



## IL-6 SERUM LEVELS IN RHEUMATOID ARTHRITIS: RELATION TO DISEASE CHARACTERISTICS AND TREATMENT

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### ABSTRACT

**Purpose:** Dysregulation of cytokine production plays a central role in the pathogenesis of rheumatoid arthritis (RA). This study aimed to analyse serum levels of IL-6 in patients with RA in relation to clinical and laboratory disease characteristics and treatment regimens.

**Methods:** Serum levels of IL-6 were measured using commercially available ELISA kits in 79 female RA patients and compared to 50 healthy women. Disease activity was assessed by Disease Activity Score-28 with CRP (DAS28-CRP). Patients were stratified into three treatment subgroups: group 1 (n = 26), receiving a low dose of systemic glucocorticoids; group 2 (n = 26), treated with conventional synthetic disease-modifying anti-rheumatic drugs (csDMARDs); and group 3 (n = 18), receiving IL-6R inhibitor Tocilizumab (TCZ).

**Results:** Serum IL-6 was significantly higher in female RA patients compared to healthy women (median [IQR]: 8.09 [20.59] pg/ml vs. 0.91 [1.85] pg/ml;  $p < 0.001$ ). Patients with active disease (DAS28-CRP > 3.2) had markedly higher IL-6 levels than those with low disease activity (DAS28-CRP ≤ 3.2) ( $p < 0.01$ ). Among treatment groups, TCZ-treated patients exhibited the highest IL-6 levels (17.2 [34.67] pg/ml;  $p = 0.015$ ). No significant differences in IL-6 levels were observed based on autoantibody status.

**Conclusions:** Our findings demonstrate elevated IL-6 levels in women with active rheumatoid arthritis, supporting the role of IL-6 as a key cytokine in the pathophysiology of this chronic inflammatory disease. Furthermore, the increased IL-6 levels observed during TCZ treatment might reflect ongoing endogenous IL-6 production and persistent disease activity.

**Keywords:** IL-6, rheumatoid arthritis, disease activity, therapy,

### INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, progressive, immune-mediated disease characterised by a strong female predominance and affecting up to 1% of the population worldwide [1]. Although RA is a heterogeneous disease, its most distinctive features include chronic hyperplastic synovitis, bone and cartilage erosions and destructions, autoantibody production, and systemic extra-articular manifestations [2]. The immunopathogenesis of RA involves a series of events linked to the loss of peripheral self-tolerance, as well as the overactivation of multiple cell types and pro-inflammatory cytokine pathways [3]. Among the numerous cytokines present in both the blood and synovial fluid of affected joints, interleukin-6 (IL-6) appears to be the most pleiotropic cytokine, with the greatest number of downstream influences [4].

IL-6 is primarily produced by monocytes and macrophages; however, various cell types, including hematopoietic and non-hematopoietic cells such as lymphocytes, endothelial cells, fibroblasts, and some epithelial cells, can also secrete it. IL-6 is involved in multiple signaling pathways, including classical signaling, trans-signaling, and the recently discovered trans-presentation [5]. The signaling mechanism requires the IL-6-binding alpha chain (IL-6R) and a signal-transducing beta chain (gp130). IL-6 binds to either membrane-bound IL-6R (mIL-6R) or the soluble form of IL-6R (sIL-6R), and the IL-6/IL-6R complex associates with gp130, forming a hexamer. The activation of gp130 by IL-6/mIL-6R is called classical cis-signaling, whereas activation of gp130-expressing cells via IL-6/sIL-6R is known as trans-signaling. The expression of mIL-6R is limited to hepatocytes, neutrophils, monocytes, activated B cells, and CD4 T cells, whereas gp130 is expressed by many

cell types, which explains the pleiotropic effects of IL-6.

It has been found that IL-6 plays a key role in the induction of immunological abnormalities and the development of joint and systemic inflammation in RA, as recently reviewed [4, 6]. IL-6 promotes synovial inflammation and joint damage by stimulating migration, proliferation, and activation of neutrophils, as well as the proliferation and activation of synovial fibroblasts. It increases the production of chemokines and metalloproteases, and stimulates osteoclast maturation and pannus formation. It also plays a role in initiating and sustaining the autoimmune process by promoting the differentiation of T lymphocytes into Th17 cells and B lymphocytes into antibody-producing plasma cells. Additionally, IL-6 mediates many systemic effects of RA, including the induction of the acute-phase response, causing anaemia, inducing fatigue, and contributing to osteoporosis due to an imbalance between osteoblasts and osteoclasts [6]. IL-6 receptor inhibitor therapy with the humanised monoclonal antibody tocilizumab (TCZ) has demonstrated efficacy in treating RA in many clinical trials [7, 8]. In the current study, we aimed to analyse the serum levels of IL-6 in women with rheumatoid arthritis in relation to disease characteristics and treatment regimens.

