential for the differentiation of Th17 lymphocytes, a subtype of T lymphocyte implicated in chronic inflammatory/autoimmune mediated diseases. IL-23 and Th17 correspond to a new axis that drives immune activation and chronic inflammation through the differentiation and activation of Th17 cells. IL-23 promotes Th17 cells producing Tumour Necrosis Factor  $\alpha$  (TNF $\alpha$ ), IL-17, IL-6, IL-22, GM-CSF, and other

novel factors, which are associated with the induction of autoimmune inflammation [6,8,9]. Animal models of chronic inflammatory diseases such as chronic joint diseases, inflammatory bowel diseases and demyelinating diseases strongly suggest the involvement of this cytokine pathway. Thus, IL-23/Th17 is considered as a relevant therapeutic target in autoimmune driven diseases, and biological agents blocking IL-23 or IL-17 are currently being developed. Targeting the IL-23/Th17 axis seems to be a relevant and realistic therapeutic approach and these new agents pave the way for additive and alternative treatments to currently available biologics in chronic inflammatory diseases [9]. This study was carried out to determine whether mucosal expression of IL 23 p 19 has a role in the pathogenesis of ulcerative colitis and to elucidate its relation to disease severity.

### Materials and Methods

#### Patients and methods

This study was carried out on 50 patients with ulcerative colitis and 10 individuals whose colonoscopic and histopathologic findings were normal as control. The studied subjects were selected from the inpatient and outpatient clinics of tropical medicine and internal medicine departments, Tanta University Hospital, Tanta, Egypt, in the period between November 2012 and October 2013. Ulcerative colitis patients were diagnosed on the basis of clinical, endoscopic and

histological manifestations according to the criteria of American Gastroenterology Association [10]. They were divided according to endoscopic and histopathological findings into: Group I: 27 patients with mild to moderate ulcerative colitis disease. Group II: 23 patients with severe ulcerative colitis disease. Group III: 10 individuals whose colonoscopic and histopathologic findings were normal as control. The following categories were excluded from the study: Pregnancy, malignancy, heart failure, renal failure, thyroid disorders, acute infection and stroke and patients with immunosuppressive drugs.

All patients and control were subjected to complete history taking and thorough clinical examination. Laboratory investigations including, complete blood picture, blood urea and serum creatinine, erythrocyte sedimentation rate and stool examination to exclude bacterial causes of colitis. Colonoscopy was performed in all groups and the severity of the disease was determined. An endoscopic scoring system for UC of Pineton de Chambrun et al., [11] was used. Score 0: Normal or inactive disease. Score 1: Mild disease (erythema, decreased vascular pattern and mild friability). Score 2: Moderate disease (marked erythema, increased vascular pattern, friability and erosion). Score 3: Severe disease (spontaneous bleeding and ulceration). Endoscopic findings were recorded and multiple biopsies were taken for histopathology, IL-23p19 immunohistochemical staining and IL-23 R expression by flow cytometry.

### Histopathological study

4- $\mu$ m-thick serial sections of formalin fixed, paraffin-embedded tissue were cut and stained by Hematoxylin and Eosin for histopathological evaluation and grading of the groups.

A six grade classification system for inflammation was used. The grades were: 0, structural change only; 1, chronic inflammation; 2, lamina propria neutrophils; 3, neutrophils in epithelium; 4, crypt destruction; and 5, erosions or ulcers [12].

### IL-23p19 Immunohistochemical staining

4- $\mu$ m-thick serial sections of formalin fixed, paraffin-embedded tissue were cut and mounted on positively charged glass slides. After incubation at 60°C overnight and deparaffinization, sections placed in 0.01 M sodium citrate buffer (pH 6.0) and heated twice for 5 minutes in a microwave oven. After inactivation of endogenous peroxidase with 0.5% metaperiodic acid in phosphate-buffered saline (PBS) for 10 minutes, sections were incubated with 10% horse serum in PBS for 1 hour. Sections were incubated at 4°C overnight with 100 $\times$  diluted primary goat anti-mouse IL-23 p19 antibody (R&D Systems, Inc.). The standard avidin-biotin peroxidase complex (ABC) technique was performed using the LabVision Secondary Detection Kit (UltraVision Detection System Anti-polyvalent, HRP). The color was visualized by incubation with chromogen 3, 3' diaminobenzidine for 5 minutes. The slides were then counterstained with Mayer hematoxylin and cover slipped with Permount (StatLab, McKinney, TX). Negative controls were set for each test without the primary antibodies.

