



Evaluation of Serum NF- κ B, IL-6, IL-8 and their Relation to Leptin in Patients with Active Behcet's Disease

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BEH CET'S DISEASE (BD) is a chronic systemic inflammatory autoimmune disease of unknown cause. It is characterized by elevated levels of proinflammatory cytokines that have been previously reported, yet the pathogenesis is still unknown.

Nuclear factor kappa B (NF- κ B) is a transcription factor that remain latent in the cytoplasm and shuttles to the nucleus whenever activated. On activation, NF- κ B regulates the enzymes cytokines (IL-6, IL-8). NF- κ B has been contributing to a wide variety of autoimmune diseases amongst which is BD. NF- κ B pathways were involved in the anti-apoptotic activity of leptin in neutrophils in vitro. The present study was conducted to determine serum NF- κ B levels, and its role in modulating the expression of different cytokines (IL-6, IL-8) in active BD patients. This is in addition to studying the relationship of leptin with NF- κ B, IL-6 and IL-8.

The current study included 25 patients with active BD and 25 healthy controls. Serum IL-6, IL-8 and NF- κ B levels were measured with quantitative sandwich ELISA kit enzyme-linked immunosorbent assay (ELISA) method, while leptin serum levels were measured using specific radioimmunoassay (RIA) kits.

IL-6, IL-8, NF- κ B and leptin were significantly higher in BD group than in the control group. Also, there was a positive correlation between IL-8, IL-6, NF- κ B and leptin.

New insights of the role of leptin in the autoimmune pathophysiology was studied, and its relationship with NF- κ B, IL-6 and IL-8 in these patients was detected and might help in elucidation of its pathogenesis. Further studies, are required to encourage the use of anti- NF- κ B and anti-Leptin as recent treatment modalities in BD.

Keywords: Behcet's disease, IL6, IL8, NF- κ B, Leptin.

Introduction

Behcet disease (BD) is one of the chronic inflammatory diseases which is characterized by persistent oral and genital ulcers, skin wounds and uveitis (Cho & Bang, 2012).

BD also affects all types and sizes of arteries, veins and capillaries, as well as the joints, central nervous system, lungs and gastrointestinal tract. In spite of the fact that the etiology of BD isn't

completely understood, neutrophil hyperfunction, vasculitis, and autoimmune & inflammatory responses are the major pathological features of BD (Türkcu et al., 2013).

The etiology and course of the disease are unknown, but considerable data indicate that immunologic abnormalities are important. Several humoral and cellular variations from the normal in patients with BD have been described, and high levels of serum interleukin-2 (IL-2), interferon- γ

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(IFN γ), IL-1 β , IL-6, tumor necrosis factor α (TNF α), and IL-8 have been detected (Coelho et al., 2013).

A few genes were appeared to be linked with existence, progress and seriousness of BD. Behcet's disease is known to be interrelated to apoptosis resistance in T cell subsets and linked with the upregulation of antiapoptotic variables, and this relationship is based on the modulation and the expression of antiapoptotic genes by the activation of NF- κ B (Lee & Song, 2018).

NF- κ B could be a family of proteins that mediate transcriptional control significant to many biological functions (Zhang et al., 2017). NF- κ B was known and named for its part in controlling κ light chain expression in B cells (Oh & Ghosh, 2013). Normally, NF- κ B proteins are latent within the cytoplasm, balanced for speedy reactions after temporarily removing their inhibition. Uninhibited NF- κ B molecules then shuttle between nucleus and cytoplasm as transcriptionally active homo- and heterodimers. (Vallabhapurapu & Karin, 2009).

Furthermore, many components of NF- κ B, including both positive and negative regulators, are under transcriptional regulation by NF- κ B itself. In spite of this complex regulatory network, specific defects in individual molecules inside the NF- κ B path have been proven to disrupt cellular homeostasis, and immune pathology is an important consequence (Afonina et al., 2017). NF- κ B contributes to immunological self-tolerance, and defects in NF- κ B contribute to autoimmune diseases. Defects in NF- κ B have also lead to immune deficiency and autoinflammatory diseases, for which variable reviews are available (Miraghazadeh & Cook, 2018).

NF- κ B is the main transcription factor of M1 macrophages and it induces large numbers of inflammatory genes, including those producing inflammatory cytokines (e.g., IL8, IL-6, and chemokines), adhesion molecules and inflammatory enzymes (e.g., COX-2, 5-LOX), (Serasanambati & Chilakapati, 2016).

