Serum Level of Interleukin-37 and Expression of Its mRNA in Ankylosing Spondylitis Patients: Possible Role in Osteoporosis

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Ankylosing spondylitis (AS) is a chronic inflammatory disease of the axial skeleton.Interleukin-37 (IL-37) is a member of IL-1 family cytokines, that downregulate expression of pro-inflammatory cytokines in chronic inflammatory diseases. The aim of the work is to investigate role of IL-37 in AS disease activity and osteoporosis. Twenty-five patients with AS and 25 controls were enrolled into this study. They were subjected to full clinical examination including assessment of disease activity according to the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI). Serum IL-37 levels and IL-37 mRNA relative concentration were measured by enzyme-linked immunosorbent assay (ELISA) and quantitative reverse transcriptase- polymerase chain reaction (RT–PCR) respectively. Bone mineral density (BMD) was determined using dual energy X-ray absorptiometry (DEXA). Spine radiographs were scored using the modified Stoke Ankylosing Spondylitis Spine Score (mSASSS). Mean serum IL-37 level was significantly higher in AS patients compared with the controls (*P*<0.001) and significantly elevated in AS patients with osteoporosis (*P*<0.05). IL-37 mRNA gene expression showed a significant increase expression in active AS patient (25 folds) as well as in inactive patient (12 folds) as compared to controls. In conclusion, serum IL-37 and its mRNA expression is increased in AS patients with special consideration in patient with Osteoporosis and correlates with disease activity and BMD which indicate that IL-37 may provide a novel research target for pathogenesis and therapy of AS.

Ankylosing spondylitis (AS) is a chronic inflammatory rheumatic disease of the axial skeleton, which is characterized by low back pain and stiffness for more than 3 months that improves with exercise. Other important symptoms are restriction of motion of the lumbar spine and limitation of chest expansion (Dean *et al.,* 2014). It is two to three times more common in men than women. Symptoms commonly begin in late adolescence and early adulthood (Braun *et al.,* 1998).

Although the etiology of AS is unclear, accumulating evidence has underlined that the levels of pro-inflammatory cytokines (TNF-α, IL-6, IL-17 and IL-23) were significantly increased in the peripheral blood of AS patients. Clinical trials suggested that blocking these cytokines could partly relieve inflammatory symptoms of AS and also appears to reduce disease severity (Rajalingham & Das, 2012).

IL-37 is a newly defined member of the IL-1 cytokine family, and it is a key cytokine in regulating inflammation. IL-37 is originally defined as IL-1 family member 7 (IL-1F7). The anti-inflammatory cytokines TGF-β, IL-10, several toll-like receptor (TLR) ligands and pro-inflammatory cytokines such as IL-1β, TNF-α, IFN-γ and IL-18 induce IL-37 production in PBMCs (Braun & Sieper, 2007). Expression of IL-37 in macrophages or epithelial cells almost completely inhibits the synthesis of pro-inflammatory cytokines. IL-37 acts as an essential inhibitor of inflammation and innate immunity in various diseases (Sieper *et al.,* 2002).

Osteoporosis (OP) is a common complication of AS with incidence between 18.7% and 62%. It is greater in males, and increases with increasing patient's age and disease duration (Singh *et al.,* 2013). BMD may be affected in the early stages of the disease, the spine and hip BMD decreased predominantly in patients with active disease (Maillefert *et al.,* 2001). Bone loss in AS appears to be multifactorial; several studies have shown a significant correlation between markers of bone turnover, levels of pro-inflammatory cytokines, and acute phase reactants such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) suggesting that systemic inflammatory mediators may be involved in the pathogenesis of OP. (Lang *et al.,* 2000). Also mechanical factors (*i.e.*, rigidity of the spine, vertebral deformities) and decrease in physical activity or mineralization defects due to subclinical gut involvement (Ulu *et al.,* 2013). Osteoporosis (OP) may contribute to spinal fractures and progressive spinal deformity (Singh *et al.,* 2013).

Dual Energy X-ray Absorption (DEXA) is the most reliable technique for measurement BMD, it can be considered as an accurate, non-invasive, repeatable and quantitative method to assess BMD at the spine and hip (Mazess, 2000). However several studies have indicated that DEXA may be a misleading method to assess BMD in advanced AS particularly at the AP lumbar spine due to the aberrant ossification or degenerative changes. New bone formation and aberrant hyperostosis inevitably cause a pseudo increase in bone density. Despite its limitations, DEXA measurements with lateral spinal projections or (QCT) quantitative CT may be a solution to this problem in patients with advanced disease (El Maghraoui *et al.,* 2005).

