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High IL-23 level is a Marker of Disease Activity in Rheumatoid Arthritis

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Rheumatoid arthritis (RA) is a chronic autoimmune systemic disorder characterized by inflammatory responses mainly affecting the synovial joints. Interleukin-23 (IL-23) is a heterodimeric pro-inflammatory cytokine secreted by activated dendritic cells and activated macrophages. IL-23 is the key cytokine controlling inflammation in peripheral tissues leading to the development of autoimmune diseases. The objective of our study was to determine the relationship between the IL-23 level and disease activity in RA patients. Sixty RA patients were included in the study with mean age of 40 years; they included 44 (73.3 %) females and 16 males (26.7 %). The clinical parameters of disease activity were determined, including the 28-joint disease activity score (DAS28), serum levels of C-reactive protein (CRP), Anti-citrullinated peptide antibody (ACPA), rheumatoid factor (RF), and TNF-α and the degree of bony erosions based on X-rays. Patients were subdivided into active disease group (n=30) with DAS28 score higher than 5.1 (Group I); and remission group (n=30) with DAS28 score less than 2.6 (Group II). Thirty healthy individuals in the same age group of RA patients including 22 (73.3%) females and 8 males (26.7%) were randomly selected as the control group (Group III). The levels of IL-23 were determined by enzyme-linked immunosorbent assay (ELISA) and the correlations between the serum levels of IL-23 and disease activity parameters of patients with RA were determined. Serum levels of IL-23 were significantly higher in RA patients during active stage of the disease in comparison to the patients in remission and the control group. There was a significant positive correlation between serum IL-23 levels in RA patients and individual disease activity parameters. It is concluded that elevated serum IL-23 level may be a useful marker to detect active RA and disease progression in patients with RA.

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by progressively destructive joint inflammation, destruction of articular cartilage and bone and synovial hyperplasia (Kim *et al.,* 2007). The chronic inflammation process is responsible for stimulating destructive mechanisms in the joints, causing structural damage and leading to functional disability and declines (Bingham, 2002). Cytokines play a fundamental role in the processes that cause inflammation, articular destruction and extra-articular manifestations associated with RA (Brennan & McInnes, 2008). The list of potential proinflammatory cytokines important in the pathogenesis of RA and other autoimmune and inflammatory diseases has been extended by another novel cytokine; interleukin (IL)-23. This cytokine plays a potential role in immune modulation of different subpopulations of T helper (Th) cells in concert with IL-12 (Lupardus & Garcia, 2008). IL-23 is a heterodimeric pro-inflammatory cytokine belonging to the IL-6/IL-12 cytokine family. IL-23 consists of two subunits; a unique IL-23 p19 chain and an IL-12 p40 chain. Like IL-12, IL-23 is produced predominantly by macrophages and dendritic cells and, together with IL-12, drives the Th1 response (Brombacher *et al.,* 2003). IL-12/23 is now believed to play a crucial role in inflammation and development of autoimmune diseases, since blocking of IL-12 p40 expression or knocking out of the p40 gene appears to prevent inflammation in various disease models (Kopp *et al.,* 2003). IL-23 can induce chronic inflammation through two independent pathways. A first pathway is by the activation of Th17 cells and the second by the induction of the secretion of

IL-17 by non-T cells (Izcue *et al.,* 2008). IL-23 promotes Th17 cells producing Tumour necrosis factor-α (TNF- α), IL-17, IL-6, IL-22, and GM-CSF which are associated with the induction of autoimmune inflammation in RA disease (Brennan & McInnes, 2008). These cytokines, especially IL-17, have a central role in autoimmune tissue inflammation in the joints and T cell-mediated activation of osteoclastogenesis (Kim *et al.,* 2007). Recent reports have indicated that in humans; increased amounts of IL-23 have been associated with several autoimmune disease including RA, Lyme arthritis, inflammatory bowel disease, Crohn's disease (CD), psoriasis and multiple sclerosis (MS) (Brentano *et al.,* 2008). Guo *et al.,* (2013) confirmed that the circulating IL-23 concentration was much higher in RA patients than healthy controls, which suggests that IL-23 may be a useful index for the diagnosis of RA. The serum IL-23 levels in RA were correlated with the CRP and the DAS28 score. Thus, the levels of IL-23 reflect RA disease activity (Guo *et al.,* 2013). In this work, we address the biologic contribution of IL-23 to RA pathology as we determined whether or not serum levels of IL-23 are increased in patients with RA in comparison to the healthy controls, and whether or not the increased levels of IL-23 correlate with disease activity.