IL-8 which is mainly produced by macrophages, additionally recognized as neutrophil activating factor, is a main cytokine which attracts and activates leukocytes strongly. It has a main role in the transformation of mononuclear to granulocytic

infiltration and causes increased adhesion of peripheral blood leukocytes to endothelial cells, hence connecting the activation of immune system with the endothelial variations in BD. Moreover, IL-8 activated neutrophils are a primary source of the enzymes included in tissue destruction in BD (Pineton de Chambrun et al., 2012).

However, serum IL-6 & IL-8 are considered as activity markers in (BD), some studies have revealed conflicting outcomes for IL-6 (Adam & Calikoglu, 2004).

There were many studies carried out to understand the basic function achieved about leptin molecule, a molecule essential and considered as a cornerstone in the study field of obesity, diabetes, inflammation, and immune regulation. Due to the pleiotropic actions and the secretion of leptin on a circadian rhythm, it is considered as a hormone (Vadacca et al., 2011). Leptin is considered an adipokine member as it is synthesized normally by adipose tissue, among visfatin, adiponectin (APN), chemerin, resistin, high molecular weight adiponectin (HMW-APN), etc. (Raucci et al., 2013). Regarding the type of the studied molecules, the adipose tissue can be considered as a pro-inflammatory or non-inflammatory.

The polarizing macrophages response is one of a non physiological adipose tissue micro environmental hallmarks, favoring an M1 phenotype, where there is a prevalence for the attention of free fatty acids via Toll-like receptors (TLR4 and TLR2) and a pro-inflammatory cytokine reaction (Th1) mediated by using of NF- κ B, chemokine secretion, activation of T and B cells and activating an unfolded protein reaction via endoplasmic reticulum stress (Kratz et al., 2014). There are various researches that emphasized the role of leptin in chronic inflammatory process such as metabolic syndrome, obesity and diabetes to title a few, and it has developed as an open secret its participation in autoimmune rheumatic illnesses such as behcet's illness (Hrycek et al., 2018). Leptin possibly acts as a survival protein for neutrophils. In vitro NF- κ B pathways were involved within the anti-apoptotic action of leptin in human neutrophils (Bruno et al., 2005).

Gaber et al. (2017) proved within the latest years that the role and differentiation of immune cells is directly determined by their metabolic

status, and thus touching tolerance and immunity, in addition to the failure of the immune system response in autoimmune diseases.

As leptin is the mainstay of adipokine family, it is considered as the sensor key of energy metabolism and a keystone within the guideline of metabolism-immune system interaction. In addition, leptin modulated the built-up of inflammatory mediators by means of immune cells. Specifically, the assembly of IL-8 & IL-6, had been increased through leptin in CD4⁺ T cells from osteoarthritis patients, however it did not increase in healthy cases (Scotece et al., 2017); therefore, signifying completely different visions regarding the effect of leptin in the immune system and autoimmune pathophysiology.

The aim of this study is to check out the presence of any associations of NF- κ B with Behçet's disease and to decide whether the inflammatory reaction in patients with Behçet's disease (BD) is associated with NF- κ B activation which was once hypothesized to have a key function in apoptosis resistance and preventing the death of T lymphocytes in BD cells.

The authors also, aim to look at the role of leptin in patients with Behçet's disease and to explore the influence of leptin on the release of inflammation-related cytokines (IL-6, IL-8), indicating an involvement of nuclear factor "kappa-light-chain-enhancer" of activated B cells (NF- κ B) in the mode of action of leptin.

Patients and Methods

Twenty five Patients with BD who attended to the Rheumatology Department and to the Dermatology Department of Cairo University, and 25 age-matched and sex-matched healthy controls were included in this study. The patients with Behçet's disease were studied during the clinically active stage. The patients were informed by the strategy of the study and a signed consent was taken from them. Clinical activity was assessed at the time of venipuncture for activity signs and symptoms (clinical criteria defined by the International Study Group for Behçet's Disease, 1990). Full history about medications should be taken from both groups because some medications especially glucocorticosteroids reduce the transcription of the pro-inflammatory cytokines (IL-6, IL-8), and such patients were not

be a part of this study (Evereklioglu et al., 2002), and those who used systemic drugs for any other reason were also not included in the study. Another exclusion criteria was obesity and the high body mass index if it is 30 or more. Subjects with renal or hepatic disorder, diabetes or elevated blood pressure were not present in the study. Because leptin is a hormone for which normal serum levels differ greatly between males and females, in the present study all patients were males (Ballou & Nelson, 1997).