The aim of the work was to investigate role of IL-37 in AS disease activity and osteoporosis.

Subjects and Methods

Study Approval

This study was approved by the Ethical Committee of Benha University institution.

All subjects gave their written informed consent before participation in this study.

Subjects

Twenty-five AS patients diagnosed according to modified New York Criteria (Van der Linden *et al.,* 1984) were recruited from the inpatients' and outpatients' clinic of the Rheumatology, Rehabilitation & Physical Medicine, department of Benha University and the Health Insurance hospitals in the period between October 2014 and April 2015. Inclusion of patients was based on the modified New York criteria:

- Requirements

A definite diagnosis of ankylosing spondylitis requires the radiological criterion and at least one clinical criterion to be satisfied as defined below.

- Radiological criterion

Sacroiliitis grade 2-4 bilaterally or grade 3 or 4 unilaterally.

- Clinical criteria

- Low back pain and stiffness for more than 3 months that improves with exercise but is not relieved by rest.

- Limitation of motion of the lumbar spine in both the sagittal and frontal planes.

- Limitation of chest expansion relative to normal values correlated for age and sex.

(Van der Linden *et al.,* 1984).

Twenty age and sex-matched volunteers were recruited from Benha University Hospitals as healthy controls. We excluded post-menopausal females and patients with other rheumatic diseases, infections or malignant tumors. Also patients with diseases (of the liver, kidney, renal stones, diabetes mellitus, alcoholism, parathyroid and thyroid) or on medications that might alter the bone metabolism.

Methods

All AS patients were subjected to: full history taking, thorough clinical examination, assessment of disease activity using the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) score (Calin *et al.,* 1999).

The BASDAI consists of a 1-10 scale measuring discomfort, pain, and fatigue (1 being no problem and 10 being the worst problem) in response to six

questions asked of the patient pertaining to the five major symptoms of AS: Fatigue, Spinal pain, Arthralgia or swelling, Enthesitis, Morning stiffness duration and Morning stiffness severity.

To give each symptom equal weighting, the average of the two scores relating to morning stiffness is taken. The resulting 0 to 50 score is divided by 5 to give a final 0 – 10 BASDAI score. BASDAI score ≥ 4 was defined as an active AS (Gratacós *et al.,* 1999).

- Laboratory procedures

The following investigations were done 1) complete blood count, 2) ESR by the Westergren method recorded in mm/1st h. 3) CRP by latex agglutination slide test, 4) liver function tests, 5) kidney function tests, 6) Parathyroid hormone, 7) T3, T4, TSH

- Sample Collection

About 5ml of venous blood was collected from each study subject by sterile venipuncture. Of these, 2 ml were allowed to clot naturally for 30 minutes; sera were separated and kept frozen at – 20^{o}C until used in ELISA. The remaining volume of venous blood was collected into an EDTA tube; 500 µl of anti-coagulated blood was added to 1.3mL RNA later solution (Applied Bio systems, USA) in 2 ml microfuge tubes and mixed thoroughly by inverting the tubes several times. Blood samples mixed with RNA later solutions were stored at -70°C until RNA extractions.

- Measurement of serum IL-37

The IL-37 was measured in the serum using a commercially available ELISA kit (Human Interleukin-37(IL-37) ELISA Kit, (PELOBIOTECH, GmbH–Germany) according to the manufacturer's protocol. In principle, it is an antigen detection immunoassay, utilizes the quantitative technique of a Sandwich enzyme-linked immunosorbent assay in which, wells of kit's microplates are already coated with antibodies (primary antibodies) specific for particular epitopes on human IL-37. Briefly, diluted serum samples were incubated in the wells which were then repeatedly washed to remove unbound proteins. Biotinylated conjugated anti-Human IL-37 antibody (secondary antibody) specific for the target epitopes were added into the microwells, incubated and then washed. Streptavidin horse radish peroxidase enzyme conjugated to the constant heavy chain of the secondary antibody was added into the microwells, incubated and the wells were washed again. The bound conjugate was developed as a coloured product (corresponding to the antigen concentration) by adding substrate. After the color is completely developed, the enzymatic reaction was terminated by addition of an acid solution. The optical density (OD) of each microwell was measured at 450 nm. Obtained optical density values, were converted into pg/ml by the Bio Rad ELISA data analysis software. The cut-off value was calculated as the mean absorbance value of the negative controls plus three standard deviations.