Patients and Methods

This study was carried out in the Department of Rheumatology and Rehabilitation and Department of Medical Microbiology and Immunology, Faculty of Medicine, Sohag University. Sixty RA patients referred to the outpatient clinic of the Rheumatology and Rehabilitation Department at Sohag University Hospital during the period from January 2012 to February 2013 were included in the study; 44 (73.3 %) of whom were females and 16 males (26.7 %). Their mean age was 40 years (range 20- 60 years). Serum samples were collected from patients clinically diagnosed and classified as RA according to the American College Of Rheumatology revised criteria for the classification of RA (Aletaha *et al.,* 2010). In addition to 30 healthy individuals in the same age group without evidence of chronic inflammatory disease; 22 (73.3 %) females and 8 males (26.7 %) have been considered as the control group (Group III). This study was approved by ethics committee of Sohag Faculty of Medicine and informed consents were taken from all the study population. All patients were evaluated clinically with 28 joints disease activity score (DAS28); depending on number of joint with tenderness and swelling (Prevoo *et al.,* 1995) and 100-mm visual analogue scales (VAS). At the same time, the erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), Anti-citrullinated peptide antibody (ACPA), serum level of TNF- α and rheumatoid factor (RF) levels were determined using standard laboratory methods. The patients were subdivided into active disease group (Group I) (n=30) with DAS28 score > 5.1, and remission group (Group II) (n=30) with DAS28 score < 2.6. Bony erosions were determined by a radiologist, who had no clinical information, based on review of X-rays of the hand joints using Larsen Score (Larsen, 1995).

Exclusion criteria

Patients found to be suffering from osteoarthritis, tubercular arthritis, infective arthritis, rheumatic fever, pulmonary tuberculosis, and any other systemic disease as renal disease, diabetes mellitus, hepatic diseases, hypertension, or ischemic heart disease.

Serum samples were collected from all subjects and stored until analysis.

Measurement of IL-23 levels

IL-23 assay was performed by measuring IL-23 in serum samples using Human IL-23 ELISA Ready-SET-Go! ® Set *(eBioscience, Inc., USA)*. The Ready-SET-Go! ® Human cytokines ELISA kit is an enzyme-linked Immunosorbent assay for the quantitative measurement of human cytokines in serum. A monoclonal antibody specific for the tested cytokine (IL-23) had been pre-coated onto a microplate. Standards and samples were pipetted into the wells and any tested cytokine could be present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme linked polyclonal antibody specific for the tested cytokine was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color develops in proportion to the amount of tested cytokine bound in the initial step. The color development was stopped and the intensity of the color was measured using a microplate reader set to 450 nm. The results were calculated using the constructed standard curve. Construction of a standard curve was made by plotting the mean absorbance for each standard on the vertical axis against the concentration of tested cytokine on the horizontal axis and drawing a best-fit curve through the points on the graph.

All tests were done according to the manufacturer's instructions using Stat Fax 2600 ELISA work station (Awareness Technology Inc., Palm City, USA).

ELISA plate coated with 100 µl/well of capture antibody in Coating Buffer. The plate was sealed and was incubated overnight at 4°C. Wells were aspirated and were washed 5 times with 250 µl/well Wash Buffer. The mixture were diluted 1 part 5X concentrated Assay Diluent with 4 parts distilled water. Wells were blocked with 200 µl/well of 1X Assay Diluent and were incubated at room temperature for 1 hour. Aspirate/wash was done as in step 2 for a total of 5 washes. Using Sample Diluent A, standards were diluted.100 µl/well of standard were added to the appropriate wells. 2-fold serial dilutions of the top standards were performed to make the standard curve. 100 µl/well of the samples were added to the appropriate wells, diluting them at least 2-fold in Sample Diluent A. The plate was covered and was incubated at room temperature for 2 hours. Aspirate/wash was done as in step 2 for a total of 5 washes. 100 µl/well of detection antibody was added diluted in 1X Assay Diluent. The plate was sealed and was incubated at room temperature for 1 hour. Aspirate/wash was done as in step 2 for a total of 5 washes. 100 µl/well of Avidin-HRP diluted in 1X Assay Diluent was added. The plate was sealed and was incubated at room temperature for 30 minutes. Aspirate/wash was done as in step 2. In this wash step, wells were soaked in Wash Buffer for 1 to 2 minutes prior to aspiration for a total of 7 washes. 100 µl/well of Substrate Solution were added to each well. Plate was incubated at room temperature for 15 minutes. 50 µl of Stop Solution was added to each well. The optical density was measured at a wavelength of 450 nm using a Stat Fax 2600 Microplate Reader (Awareness Technology Inc., Palm City, USA).

