Evaluation of Serum and Synovial Fluid Interleukin-33 Levels in Patients with Active Rheumatoid Arthritis: The Association with Disease Activity and Clinical Findings

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Abstract
Aim of work: To evaluate serum and synovial fluid (SF) interleukin-33 (IL-33) levels in patients with active rheumatoid arthritis (RA) and to investigate the association with the disease activity, clinical and laboratory findings. Patients and Methods: Fifty RA patients with disease activity score 28 (DAS28) >3.2 and 30 patients with osteoarthritis (OA) serving as a control group were included in this cross-sectional study. Matched serum and SF were obtained from both patients and controls. Measurement of serum and SF IL-33 was done using the Human IL-33 Enzyme-linked immunosorbent assay. Results: Median serum and SF IL-33 levels in RA patients were higher than OA control group (82.3 pg/ml (range 27.7-180) vs. 21 pg/ml (range 6-65) and 135.75 pg/ml (range 79.7-228.9) vs. 23 pg/ml (range 7-42) respectively) with a high statistically significant difference between them ($p<0.001$). SF IL-33 levels showed higher results than matched serum samples in the RA group with a positive significant correlation ($p<0.001$). A statistically insignificant association was found between serum and SF IL-33 levels with erythrocyte sedimentation rate, C-reactive protein, tender joint count, swollen joint count, DAS28, disease duration, anti-cyclic citrullinated peptide antibody, rheumatoid factor and modified Sharp Score. Conclusion: IL-33 levels showed an increase in serum and SF in RA patients supporting the notion of its involvement in the pathogenesis of RA; however, it may not be a sensitive marker of disease activity.
Keywords: Rheumatoid Arthritis; Interleukin-33; DAS-28

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disorder in which joint inflammation leads to damage to cartilage and bone, ultimately leading to disability [1]. In addition, systemic involvement occurs including lung and cardiovascular disorders [1].
RA affects approximately 1% of the population worldwide with females being affected more frequently than males at 40 to 60 years of age [2]. Common symptoms include joint pain, swelling and stiffness [3].
Cytokines have a fundamental role in RA pathogenesis [4]. Leukocytes infiltrate the synovial joints; pro-inflammatory mediators fill the synovial fluid thereby initiating an inflammatory cascade [4].
When first discovered, interleukin-33 (IL-33) was believed to be a nuclear factor present in endothelial cells [5]. It was not until later that it was identified as part of the IL-1 family; inducing cytokine production including IL-4, IL-5 and IL-13 and mediating inflammation by binding to its receptor ST2 [6]. Many immune and non-immune cells produce IL-33 including mast cells, dendritic cells, macrophages, smooth muscle cells, endothelial and epithelial cells and fibroblasts [7]. It is mainly confined to the nucleus but as a result of inflammation it is either released in a passive manner from necrotic cells or secreted extracellularly from healthy cells [8].
Over the years, evidence of IL-33 involvement in RA has been appreciated. Several studies reported the increase of serum and synovial fluid IL-33 in patients with RA [9-11]. Furthermore, introduction of an anti-ST2 antibody at disease onset decreased the severity of arthritis leading to reduction of joint destruction and cartilage erosion and diminished neutrophil infiltration, suggesting the possible contributory role of IL-33 in the pathogenesis of joint destruction [12].
IL-33 has also been shown to inhibit apoptosis of fibroblast-like synoviocytes in RA and promote their proliferation consequently leading to an increase inflammatory cytokines [13].
Our study aimed to evaluate serum and synovial fluid interleukin-33 levels in patients with active RA and to investigate the association with the disease activity, clinical and laboratory findings.