### Immunohistochemical evaluation

Results were expressed semi-quantitatively. Positively stained cells were counted by examining at least 10 random fields (X200) in each section and expressed as the percentage of positive cells over total cell number [13].

### IL-23 R expression by flow cytometry

#### Peripheral blood mononuclear cells (PBMC)

**CD4+ T isolation from blood samples:** Lymphocytes were isolated from peripheral blood by incubation with Rosette Sep Human CD4+T cells enrichment cocktail (Stem Cells Technologies, Grenoble, France) followed by centrifugation on a density gradient (Lymphoprep, PAA, Pasching, Austria). Lymphocytes were purified by centrifugation through Lymphoprep.

**For peripheral CD4+:** For cellular surface staining the following antibodies and secondary reagents were used in different combinations: biotinylated goat anti-human IL-23R (BAF1400, R&D System, Minneapolis, MN). Streptavidin-APC (BD Bioscience, San Jose, CA), CD3-FITC (eBioscience, San Diego, CA), CD4 PE-Texas Red (Invitrogen, Carlsbad, CA), CD45RO-FITC (Dako, Glostrup, Denmark), CD45RO-Pacific Blue (BioLegend, San Diego, CA), CCR6-PE (BD Bioscience), CD45RA-PE (Invitrogen), CD45RA-PE-Cy7 (eBioscience), plus matched isotypes as controls. Cells were acquired on a BD FACSAria II (BD Bioscience). Analysis of FACS data was performed by FlowJo (TreeStar, Ashland, OR) software.

#### Lamina propria mononuclear cells (LPMC)

**Isolation of intestinal lymphocytes (IEL):** For IEL isolation, endoscopic procedure was done, tissue (dissected mucosa) was placed and stirred for 15 minutes at room temperature in prewarmed (37°C) 1 $\times$ HBSS containing 10% fetal bovine serum (Gibco catalog no. 10082), 0.015 M HEPES, and 5 mM EDTA and stirred for 15 minutes at 37°C, followed by three 15-minute washes with buffer adjusted to room temperature. The supernatant from each wash was pooled and poured through a nylon wool column to enrich for T cells and remove mucus. The resulting cell suspension was used to analyze IEL.

**For IEL:** The IEL suspensions containing approximately 4 $\times$ 10<sup>6</sup> cells each were resuspended in cold phosphate-buffered saline and stained with Aqua Live/Dead cell discriminator (Invitrogen catalog no. L34597) according to the manufacturer's protocol. Cells were then stained for 1 hour in the dark at 4°C with optimized concentrations of anti-CD3 Alexa750-APC (eBioscience clone 17A2), anti-CD8 Alexa700 (eBioscience clone 53-6.7), anti-CD4 Pacific Blue (eBioscience clone RM4-5), anti-TCR GD R-PE (BD Pharmingen clone GL3), and biotinylated polyclonal anti-IL-23R (BAF1686; R&D Systems). Cells were washed twice with PBS containing 1% bovine serum albumin (fluorescence-activated cell sorting [FACS] buffer). Cells were then stained for 1 hour with streptavidin-conjugated Qdot 605 (Q10101MP; Invitrogen). Stained cells were washed once in FACS buffer and subsequently fixed in 4% formalin for 1 hour. Cells were then washed once and resuspended in FACS buffer and analyzed using an LSR II flow cytometer (Becton Dickinson, San Jose, CA). The data were analyzed by using FlowJo software (TreeStar, Inc., Ashland, OR). Gates were set on singlets and then on live lymphocytes. Subsequent gates were based on Fluorescence-Minus-One and unstained controls.