Measurement of TNF-α levels

The serum concentration of TNF-α was measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (AviBion Human TNF-α ELISA kit, Orgenium Laboratories Business Unit, Finland). All tests were done according to the manufacturer's instructions using Stat Fax 2600 ELISA work station (Awareness Technology Inc., Palm City, USA).

50 µl standard, test samples and sample diluents were added in each well of a 96-well plate coated with TNF-α monoclonal antibody. The mixture was then incubated for one hour at room temperature, and each well was washed five times with wash solution. 50 µl ready for use biotin antibody was added to each well. The mixture was then incubated for 30 minutes at room temperature, and each well was washed five times with wash solution. Subsequently, 50 µl ready for use horseradish peroxidase (HRP)-Streptavidin solution was added to each well. The mixture was then incubated for 30 minutes at room temperature, and again, each well was washed five times with wash solution. 50 µl TMB substrate solution was added to each well and the mixture was then incubated for 20 minutes at room temperature. The reaction was quenched by the addition of 25µl stop solution. Immediately; the optical density was measured at a wavelength of 450 nm using a Stat Fax 2600 Microplate Reader (Awareness Technology Inc., Palm City, USA). The serum concentration of TNF-α was determined based on a standard concentration curve.

Statistical Analysis

To determine statistical differences by using analysis of variance (ANOVA) and Student´s t-test. All values were expressed as means ± SD; P values < 0.05 were considered significant. The relationship between studied measures was assayed by Pearson's linear correlation coefficient.

Results

The present study was carried out in Sohag University Hospital during the period from January 2012 to February 2013. Sixty RA patients were included in our study; 44 (73.3 %) females and 16 males (26.7 %). All patients were subjected to complete clinical assessment and routine laboratory investigations including; ESR, RF, CRP, and ACPA. X-rays of the hand joints were done to all patients. The demographic and clinical data of the patients are shown in Table 1.

Table 1. Demographic and Clinical data of patients with Rheumatoid Arthritis

Indices	Values
Age (years)	40
Gender (females/males, *n*)	44/16
Disease duration (years)	10 ± 2
Morning stiffness (hours)	1± 0.5
Tender joint count	7.3 ± 1.5
Swollen tender joint count	5.4 ± 2.3
DAS28	5.2 ± 1.1
Rheumatoid factor (IU/ml)	32.6 ± 15.2
C-reactive protein (mg/L)	24 ± 6
Anti-citrullinated peptide antibody (ACPA) (IU/ml)	200 ± 25
X-ray classification (I/II/III/IV, *n*)	12/18/15/15

Serum level of IL-23 in RA patients and healthy controls

The mean serum level of IL-23 was (420.5± 15.3) ng/ml in the patient group and (135 ± 12) ng/ml in the healthy control group. The level was significantly elevated in the patient group compared with the healthy control group (P <0.007). (Table 2, Fig 1)

Table 2. Serum level of IL-23 in RA patients and healthy controls.

Parameter	RA patients (60)	Healthy control group (30)	**P* value
Serum level of IL-23 (ng/ml)	420.5± 15.3	135 ± 12	<0.007