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2. Material and Methods

A cross-sectional study was conducted on a series of 50 active RA patients with disease activity score 28 (DAS28) >3.2 [14] fulfilling the 2010 American College of Rheumatology/European League against Rheumatism (ACR-EULAR) classification criteria for rheumatoid arthritis [15]. In addition, 30 control subjects were recruited from osteoarthritis (OA) patients. Matched serum and synovial fluid (SF) were obtained from patients and controls attending the outpatient clinic of the Rheumatology and Rehabilitation Department, Cairo University Hospitals. Informed consent was obtained from all subjects and the study was approved by Cairo University Ethics Committee according to the Declaration of Helsinki. RA patients receiving biologic agents; anti-tumor necrosis factor drugs or anti-interleukin 1 drugs, and control OA patients receiving anti-interleukin 1 drugs were excluded to avoid affecting IL-33 results [10].

Full history was taken from all patients and clinical examination was performed. Complete blood count (CBC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), liver and kidney function tests were done. Rheumatoid factor (RF) was assayed by immunonephelometry and anti-cyclic citrullinated peptide (anti-CCP) antibodies were measured using the chemiluminescent microparticle immunoassay method. IL-33 was assessed by Enzyme-Linked Immunosorbent Assay (ELISA) in both patients and controls. Plain radiographs of both hands and feet and chest x-ray were done. Disease activity was calculated using DAS28.

2.1. Quantitative measurement of Interleukin-33 (Serum and synovial)

Human IL-33 ELISA kit was used according to the manufacturer's instructions (Wuhan EIAab Science Co., Ltd, China). Detection range was 15.6 picograms/milliliter (pg/mL) -1000 pg/mL. The microtiter plate provided in this kit has been pre-coated with an antibody specific to IL-33. Standards or samples were then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for IL-33 and Avidin conjugated to Horseradish Peroxidase was added to each microplate well and incubated. Then a TMB substrate solution was added to each well. Only those wells that contained IL-33, biotin-conjugated antibody and enzyme-conjugated Avidin exhibited a change in color. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm ±2 nm. The concentration of IL-33 in the samples was then determined by comparing the optical densities of the samples to the standard curve.

Statistical analysis

Data was coded and entered using the statistical package SPSS version 15. Data was summarized using number and percent for qualitative variables. Mean and standard deviation were used for normally distributed quantitative variables, while median and range were used for quantitative variables not normally distributed. Comparison between groups was done using chi square test for qualitative variables, independent sample t test for quantitative variables which were normally distributed while non-parametric Mann-Whitney U test for quantitative variables not normally distributed. Correlations were done to test for linear relations between variables using Spearman's rank correlation test. P-values less than or equal to 0.05 were considered statistically significant.

3. Results

Fifty RA patients and 30 OA controls were included in the study. The patients in the RA group were 37 (74%) females and 13 (26%) males ages ranging from 27-65 years (mean 48.38 ± 11.14 years). In the OA group, there were 21 (70%) females and nine (30%) males with age range from 35-61 years (51.23 ± 7.84 years). A statistically insignificant difference was found between patients and controls regarding age and sex with a p-value > 0.05. Clinical, radiological and laboratory characteristics of patients are presented in **Table 1**.

Table 1: Clinical, radiological and laboratory characteristics of the studied RA patients.

Clinical data	Range	
Disease duration(years)	1-30	7*
Morning stiffness(minutes)	0-300	30*
TJC	1-25	5*
SJC	1-15	3*
MSS erosions	1-163	10*
MSS JSN	6-135	40*
Total MSS	8-298	48.50*
DAS 28	3.55-7.59	5.35 ± 0.8 **
ESR (mm/hour)	12-112	74 *
CRP (mg/l)	4.3-74.3	15.45 *
Anti-CCP (U/ml)	1-265	84.4 *

*: values expressed as median **: values expressed as mean ± SD (standard deviation).
TJC: tender joint count; SJC: swelling joint count; MSS: modified Sharp score; JSN: joint space narrowing; DAS: Disease Activity Score; TLC: total leucocytic count; ALT: Serum alanine aminotransferase; AST: Serum aspartate aminotransferase; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; Anti-CCP: Anti-cyclic citrullinated peptide.