- RNA extraction and real-time polymerase chain reaction (RT-PCR)

a-Total RNA Extraction

Total RNA was extracted from blood samples mixed with RNA later solution using GeneJET RNA Purification Kit (Fermentas, EU) following the manufacturer instructions and the standard protocol.the eluted RNA was collected immediately, placed in ice or stored at -20ºC for further processing.

The quantity and purity of RNA were then detected by Spectrophotometer System (The Thermo Scientific NanoDrop™ 1000 Spectrophotometer, USA) at 260 nm and 280 nm using the formula: RNA concentration (µg/ml) = OD260 (optical density) x100 (dilution factor) x 40 µg/ml /1000.Samples with ratios from 1.8 to 2.0 were accepted for subsequent reverse transcription reaction (Baine *et al.,* 2013).

b- Reverse transcription & cDNA synthesis

cDNAs were obtained using Maxime RT PreMix Kit tubes (iNtRON BIOTECHNOLOGY). Each tube contains OptiScriptTM RT System, RT-PCR buffer (10Ý) and dNTPs in a dried pellet. The total reaction volume is 20 µl with 1 µg of total RNA, the cDNA synthesis reaction was performed as follows: cDNA synthesis occurs at 45^{0}C for 60 min. followed by RTase inactivation step at 95^{0}C for 5min in a thermal cycler (Biometra, Goettingen, Germany).

- Quantitative PCR analysis of IL-37 using SYBR GREEN

The real time PCR was performed in a total reaction volume of 20 µl. The specific primers used were as follows, IL-37, forward 5- AGTGCTGCTTAGAAGA CCCGG -3 and reverse, 5-AGAGTCCAGGACCAGT ACTTTGTGA -3; (Bioneer), (Chen *et al.,* 2015). The sequences of Beta–actin (act as housekeeping gene or endogenous control) forward 5-GATCATTGCTCCTC CTGAGC-3'and reverse 5-ACTCCTGCTTGCTGATC CAC-3. The PCR mixture contains 10 µl Super Real Pre Mix Plus SYBR Green, master mix (TIANGEN Biotech, Beijing), 2 µl of Rox dye, 0.6 µl of each primer, 2 µl of template DNA, nuclease-free water were added to achieve a reaction volume of 20 µl. In ABI7900HT (Applied Biosystems, USA) with the following instrument settings: 95°C for 2 min (initial denaturation) followed by 40 amplification cycles

(95°C for 10 Sec. and 58°C for 20 Sec.) for annealing and elongation respectively. Negative control that contains 2 μL nuclease free water instead of sample DNA was included in the run. Amplification specificity was checked by melting-curve analysis. the relative endogenous quantities of the IL-37 gene are normalized Against, the relative Quantities of the control Beta-actin gene fold expression changes are calculated using the equation $2^{-\Delta\Delta CT}$ (Livak & Schmittgen, 2001).

Radiological Evaluation

Lateral radiographs of the cervical, thoracic and lumbar spine were acquired and changes related to AS were assessed using the modified Stoke Ankylosing Spondylitis Spine Score (mSASSS). In the mSASSS, the anterior vertebral corners of the cervical and lumbar segments (lower border of C2 to upper border of T1andlower border of T12 to upper border of S1; measure a total of 24 VCs) are scored for the presence of erosions and/or sclerosis and/or squaring (1 point), syndesmophyte (2 points) and bridging syndesmophyte (3 points). The total score ranges from 0 to 72 (Creemers *et al.,* 2005).

BMD was determined for the lumbar spine and femur using dual X-ray absorptiometry (DEXA).The World Health Organization (WHO) definition of osteopenia and osteoporosis were used: osteopenia, T-score < -1 to > -2.5 SD and osteoporosis, T-score ≤ -2.5 SD. The lowest value of BMD measured in the lumbar spine, total hip or femoral neck was used (WHO, 1994).