**P*<0.05 is significant

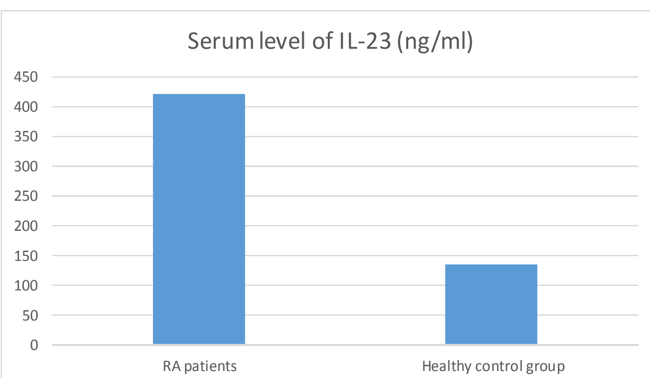

Figure 1. Serum level of IL-23 in RA patients and healthy controls

Serum level of TNF-α in RA patients and healthy controls

The serum level of TNF-α was (210 ± 10.3) ng/ml in the patient group and (22.2 ± 3.5) ng/ml in the healthy control group. The serum level of TNF-α was significantly elevated in the patient group compared with the healthy control group (P =0.006). (Table 3)

Table 3. Serum level of TNF-α in RA patients and healthy controls

Parameter	RA patients (60)	Healthy control group (30)	**P* value
Serum level of TNF-α (ng/ml)	210 ± 10.3	22.2 ± 3.5	0.006

**P*<0.05 is significant

The relationship between IL-23 levels and disease activity

The serum level of IL-23 was (375± 10.2) ng/ml in patients with active disease (30 patients) and (201 ± 5.8) ng/ml in patients in remission (30 patients). The serum level of IL-23 was significantly elevated in patients with active disease compared to patients in remission (P = 0.01). The serum concentration of IL-23 had positive correlation with the CRP level (r =0.381, P=0.005), DAS28 (r =0.683, P=0.01), and TNF-α level (r =0.596, P=0.01), (Table 4, Fig. 2).

Serum level of IL-23 in RA patients with X-ray classification phases I, II, III, and IV were significantly elevated (P =0.04), (Table 5, Fig. 3).

Table 4. The relationship between IL-23 levels and disease activity

Indices	active disease group (30 patients)	patients in remission group (30 patients)	**P* value
Serum level of IL-23 (ng/ml)	375± 10.2	201 ± 5.8	0.01
DAS-28	> 5.1	< 2.6	
Serum level of TNF-α (ng/ml)	200± 10.3	85± 5.5	0.01

**P*<0.05 is significant

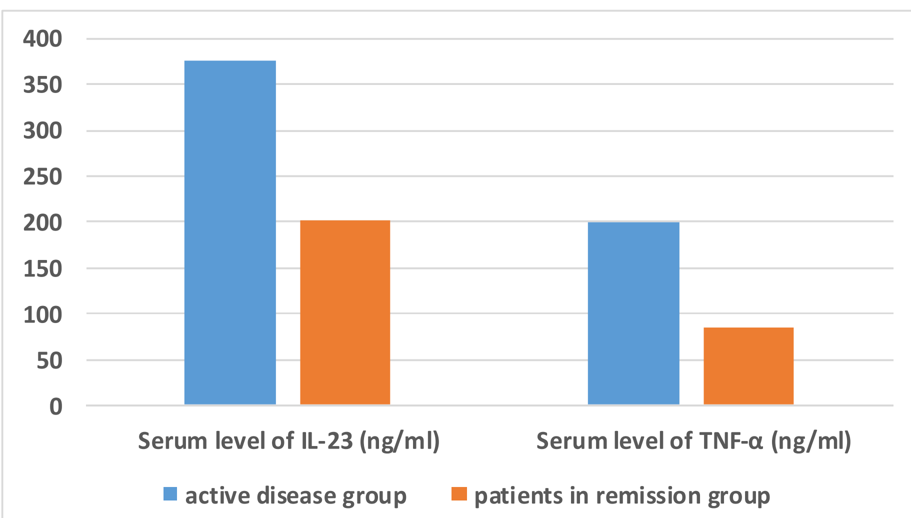

Figure 2. The relationship between IL-23 levels and TNF-α levels

Table 5. Relationship between IL-23 serum levels and X-ray classification

X-ray classification	N of patients	Serum level of IL-23 (ng/ml)
I	12	135 ± 13
II	18	250 ± 25
III	15	305 ± 20
IV	15	400 ± 10

P=0.04 (*P*<0.05 is significant)