IL-33 serum and synovial fluid concentrations were higher in RA patients compared with controls. The

median serum IL-33 level in patients was 82.3 pg/ml (range 27.7-180) while in controls it was 21 pg/ml (range 6-65). Comparison of both groups showed a high statistical significance (p<0.001) (Fig.1).

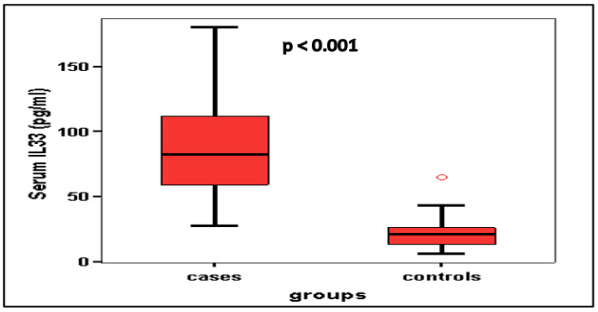

Fig. 1 Comparison of serum interleukin-33 (pg/ml) in cases and controls

The median synovial fluid IL-33 level in patients was 135.75 pg/ml (range 79.7-228.9) while in controls it was 23 pg/ml (range 7-42). Comparison of both groups showed a high statistical significance (p<0.001) (Fig. 2).

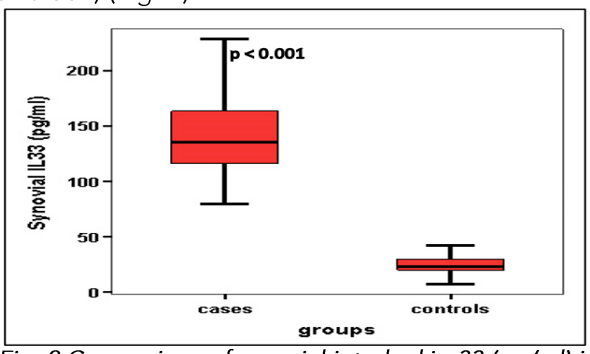

Fig. 2 Comparison of synovial interleukin-33 (pg/ml) in cases and controls

Synovial IL-33 was higher than serum levels in RA patients where a positive significant correlation was found with a p-value of <0.001 (Fig. 3).

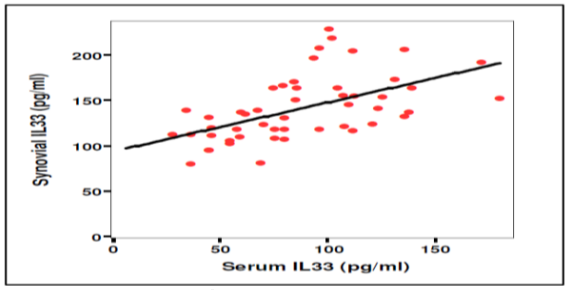

Fig. 3 Correlation of serum and synovial interleukin-33 in RA patients

RF was positive in 35 patients (70%) and negative in 15 patients (30%). When comparing serum and synovial fluid IL-33 concentrations in both groups, no statistical significance was found (p > 0.05). Anti-CCP antibodies were positive in 38 patients (76%) and negative in 12 patients (24%). Comparing serum and synovial fluid IL-33 in both groups also showed no statistical significance (p > 0.05).

In regard to disease activity that was measured by DAS28, 36 (72%) patients had high disease activity and 14 (28%) had moderate activity. In the high disease activity group, the median serum IL-33 level was 79.95 (range 27.7 – 180) pg/ml and in the moderate disease activity group, it was 85.25 (range 44.7-136.2) pg/ml. Comparison between the median serum IL-33 levels and DAS28 showed no statistical significance (p=0.72).

In the high disease activity group, the mean ± SD synovial IL33 level was 140.66 ± 36.86 (range 79.7-228.9) pg/ml, and in the moderate disease activity group, the mean ± SD synovial IL33 level was 141.51± 33.53 (range 94.7-206) pg/ml. Comparison between the mean synovial IL-33 levels and DAS28 showed no statistical significance (p=0.94).