Statistical Analysis

Collected data were tabulated, coded then analyzed using the computer program SPSS (Statistical package for social science) version 20, qualitative date were expressed in number and percent, quantitative data were expressed in mean and standard deviation. In the statistical comparison between the different groups, the significance of difference was tested using ANOVA (analysis of variance) to compare between more than two groups of numerical parametric data, kruskal wallis test was used in non-parametric data, post-hoc test was used to detect significance difference in-between groups, Student "t" test was used to compare between two groups of numerical parametric data, Mann-Whitney test was used in non-parametric data. Correlation coefficient (r) test was used correlating different parameters. P value <0.05 was considered statistically significant.

Results

This study included 25 AS patients and 20 apparently healthy volunteers as control group. Patients' and controls' data were summarized in Table 1. There were no significant differences between patients and controls as regards to age and sex (P>0.05). Associated clinical and laboratory characteristic of AS patients were summarized in Table 1.

Serum IL-37 levels were significantly higher in AS patients compared with the controls (P<0.001), (table 2). There is a significant elevation of serum IL-37 levels in active AS patients compared with inactive AS patients and controls (P<0.001). There is no statistical significant difference in the mean serum IL-37 levels between patients with inactive AS and controls (Figure 1A).

The expression of IL-37 mRNA from AS patients was higher than the controls (P<0.001) (Table2). In active AS mean expression of IL-37 showed significant increase compared with those with inactive AS and healthy controls (P<0.001).

IL-37 mRNA gene expression showed a significant increased expression in active AS patient by 25 folds increase vs. 12 folds for inactive AS respectively compared to control. No difference was observed in IL-37 mRNA levels between inactive AS patients and healthy controls (Figure 1B).

Table 1. Clinical and laboratory characteristics of the AS patients and controls

Characteristic	Active AS (N=15)	Inactive AS (N=10)	AS (N=25)	Healthy Control (n=20)	*P-value
Age (mean ±SD years)	31.9±9.9	29.3±8.6	30.5±10.3	35.3±9.3	NS
Sex No. (%) Male	12(80%)	7(70%)	19 (76%)	12 (60%)	NS
Sex No. (%) Female	3(20%)	3(30%)	6(24%)	8(40%)	
Disease duration(years)	5.73±3.5	6.46±4.78	6.09±3.95		
Back pain and morning stiffness	15	10			
Fatigue	13	4			
Arthralgia	9	5			
Arthritis	7	3			
Enthsitis	12	6			
Osteoporosis	9	4	13		
Spinal fracture	1	0	1		
Neurological manifestations (tingling and numbness)	1	1	2		
Cardiac manifestations (arrhythmias)	2	0	2		
Pulmonary manifestations (Dyspnea, chest infection)	1	0	1		
Anterior uveitis (eye redness, pain, floaters and sensitivity to light)	7	3			
ESR (mm/1st hour)	32.1±16.87	9.5±4.12	20.8± 12.53	6.7±1.06	<0.001
CRP (mg/L)	18.9±4.01	6.8±3.41	12.85±3.6	4.2±1.1	<0.001

**P* value >0.05 was considered not significant (NS).

Table 2. Mean serum levels of IL-37 and Expression of IL-37 mRNA in AS Patients and Controls.

	AS (n=25)	Controls (n=20)	*P-value
IL-37 (pg/ml) (mean ±SD)	257.8±125.3	149.3±87.1	<0.01
IL-37 mRNA	686542.3± 134578.6	19316.3±4573.1	<0.001