#### Serum IL-23 by ELISA

IL-23 cytokine level was assayed using a commercially available IL-22 ELISA kit (R&D Systems) according to manufacturers' instructions. All patients gave their informed consents and the study was approved by Ethical, and Research Committee, Tanta Faculty of Medicine, Tanta, Egypt.

### Statistical analysis

The statistical data are reported as the mean ± SD, frequencies (number) and percentages when appropriate. A comparison of numerical variables between the study groups was performed using Mann–Whitney U test to compare independent samples from two groups. One-way analysis of variance test was used to compare between more than two groups when data were normal and the Kruskal–Wallis test when the data were not normal while qualitative data compared with chi square test. Spearman’s rank correlation was used to quantify the association between continuous or ordered categorical variables. A P-value less than 0.05 were considered

statistically significant. All statistical calculations were performed using the computer program SPSS (Statistical Package for the Social Science; SPSS, Chicago, IL, USA) version 15 for Microsoft Windows.

### Results

Significant higher levels of Erythrocyte Sedimentation Rate (ESR) and disease duration were detected in patients with severe disease compared to patients with mild to moderate ulcerative colitis disease P<0.05. Abdominal pain, diarrhea, blood in the stool and ESR were significantly higher in ulcerative colitis patients compared with control (Tables 1 and 2).

Parameters	Mild-moderate UC Group I (n=27)	Severe UC Group II (n=23)	Control Group Group III (n=10)	P-value
Age (ys)	36.1 ± 13.4	41.5 ± 12.7	35.2 ± 12.8	0.27
Sex (m/f)	16/11	14/9	4-Jun	1
Disease duration(ys)	2.6 ± 1.7	4.2 ± 3.1 <sup>b</sup>	-	0.042 <sup>b</sup>
Abdominal pain	20/27	18/23	3 <sup>a</sup>	0.083
Diarrhea	18/27	19/23	2 <sup>a</sup>	0.017 <sup>*</sup>
Blood in stool	19/27	21/23	-	0.111
ESR (mm/hr)	21.8 ± 7.9	33.6 ± 8.5 <sup>b</sup>	10.9 ± 1.9 <sup>a</sup>	<0.001 <sup>*</sup>
Hb (gm/dl)	12.1 ± 1.3	11.6 ± 1.6	12.6 ± 1.5	0.197

P<0.05<sup>\*</sup>significant between all groups; <sup>a</sup>sig between patients & control; <sup>b</sup>sig between GI & GII. UC, ulcerative colitis

**Table 1:** Clinical and laboratory data of the studied group

Studied groups	Histopathological grading						P -value
	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5	
Mild-moderate UC Group I (n=27)	5	12	6	4	0	0	0.02 <sup>*</sup>
Severe UC Group II (n=23)	0	0	2	11	6	4	

P<0.05 <sup>\*</sup>significant; UC, ulcerative colitis.

**Table 2:** Correlation between histological grading and endoscopic severity in the UC patients

The studied 50 cases of UC showed different grades of severity 5 cases were grade 0 (10%); 12 cases were grade 1 (24%); 8 cases were grade 2 (16%), 15 cases were grade 3 (30%) (Figure 1a); 6 cases were grade 4 (12%) (Figure 1b) and only 4 cases were grade 5 (8%) (Figure 1c). In correlation with the endoscopic grouping; in group I; the cases distributed among grades 0-3; while in group II; the cases were distributed among grades 2-5 with statistical significance (P=0.02) (Table 3).

Table 3 shows significant increase in serum level of IL-23 and IL-23R expression in both peripheral blood and lamina propria lymphocyte (CD4) in ulcerative colitis patients compared with control group. This increase was more in patients with severe disease compared with patients with mild to moderate disease. IL-23 p 19 Immunohistochemical evaluation showed that IL23p19 was expressed in the lamina propria macrophages. The mean number of the cells expressing IL23p19 in group I was 11.6 ± 3.4 (Figure 2a) while in group II it was 19.6 ± 5.5 (Figure 2b) with statistical difference between the two groups. On the other hand, it was only 3.5 ± 1.6 in group III (control group) (Figure 2c) with statistical difference between the three groups.