Correlation between serum and synovial IL-33 levels with various clinical, radiological and laboratory variables all showed no statistical significance **(Table 2).**

Table 2: Correlation between serum and synovial IL-33(pg/ml) levels and clinical, radiological and laboratory variables.

Variable	Serum IL33		Synovial IL33	
	r	P	r	p
Age(yr)	0.018	0.902	0.084	0.560
Disease duration(yr)	0.005	0.971	0.139	0.336
Morning stiffness	-0.095	0.511	0.074	0.608
SJC	-0.155	0.283	-0.102	0.482
TJC	0.085	0.559	0.175	0.225
DAS28	0.26	0.856	0.072	0.620
ESR mm/h	-0.035	0.807	-0.086	0.553
CRP mg/l	0.083	0.567	-0.031	0.832
Anti-CCP U/ml	0.256	0.072	0.057	0.696
MSS erosion	0.051	0.725	0.137	0.343
MSS JSN	-0.257	0.071	0.106	0.464
Total MSS	-0.185	0.199	0.113	0.435

R: correlation coefficient

SJC: swelling joint count; TJC: tender joint count; ESR: erythrocyte sedimentation rate; DAS28: disease activity score 28; CRP: C-reactive protein; anti-CCP: anti-cyclic citrullinated peptide; MSS: modified Sharp score; JSN: joint space narrowing

4. Discussion

Interleukin-33 is now a well-substantiated member of the IL-1 family. It is present in many tissues; however its cellular distribution is limited to epithelial cells, smooth muscle cells fibroblasts, dendritic cells and macrophages [16]. Over production of pro-inflammatory cytokines by IL-33 advocates its role in

pathogenesis of RA [17].

In our study, serum IL-33 in RA patients was significantly higher compared to controls ($p < 0.001$). In agreement with our study, Mu et al. detected IL-33 in 94 out of the 223 patients with RA [18]. In their study they stated that IL-33 serum concentration showed a significant increase in patients with RA than in 71 healthy controls and 40 OA patients. In addition, they demonstrated a decrease in serum IL-33 concentration after treatment with anti-tumor necrosis factor (TNF) which lead them to the assumption that TNF-α is involved in excess IL-33 production in RA [18].

Matsuyama et al. also reported elevation of serum IL-33 level in 30 out of 59 RA patients and this increase was statistically significant compared to patients with infectious diseases and healthy controls [9]. In 121 RA patients, Xiangyang and his colleagues found a statistically significant increase in serum IL-33 compared to 47 controls [19].

In consistence with previous studies, our results showed a significant increase in SF IL-33 in RA patients as compared to controls ($p < 0.001$). Matsuyama et al. found synovial IL-33 to be elevated in all 15 cases of RA patients, while it was undetected in 11 out of 13 OA cases [9]. Hong et al. measured SF IL-33 in 16 RA patients and 10 OA patients; results showed a significantly higher level in RA than OA [11]. In the study by Tang and coworkers they concluded that SF IL-33 was significantly higher in RA than OA patients [20].

In the present study we showed that matched serum and SF samples in the RA group showed a significantly higher SF IL-33 concentration than that of serum ($p<0.001$). In concordance with this result, Matsuyama et al. demonstrated an increase in SF IL-33 concentration greater than that of serum in all 7 RA patients measured simultaneously. They proposed that IL-33 behaves as a cytokine by binding to its receptor ST2 in the inflamed joint [9].

In contrast to these findings however, Mu et al. found comparable levels in matched serum and synovial fluid IL-33 concentrations in 17 RA patients [18]. In a study performed on 10 RA patients, Talabot-Ayer et al. also reported comparable IL-33 concentrations in matched serum and synovial samples [21]. The discrepancy between these results and our study could be explained by the smaller sample size of these studies in comparison to ours.