**P* value <0.05 was considered statistically significant.

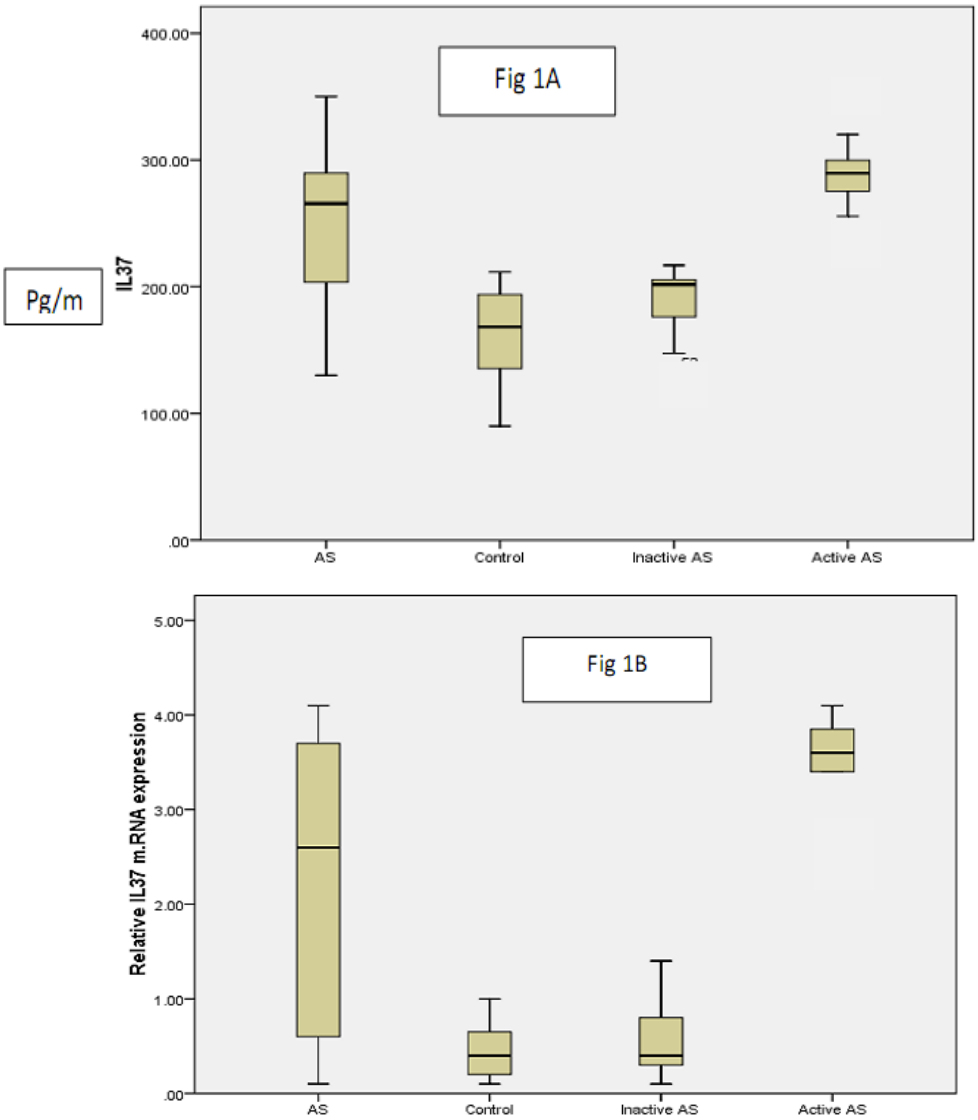

Figure 1 A & B. Mean serum levels of IL-37 and IL-37 mRNAs expression between AS patients and controls.

IL-37 mean serum levels were significantly elevated in AS patients with osteoporosis (P<0.05), while there were no significant increase in IL-37 serum levels with other clinical manifestations (P> 0.05) (Table 3).

The mean serum levels of IL-37 were higher in active AS patients with OP than active patients without OP, but this increase was statistically insignificant (P>0.05) (Table 4).

Table 3. Mean serum levels of IL-37 according to clinical manifestation of AS patient

		Mean ± SD	*P-value
Osteoporosis	Present (n=13)	251.8±119.3	<0.05
	Absent (n=12)	158.5±88.2	
Neurological manifestations	Present (n=2)	221.4±97.1	NS
	Absent (n=23)	207.2±81.2	
Cardiac manifestations	Present (n=2)	219.2±85.1	NS
	Absent (n=23)	202.3±99.3	

**P* value >0.05 was considered not significant (NS).

Table 4. Mean serum levels of IL-37 in AS patients with and without osteoporosis

	Active AS with osteoporosis(n=9)	Active AS without Osteoporosis (n=6)	*P-value
IL-37(pg/ml)(mean± SD)	273.36±112.3	189.96±88.1	NS

**P* value >0.05 was considered not significant (NS).

There was a significant positive correlation between the BASDAI score and serum IL-37 levels (r=0.9, P<0.001), (Figure 2). Figure 3 shows that mSASSS significantly correlated with femoral BMD (r= -0.8, P< 0.001).