Both clinical and laboratory findings were utilized for the determination of active Behçet's patients. During clinical assessment, any deterioration of the clinical symptoms at any time of the study and having as a minimum three of the major symptoms (genital ulcers, oral ulcers, skin lesions and uveitis) were considered to be in the active period of the disease.

Body mass index (BMI), height, weight, sex and age are taken. C reactive protein (CRP), erythrocyte sedimentation rate (ESR) and Total leucocytic count (TLC), were measured. Blood samples were taken after 12 h fasting, serum was collected and centrifuged at 1000 \times g (or 3000 rpm) for approximately 20 minutes and stored at -20°C or -80°C. Serum IL-8 was determined by Human Interleukin 8 (IL8) quantitative sandwich ELISA kit. The sensitivity of this kit is 1.0 pg/ml, the detection range of this kit is 6.25 pg/ml-200 pg/ml. Standard Concentration Gradients (S6 to S1) were 200,100,50,25,12.5,6.25 pg/ml.

Serum IL-6 was determined by Human Interleukin 6 (IL-6) ELISA Kit. The principle of this kit is Double Antibody Sandwich in which the tested antigen appear in more than two ways that can similarly recognize coated antibody and detect antibody. As respects affectability, the least perceptible human IL-6 up to 1pg/ml. (Intra assay Precision) \leq 8% (Inter assay Precision) \leq 12%.

Serum NF- κ B was determined by Human Nuclear Factor Kappa B (NF- κ B) ELISA Kit Catalog No: MBS450580, The kit is a sandwich enzyme immunoassay for the in vitro quantitative measurement of NF- κ B in human biological fluids, all reagents, samples and standards were prepared, 100 μ L standard or sample was added to each well.

Leptin, was assayed using commercial specific

RIA kits (Linco Research, Inc., St. Charles, MO, USA), with a range of detection from 0.5 to 50 ng/ml, the intra-assay and inter-assay coefficients of variability were 6.6±0.8% and 5.5±0.9%, respectively.

Statistical methods

Statistical analysis was performed using SPSS (Statistical Package for Social Sciences) software version 20 and a P-value of < 0.05 was considered statistically significant. All the results were defined as mean±standard deviation. The independent sample-t test was applied to compare the leptin levels, and the other measured parameters between (BD) group and control group. One-way ANOVA test was applied for comparison between mild, severe subgroups of patients and the control group. While correlations were conducted using Pearson correlation. ROC curve was used to evaluate the performance of different tests in differentiating Behcet's disease group from the control group, the optimum cutoff

point was selected based on the highest Youden's index.

Table 1 shows that CRP, IL-6, IL-8, NF-κB and leptin were significantly higher in Behcet's disease group than in the control group. Table 2 shows characteristics of Behcet's disease group.

Table 3 shows that no significant differences were found between severity grades regarding age, onset and duration and laboratory findings. Table 4 shows that there were significant positive correlations between each of IL-6, IL-8, NF-κB and leptin.

Figure 1 shows the positive correlation between leptin and IL-6 in Behcet's disease, Fig 2 shows the positive correlation between leptin and IL-8 in Behcet's disease and Fig 3 shows the positive correlation between leptin and NF-κB in Behcet's disease.

TABLE 1. CRP, IL-6, IL-8, NF-κB and Leptin levels in patients with Behçet's disease and control subjects

Variables	Control (n=25)	Behçet's disease (n=25)	^P-value
Age (years)	32.7±8.1	34.6±11.4	0.496
CRP (mg/L)	3.4±0.8	33.7±5.0	<0.001*
IL-6 (pg/ml)	37.8±8.7	108.2±23.0	<0.001*
IL-8 (pg/ml)	91.1±12.5	291.7±75.1	<0.001*
NF-κB (ng/ml)	30.4±10.4	144.8±28.3	<0.001*
Leptin (ng/ml)	11.7±2.1	20.5±2.4	<0.001*

^Independent t-test. *Significant

TABLE 2. The clinical data and laboratory investigations of Behçet's disease group

Variables	Mean ± SD	
Onset (years)	26.4±9.0	
Duration (years)	8.2±7.4	
ESR (ml/h)	15.9±9.8	
Hemoglobin (gm/dL)	11.5±1.4	
TLC (x10 ³ /ml)	9.7±3.5	
Platelets (x10 ³ /ml)	258.1±69.4	
ALT (U/L)	30.6±22.2	
AST (U/L)	23.6±10.8	
Creatinine (mg/dL)	0.82±0.17	
	n	%
Severity		
• Mild	11	44.0
• Moderate	9	36.0
• Severe	5	20.0

Total=25.