Table 4 shows significant positive correlation between both endoscopic severity and histological grading of ulcerative colitis with each of IL-23p19 expression (r=0.591, p=0.038) and, (r=0.612, p=0.047) respectively, IL-23 R expression (r=0.542, p=0.015) and (r=0.526, p=0.031) respectively, serum level of IL-23 (r=0.637, p=0.024) and (r=0.551, p=0.019) respectively.

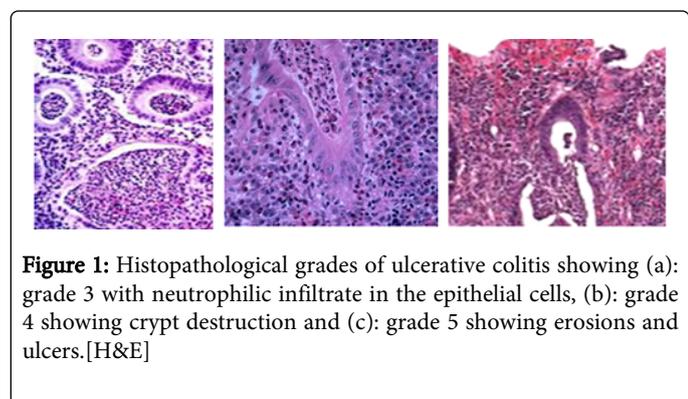
### Discussion

Patients with UC are at increased risk of inflammation. IL-23 is a newly identified cytokine with increased expression in inflamed biopsies of colon mucosa in patients with celiac disease (CD); however there is inconsistent evidence on its role in ulcerative colitis [14]. IL-23 is a heterodimeric cytokine that shows similar function to IL-12 in

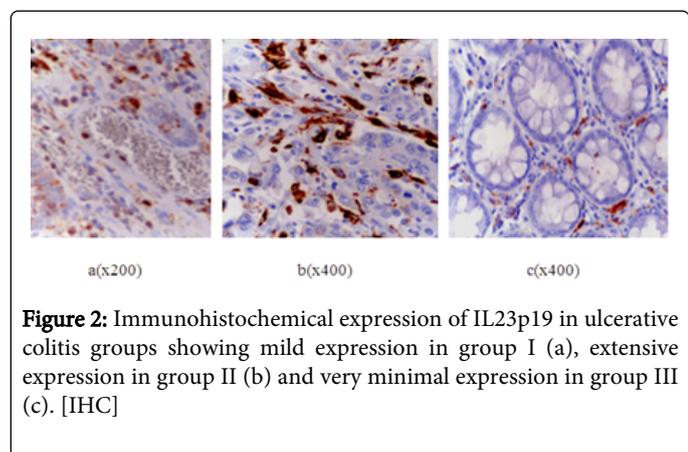
promoting cellular immunity and enhancing lymphocyte proliferation [15].

Parameters	Mild-moderate UC	Severe UC	Control Group	P-value
	Group I	Group II	Group III	
	(n=27)	(n=23)	(n=10)	
-IL-23 R expression %				
.PBMC	6	12	1c	0.037*
.LPMC	20	29	6a	0.001*
-S IL-23 (pg/ml)	48.9±7.6	137.4 ± 45.5b	23.2 ± 9.4a	<0.001*
-IL-23 p 19 expression	11.6±3.4	19.6 ± 5.5b	3.5 ± 1.6a	<0.001*
Immunohistochemistry% of +ve cells				
P<0.05*significant between all groups; asig between patients & control; bsig between GI & GII. csig between GII & control; UC: ulcerative colitis; PBMC: peripheral blood mononuclear cells (lymphocyte) LPMC: lamina propria mononuclear cells (lymphocyte).				