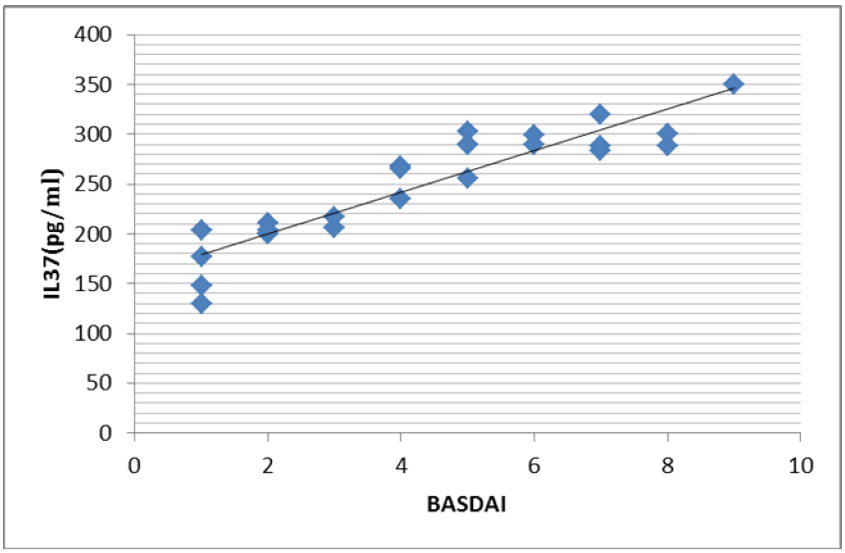

Figure 2. Correlation between BASDAI score and serum IL-37 levels

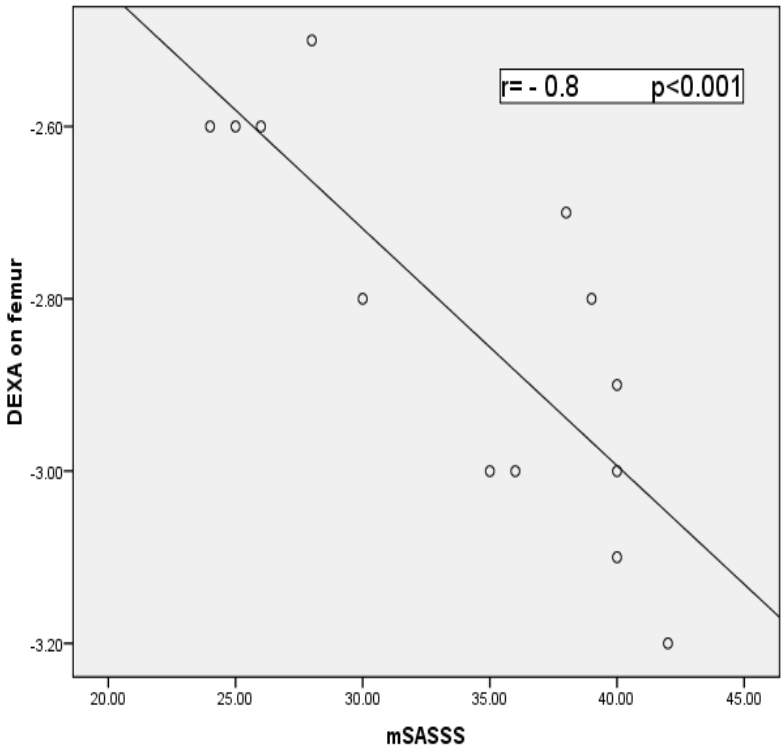

Figure 3. Correlation between femoral BMD and mSASSS

Discussion

Ankylosing spondylitis (AS) is a prevalent chronic inflammatory disease characterized by chronic inflammation in the axial skeleton and peripheral joints respectively and leading to bone erosion, which seriously influences the quality life of patients (Dean *et al.,* 2014). The onset, pathogenic process and severity of AS are depended on the degrees of inflammation in the disease (Braun *et al.,* 2007).

IL-37 is highly concentrated in the testis, thymus and uterus, and can be induced in various types of cells such as peripheral blood mononuclear cells (PBMCs), epithelial cells, dendritic cells, monocytes and keratinocytes (Klingberg *et al.,* 2012). Up-regulated expressions of IL-37 in serum have been reported in many inflammation-related disorders (Ye *et al.,* 2014).