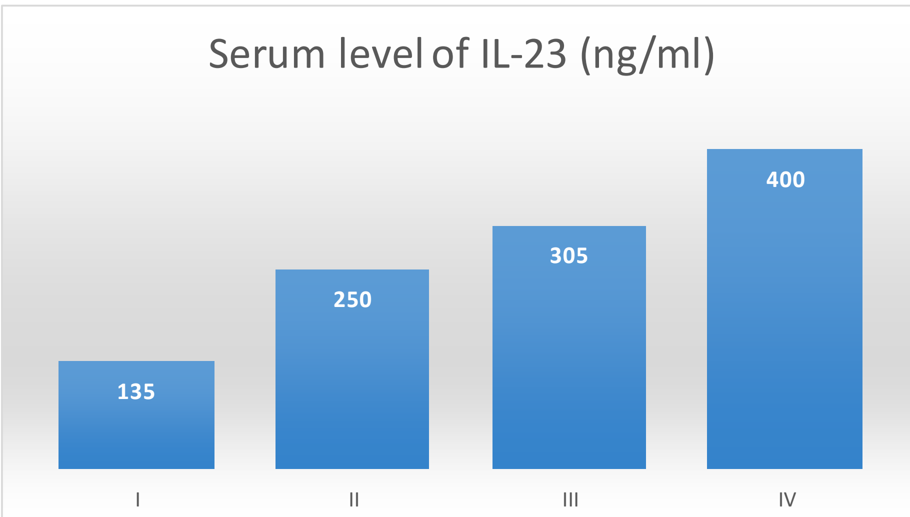

Figure 3. The relationship between IL-23 and X-ray classification

Discussion

Rheumatoid arthritis (RA) is a systemic inflammatory disease, of autoimmune origin (Piccoli *et al.,* 2011). In RA, several cytokines, e.g. IL-1, IL-6, IL-17, IL-23, and tumour necrosis factor-α (TNF-α) are involved in almost all aspects of articular inflammation and destruction (Liu *et al.,* 2007). IL-23 is one of the essential factors required for the survival and / or expansion of Th17 cells, which produce IL-17, IL-17F, IL-6 and TNF- α and is thus believed to contribute to inflammatory conditions and in the pathogenesis of autoimmune diseases including RA (Paradowska *et al.,* 2010). In the current study we determined the serum concentration of IL-23 by ELISA in RA patients in comparison to healthy individuals and we determined the relationship between the IL-23 level and disease activity in RA patients by confirming the correlation of IL-23 RA disease activity markers as CRP, ESR and TNF- α levels and the DAS28 score. Our study showed that levels of IL-23 were significantly increased in RA patients in comparison to healthy individuals. We also found that the serum IL-23 levels in RA were correlated with the CRP and the DAS28 score. Thus, we found a significant positive correlation between the serum levels of IL-23 in RA patients and disease activity. Our results are in agreement with results of Rasmussen *et al.,* (2010) which stated that the plasma levels of IL-23 are increased in RA patients and these levels were correlated with disease activity. Similar results are reported by Melis *et al.,* (2010) who reported that increased levels of IL-23 in plasma of patients with chronic RA are associated with the pathogenesis and disease activity. IL-23 production appears to be of great importance in the inflammatory reaction in RA, because its biologic activity causes the activation of Th17 cell, producing IL-17, a proinflammatory cytokine that induces other proinflammatory cytokines (Kim *et al.,* 2013). Th17 cells stimulated by the IL-23 promote osteoclastogenesis. Clinical trials of anti-IL-23 in humans have not yet been reported, but trials of anti-p40 in Crohn's disease and psoriasis were shown to be efficacious (Hillyer *et al.,* 2009). From all of these results we can deduced that; IL-23 has an important role in the development of autoimmune pathology of RA and so, we can suggest that the use of IL-23 blockers as a modality for treatment of RA. To sum-up Rheumatoid arthritis is a debilitating, chronic multisystem autoimmune disease. It is now clear that; a large number of cytokines are active in the joints of patients with rheumatoid arthritis (RA). These cytokines play a fundamental role in the processes that cause inflammation, articular destruction, and the comorbidities associated with RA. The results of our study indicated that the novel proinflammatory cytokine; IL-23 can contribute in the pathogenesis of RA and may be considered as a marker of disease activity. These results may be useful in the management of new modalities of therapy for the treatment of RA using direct IL-23 blockade which may be an important key in treating such inflammatory autoimmune diseases.

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