TABLE 3. A comparison between mild BD subgroup (n=11), moderate BD subgroup (n=9) and severe BD subgroup (n=5) in clinical and biochemical data

Variables	Mild (n= 11)	Moderate (n= 9)	Severe (n= 5)	P-value
Age (years)	37.8±12.0	34.6±11.2	27.8±9.1	0.274
Onset (years)	26.8±9.3	26.9±10.2	24.8±7.5	0.909
Duration (years)	11.0±9.2	7.7±5.3	3.0±2.3	0.130
ESR (ml/h)	13.5±8.3	17.6±9.1	18.4±14.5	0.550
Hemoglobin (gm/dl)	11.1±1.4	11.9±1.3	11.6±1.7	0.465
TLC (x10 ³ /ml)	10.1±2.1	10.0±4.5	8.4±4.4	0.656
Platelets (x10 ³ /ml)	260.5±65.6	249.8±83.8	267.8±61.4	0.895
ALT (U/L)	26.5±20.2	29.2±16.2	42.2±34.7	0.429
AST (U/L)	22.2±12.0	23.4±9.6	26.8±11.6	0.746
Creatinine (mg/dL)	0.79±0.19	0.83±0.17	0.84±0.18	0.828
CRP (mg/L)	34.8±4.9	33.1±5.3	32.6±5.2	0.661
IL-6 (pg/ml)	107.3±13.6	110.5±29.4	106.0±30.8	0.932
IL-8 (pg/ml)	273.9±45.0	308.2±83.3	301.4±115.5	0.587
NF-κB (ng/ml)	144.5±26.2	139.5±27.5	155.1±37.3	0.633
Leptin (ng/ml)	20.2±2.0	20.6±2.5	21.2±3.5	0.746

ANOVA test

TABLE 4. Correlations between leptin with IL-6, IL-8 and NF-κB in Behcet's disease group

Behcet's disease (n= 25)			
		Leptin	NF-κB
IL-6	r	0.674	0.677
	P-value	<0.001*	<0.001*
IL-8	r	0.551	0.685
	P-value	0.004*	<0.001*
NF-κB	r	0.723	
	P-value	<0.001*	

Pearson correlation, r: Correlation coefficient. *Significant

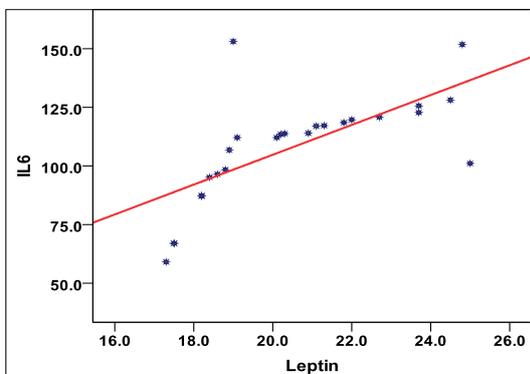


Fig. 1. The positive correlation between leptin and IL-6 in Behcet's disease

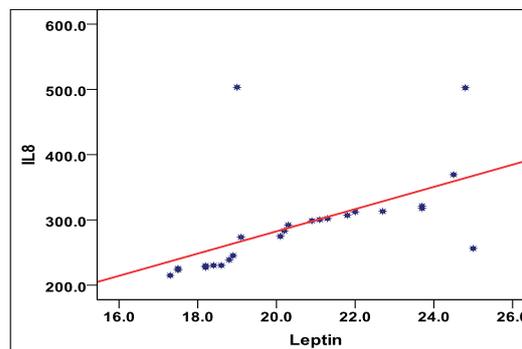


Fig. 2. The positive correlation between leptin and IL-8 in Behcet's disease

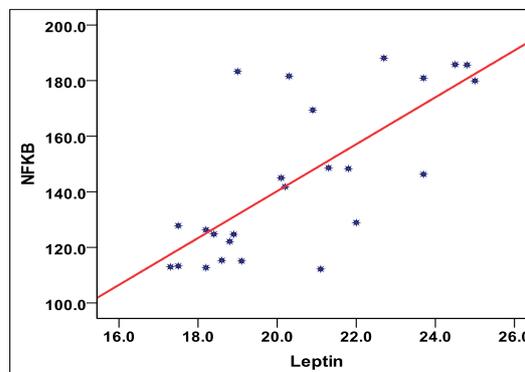


Fig. 3. The positive correlation between leptin and NF-κB in Behcet's disease

Discussion

Behcet disease is considered a chronic inflammatory disease with multi-systemic involvement that have a relapsing inflammatory course. While the etiology and pathogenesis of the disease till now has not been explained, different mechanisms such as genetic disorder, infections and autoimmunity have been claimed (Akdeniz et al., 2004).