The results of our study demonstrated that IL-37 mRNAs expressions and serum protein levels were significantly higher in AS patients than in controls. Further investigation revealed that IL-37 protein levels and its mRNAs relative expressions were significantly higher in 15 patients with active diseases than in 10 patients with inactive diseases and in 20 controls. However, there is no difference between inactive diseases and controls.

These results were in agreement with Chen *et al.,* (2015) who reported that IL-37 concentrations in the serum and IL-37 mRNA expression in PBMCs were dramatically higher in AS patients compared to the controls , and found that a significant upregulation of IL-37 mRNA expressions in 25 active AS patients compared with 21 inactive AS

patients and 46 controls . There was no difference observed in IL-37mRNA levels between inactive AS patients and controls.

Furthermore, our data showed that the IL-37 is positively correlated with the activity of AS patients (BASDAI score). This is consistent with the notion that both the levels of IL-37 and the disease activity are determined by the same underlying factors, most likely the levels of pro-inflammatory cytokines and these results imply that the inflammatory reaction of AS might stimulate the expression of IL-37. These results were in consistence with Chen *et al.,* (2015) who revealed that serum IL-37 levels were positively associated with BASDAI. Accordingly, serum IL-37 levels have a significantly positive correlation with CRP and ESR.

The anti-inflammatory mechanism of IL-37 is still not clear. There are two hypotheses: either IL-37 is secreted into the extracellular space to inhibit the actions of pro-inflammatory cytokines or their receptors Bufler *et al.,* (2002), or IL-37 translocates to the nucleus where it interacts with Smad3 to interrupt transcription of pro-inflammatory cytokine genes (Sharma *et al.,* 2008; Boraschi *et al.,* 2011).

Nold *et al.,* (2010) revealed that IL-37 can reduce the expression of STAT3 while the STAT3 have been reported to closely related to AS.

Also Tete *et al*., (2012) who reported that: IL-37 has been shown to significantly suppress IL-1b-induced expression of IL-1a, IL-8, IL-6, chemokines MIP-2/CXCL-2, MCP-5/CCL-12, and BCA-1/CXCL13, IL-23, IL-1RA, IL-17, IL-18, IFN-c and TNFa in several cell types. In contrast, pro-inflammatory cytokines IL-18, IFN-c, IL-1b and TNF-a can increase synthesis of IL-37 in human peripheral blood mononuclear cells.

In AS two enhanced but opposite bone remodeling processes are taking place in close vicinity within the spine; these are pathologic new bone formation in the cortical zone of the vertebrae, the zygapophyseal joints, and the ligamentous apparatus and excessive loss of trabecular bone in the center of the vertebral body leading to osteoporosis, (Klingberg *et al.,* 2012).

In our study 13 patients had osteoporosis (52%) and mean IL-37 serum levels was statistically significantly increased in AS patients with OP ($P< 0.05$).

These results are in hand with these reported by Chen *et al.,* 2015 who found that patients with active AS and with OP had the highest serum IL-37 levels also Başkan *et al.* (2010) revealed that the OP was related to the AS disease activity.

Klingberg *et al.,* (2012) demonstrated an increased prevalence of osteoporosis and significantly lower bone mineral density (BMD) in AS patients compared with sex and age matched controls.

Magrey & Khan (2010) revealed that OP was associated with the high levels of pro-inflammatory cytokines in AS patients. Also Chen *et al.,* (2015) had confirmed that serum pro-inflammatory cytokines levels such as TNF, IL-6 and IL-17 were significantly higher in AS patients with OP than those without OP.

We found that the higher the mSASSS score values, the lower the BMD results, Karberg *et al.,* (2005) found that patients with more syndesmophytes had a lower BMD than those without and Therefore, they suggested that bone loss and bone growth occur parallel, also even in the early stages of AS. This could not be confirmed by the study of van der Weijden *et al.,* (2012) because very few syndesmophytes were found in the early stage of disease in combination with a high prevalence of low BMD.

In conclusion, serum level of IL-37 and its mRNA expression is increased in AS patients with special consideration in patient with Osteoporosis and it is a good biological

marker in AS that well correlates with disease activity and BMD which indicate that IL-37 may provide a novel research target for the pathogenesis and therapy of AS.

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