**Table 3:** IL-23 R expression, serum IL-23 and IL-23p19 expression in the studied groups



**Figure 1:** Histopathological grades of ulcerative colitis showing (a): grade 3 with neutrophilic infiltrate in the epithelial cells, (b): grade 4 showing crypt destruction and (c): grade 5 showing erosions and ulcers. [H&E]



**Figure 2:** Immunohistochemical expression of IL23p19 in ulcerative colitis groups showing mild expression in group I (a), extensive expression in group II (b) and very minimal expression in group III (c). [IHC]

Parameters	IL-23p19 gene expression		IL-23 R expression		Serum IL-23	
	R	P-value	R	P-value	R	P-value
-Endoscopic severity	0.591	0.038 *	0.542	0.015 *	0.637	0.024 *
-Histopathological grade						
	0.612	0.047*	0.526	0.031*	0.551	0.019*
P<0.05 *significant; UC, ulcerative colitis.						

**Table 4:** Correlation between endoscopic severity, histopathological grading and studied parameters in UC patients

This study showed significant increased expression of IL-23p 19 gene in patients with UC compared with control. This increase was significantly higher in patients with severe UC disease compared with mild to moderate disease. This was similar to the results reported by Schmidt et al. [16], Zhanju et al. [13] and Kobayashi et al. [17]. We observed that IL-23 p19 positive cells by immunohistochemistry were mainly macrophages. This was in agreement with Zhanju et al. [13]. These findings suggest that IL-23 is produced by intestinal mucosal macrophages in inflamed mucosa of ulcerative colitis patients [13]. Upon stimulation by bacterial ligand, IL-23 is produced by antigen presenting cells. After binding to appropriate receptor (IL-23 R), this cytokine can stimulate the production of IL-17, TNF alpha and IL-6 from T cells. IL-17 stimulates the expression of adhesion molecules on endothelial cells, as well as the release of IL-6 and IL-8 from myofibroblast and epithelial cells. IL-8 act as chemotactic factor for neutrophil influx into the intestine. Inflammatory neutrophil release inflammatory mediators like matrix metalloproteinase and inducible nitric oxide. This sequel of pathogenic events leads to the chronic inflammation and epithelial cell damage associated with the disease [18]. Therefore, IL-23 was proposed to play an integral role in the pathogenesis of IBD [19].

This study showed the increased expression of IL-23 R in both peripheral blood and mucosal biopsy of ulcerative colitis patients were significant compared with control group. This increased expression was higher in patients with severe disease compared with those have mild to moderate disease. Also, Zhanju et al. [13] demonstrated significant increased expression of IL-23 R in peripheral blood and mucosal lamina propria cells.

In accordance with the findings reported by Mohammadi et al. [14] and Zheng et al. [20], we demonstrated increased serum level of IL-23 in ulcerative colitis patients compared with control. In addition, the increased serum levels of IL-23 were more in patients with severe disease compared with mild to moderate disease. Moreover, this study reported significant positive correlation between high serum IL-23 levels and endoscopic severity of the disease. These findings support the hypothesis that, increased IL-23 levels reflect the activity of T helper 17 in patients with ulcerative colitis and that IL-23 participate in the pathogenesis of the disease [20]. This study noticed, the increased expression of IL-23p19 and IL-23 R were significantly positively correlated with endoscopic severity of the disease. These were consistent with Schmidt et al. [16] and Zhanju et al. [13]. In consistent with experimental studies of Daniel et al. [21] and Ando et al. [22], there were significant positive correlation between histopathological severity and both IL-23p19, IL-23R expression in the mucosa of ulcerative colitis patients. Furthermore the severity of

histopathological lesions was significantly correlated with serum IL-23, similar to an experimental study of Sheikh et al. [23].

It can be concluded that the increased expression of IL-23p19 may indicate a role in the pathogenesis of ulcerative colitis; therefore targeted therapy directed against IL-23p19 may have a therapeutic role for the disease. The increased expression of IL-23p19, IL-23 R and serum IL-23 correlate with disease severity. However, due to the limited samples in this study, there is a need for a larger study to emphasize the role of IL23p19 in ulcerative colitis.

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