A broad spectrum of pathologies of innate immunity are postulated in the pathogenesis of systemic autoinflammatory diseases. Recently, several studies have reported the role of new genes in these diseases, showing new pathophysiological pathways including leptin, NF κ B as well as new-targeted therapies such as biotherapy, which has the advantage to replace non-specific anti-inflammatory drugs (Zhang et al., 2017).

NF κ B is a transcription factor that acts as a main factor for turning on certain immune and inflammatory reactions. NF κ B changes cell performance in different ways; increases cell proliferation, it prevents apoptosis (automatic cell death), increases both cell proliferation and inflammatory and immune response (Yenmis et al., 2015).

The authors of the present study measured some of the inflammatory biomarkers such as NF κ B in plasma, serum IL-6, IL-8 and leptin in patients with active Behcet's disease.

In the present study, NF κ B concentrations were measured in plasma from the normal controls and patients with active BD. Table 1 shows that there was a significant increase in plasma NF κ B concentration in patients with active BD, versus the control.

This is in agreement with a number of studies that have indicated that NF κ B was up-regulated and significantly increased in active BD cells, suggesting that NF κ B increase and activation may modulate the expression of antiapoptotic genes. Todaro et al. (2005) hypothesized that NF κ B activation has a key function in regulating expression of antiapoptotic proteins such as FLIP and Bcl-xL, preventing the death of T lymphocytes in BD cells, leading to the apoptotic resistance of activated T cells in patients with BD.

This is also in accordance with (Kurylowicz & Nauman, 2008) who detected the elevation of serum levels of pro-inflammatory cytokines, including: IL-1, TNF α and IL-6 which are well-known to be NF κ B target genes, signifying initiation of this signaling pathway in the progression of BD. These cytokines are able to activate NF- κ B in fibroblasts and innate immune cells, thus encouraging the expression of extra inflammatory chemokines and cytokines, leading to more recruitment of inflammatory immune cells and spreading of inflammation. (Simmonds & Foxwell, 2008)

Kallen (2002) detected an association of abnormal overproduction of IL-6 in certain autoimmune illnesses and chronic inflammations, in which he suggested that such excessive production might have led to abnormal differentiation of B cell and antibody production.

In this study, the serum levels of IL-6 in patients with active BD group were significantly higher as compared to the controls. These results are similar to the results of the study done by Akdeniz et al. (2004) that showed that there were significant increases in serum levels of IL-6 in patients with BD than the controls.

Similarly, Evreklioglu et al. (2002) found that there were significantly elevated IL-6 levels in patients with BD versus the control subjects, with the uppermost values detected in the active period of the disease. He stated that since IL-6 is a key activator of the acute-phase response and implicated in liver synthesis of acute-phase reactants, it is likely to play an important role in the course of BD.

On the opposite of the current results, Sadegh et al. (2017) stated that in their study, there were no significant differences in IL-6 levels among both groups (active BD Vs the control).

IL-8, also known as neutrophil activating factor, is a major cytokine, which powerfully attracts and activates leukocytes and might play a role in tissue damage in BD.

Serum IL-8 was measured in the current study, and showed that it was significantly elevated in active BD patients Vs healthy Controls.

The present results were similar to what is

reported by Akkurt et al. (2015), who found that elevation of serum levels of IL-8 were noticed in patients with active BD in their study. Similar findings were observed by Ben Ahmed et al. (2004) who reported that at the same time IL-8 is a strong activator of neutrophil function, this finding may support the suggestion of polynuclear cell hyperfunction within the pathogenesis of BD lesions. However, Sadeghi et al. (2017), who contradicted the present results, found that there was no association between levels of IL-8 and active BD in their study.

It is likely that the disease is associated with the emission of pro-inflammatory mediators by direct activation of circulating monocytes (Evereklioglu et al., 2002). Al-Dalaan et al. (1995) have also stated increased serum IL-6, IL-8 levels. The results of this study confirm these outcomes and suggest an involvement of the activation of the immune system in BD. As the results of this study showed increased levels of the cytokines in the investigated patient group, the authors of the present study suggested that these cytokines could be related to pathogenesis of the disease. These pro-inflammatory cytokines may have a role through the course of the disease and take portion in tissue damage.