In their study, Xu et al. showed that treatment with IL-33 showed aggravation of arthritis in mice [22]. Therefore, it would seem logical to assume that IL-33 interrelates with disease activity. On the contrary, we showed an insignificant statistical correlation between IL-33 concentrations and DAS28, results of which agreed with studies by Mu et al. and Xiangyang et al. [18, 19]

Other studies however disagreed with our results. Matsuyama et al. reported that DAS28-CRP showed a significant elevation in patients who demonstrated positive levels of serum IL-33 compared to those with negative levels [9]. Similarly, Abdel-Wahab et al. and Kageyama et al. also found a significant positive correlation of DAS28 with serum IL-33 [23, 24].

The differences in these results may be due to differences in the degree of RA activity in different studies as well as the difference in the sample size and the demographic features of the patients.

Correlation between IL-33 and disease duration in our study showed no significance, results of which matched a study conducted by Abdel-Wahab et al. [23] .In contrast, two studies conducted on Chinese patients found that longer disease duration was associated with higher serum IL-33 levels [18,19]. Interestingly enough, our study and the study by Abdel-Wahab et al. [23] were performed on Egyptian patients suggesting that demographic differences might be a cause of this discrepancy.

Our study showed an insignificant statistical correlation of IL-33 levels with the tender joint count (TJC) and the swollen joint count (SJC). In agreement with our results, Mu et al. found an insignificant correlation of IL-33 with TJC and SJC in the 223 RA patients they studied. Furthermore, the patients were divided into IL-33 positive and negative. Results showed no significant differences between both groups [18].

The results in the study by Kageyama et al. was in agreement with our study regarding the insignificant correlation between SJC and IL-33 levels. However, they found a positive significant correlation between the TJC and IL-33 levels [24]. Our results were not in line with Matsuyama et al. who reported significant correlations of TJC and SJC with serum IL-33 [9]. Unlike our study where we used the DAS28 joint count, they measured 46 joints in TJC and 48 joints in SJC which may be the cause of the difference in results.

RF and anti-CCP are considered poor prognostic factors in RA [19]. In our study comparison of IL-33 levels in RF positive and RF negative patients was statistically insignificant. In addition, we did not find any significant correlation between IL-33 and anti-CCP. Tang and coworkers showed similar results where they found an insignificant correlation of IL-33 with anti-CCP. However, they disagreed with us in that they found a significant correlation of both serum and synovial IL-33 with RF [20]. One study partly agreed with our study in that they showed an insignificant correlation of synovial IL-33 with RF; however, serum IL-33 correlated significantly with RF [25].

On the other hand, Abdel-Wahab and coworkers disagreed with our results as they reported a significant correlation of serum IL-33 with RF [23]. Xiangyang et al. also disagreed with our results in that they showed IL-33 to be significantly higher in the anti-CCP positive group versus the anti CCP-negative group [19].

One explanation for this difference in the studies is that anti-CCP antibody tests that detect the antibodies to citrullinated protein antigens (ACPA) consist of many generations. Consequently, not all ACPAs may be detected [26].

CRP and ESR are commonly used as markers of activity in RA [27]. Our study found an insignificant statistical correlation of IL-33 levels with CRP and ESR, results of which were in line with several other studies [9, 11, 18, 19, 25]. Although reporting an insignificant correlation between IL-33 levels and CRP, Hong et al. found a significant positive relationship between the amount of decrease of the level of IL-33 and that of CRP after introduction of disease-modifying anti-rheumatic drugs in treatment-naïve RA patients. They therefore suggested that like CRP, IL-33 could also be used as an inflammatory marker in RA [11].

In conclusion, this study supports the postulation that IL-33 may have a role in the pathogenesis of RA by means of increased serum and synovial fluid concentrations significantly. It may not be sensitive in assessing disease activity; however, our results support its pro-inflammatory role in this autoimmune disease. IL-33 may become a new target of local treatment and needs to await clinical studies to investigate the outcome of treatment by anti-IL-33 antibodies.

Conflict of interest: The authors declare that they have no competing interests

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