Leptin has pro-inflammatory criteria and several activities related to those of the acute phase reactants, and up-regulates the production of inflammatory cytokines as IL-6 (Shen et al., 2005).

Leptin was investigated in the present study, as a pro-inflammatory hormone, it was found to be significantly elevated in active BD patients Vs the control.

The current results come in accordance with Lee & Song (2018) who has shown that leptin level was increased significantly in the BD group as compared with the healthy control group. Also Nilufer et al. (2007) stated that there were higher serum leptin levels in Behcet's disease group distinguished in accordance to age, sex, and BMI versus the controls. On the contrary to the present study, other study performed by Kavuncu et al. (2005) on 28 males with active Behcet's disease compared with 15 healthy controls, and they found that there was no significant difference in leptin serum levels among the two groups.

The elevation of leptin serum levels in patients with Behcet's disease compared to healthy subjects in this study and its close link with several pro-inflammatory mediators underlines the ability of this molecule to stimulate or sustain low-grade inflammation that could be recognized to a possible role for leptin in the pathogenesis of Behcet's diseases and might eventually lead to the development of chronic disease (Lee & Song, 2018).

Leptin has a direct role in increasing the production of some pro-inflammatory cytokines, for example IL-6, and the chemokines IL-8 in peripheral blood monocytes. In addition, leptin has a role in the increase of platelet aggregation and enhancing leukocyte-endothelial cell interactions by increasing adhesion molecules expression on myeloid and vascular endothelial cells. (Pinteaux et al., 2007). TH1 and TH17 replies are improved with leptin, which can be able to stop T-cell apoptosis (Fantuzzi, 2005).

In vitro, it was found that NF- κ B pathways play a role in the anti-apoptotic activity of leptin in human neutrophils (Bruno et al., 2005). It has been proved that there is a great effect of leptin on neutrophil chemotaxis and infiltration (Francisco et al., 2018).

Specifically, the formation of IL-6 and IL-8, which were increased by leptin in CD4+ T cells from active Behcets patients, but not from healthy controls (Scotece et al., 2017); thus, displaying new visions into the role of leptin in the immune system and autoimmune pathophysiology. Literature data suggest a correlation between leptin levels and disease activity in BD (Evereklioglu et al., 2002), even though serum leptin concentrations were not associated significantly with the presence of active disease in such studies (Yalc,indag et al., 2007). Moreover, in some cases, a correlation was reported between serum levels of IL-6, IL-8 and some of the main clinical features of BD. The current data have confirmed the results, suggesting a possible role for IL-6 in the immune complex-mediated pathology (Kavuncu et al., 2005). The present results are in agreement with previously published data that detected a significant positive correlation between leptin serum levels in BD patients and between each of il6, IL-8 and NF- κ B (Cantarini et al., 2016).

Comparing between severity grades of active

Behcets disease, as regards, NF- κ B, IL-6, IL-8 and leptin, the present study, revealed no statistical significant differences between the three grades (mild, moderate and severe).

Conclusion

BD could be a disease including complex interactions of cells of the immune system. Different changes in cytokine signaling appear to play a major role in its pathogenesis. NF- κ B contributes to immunological self-tolerance, and defects in NF- κ B contribute to autoimmune diseases. In the present study it was indicated that NF κ B was up-regulated and significantly increased in active BD patients, the authors detected constitutively high serum levels of pro-inflammatory cytokines, including: IL-6 and IL-8 which are known to be NF- κ B target genes, suggesting activation of this signaling pathway in the course of BD disease.

Levels of IL-6 and IL-8 as pro-inflammatory cytokines, may be valuable in following disease activity, or in expecting patients with severer diseases.

Leptin which was investigated in the present study, as a pro-inflammatory hormone, was found to be elevated. However, a confirmatory role of leptin in autoimmunity should be further investigated. More studies would be valuable to explain the effect of leptin in a certain type of organ participation in BD and, as a significance, to explain the potential importance of leptin as a prognostic biomarker of disease activity.

It is possible that additional management modalities that interfere with cell signaling procedures may be the upcoming direction for the clinical treatment of BD. Further studies are required for development of new therapeutic approaches including anti-NF κ B and anti-Leptin, as a possible novel treatment for BD.

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