## MATERIALS AND METHODS

### Study subjects

A total of 79 female patients with RA attending the Rheumatology Clinic of the University Hospital “St. Iv. Rilski” in Sofia were prospectively included in this cross-sectional study. The patients met the ACR/EULAR 2010 RA Classification Criteria [9]. Serum levels of C-reactive protein (CRP), rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibodies (anti-CCP) were measured. Disease activity in RA was assessed using the Disease Activity Score 28, calculated based on the CRP level (DAS28-CRP) [10, 11]. Disease activity was classified as low disease activity (DAS28-CRP  $\leq$ 3.2), moderate activity (DAS28-CRP  $>$  3.2 and  $<$  5.1), and high disease activity (DAS28-CRP  $\geq$ 5.1). A total of 12 patients (15.2%) exhibited low disease activity, 48 (60.8%) presented with moderate disease activity, and 19 (24.0%) experienced high disease activity. With regard to drug therapy, patients were stratified into three subgroups: group 1 received a low dose of systemic glucocorticoids (10 mg/day) (n=32); group 2 was treated with conventional synthetic disease-modifying anti-rheumatic drugs (csDMARDs; n=27); and group 3 included those receiving TCZ (n=20) throughout the sample collection. Demographic and clinical characteristics of the RA patients are summarised in Table 1.

**Table 1.** Demographic characteristics and clinical data of RA patients.

	RA
<i>n</i>	79
Age (years)	52.3 $\pm$ 11.2 (27-75)
Disease duration (years)	8.7 $\pm$ 7.7 (0.2-31.0)
Patients with positive RF <i>n</i> (%)	59 (74.7%)
Patients with anti-CCP <i>n</i> (%)	51 (64.6%)
DAS28-CRP	4.4 $\pm$ 1.1 (2.04-6.71)
$\leq$ 3.2 - low activity <i>n</i> (%)	12 (15.2%)
3.2-5.1 - moderate activity <i>n</i> (%)	48 (60.8%)
$\geq$ 5.1 - high activity <i>n</i> (%)	19 (24.0%)

Data are presented as mean  $\pm$  SD (range); anti-CCP, anti-cyclic citrullinated peptide antibodies; DAS28-CRP, Disease Activity Score 28 calculated using C-reactive protein level; RA, Rheumatoid arthritis; RF, rheumatoid factor; SD, standard deviation.

Comparisons were made with 50 healthy women (mean  $\pm$ SD age, 53.37  $\pm$  11.5 years, range 32-77 years) without a history of autoimmune diseases. This study was approved by the institutional ethics committee (University Hospital “St. Iv. Rilski”, decision number 6, 29 November 2016) and all subjects signed informed consent in accordance with the ethical guidelines of the Helsinki Declaration.

### Quantification of serum IL-6 levels

Blood samples were collected in gel/clot activator vacutainer tubes and allowed to clot at room temperature for 30 minutes prior to centrifugation. The serum samples were removed and stored at -20 °C until analysis. Serum IL-6 levels were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen) purchased from ThermoFisher Scientific (Vienna, Austria), in accordance with the manufacturer’s instructions. A 5-parameter fit standard curve constructed with the kit’s standards was used to determine the cytokine level expressed in picograms per millilitre (pg/ml). Serum samples from patients and controls were run in duplicate and analysed together in the same analytical batch. The limit of detection of the used ELISA kit was 0.92 pg/ml.

### Statistical analysis.

Statistical analyses were conducted using SPSS version 26.0 for Windows (SPSS Inc., Chicago, IL). The normality of the IL-6 distribution was evaluated using the Kolmogorov-Smirnov and Shapiro-Wilk tests. The IL-6 levels between RA patients and healthy controls were compared using the Mann-Whitney U test. The data are presented as median with interquartile range (IQR). Re-

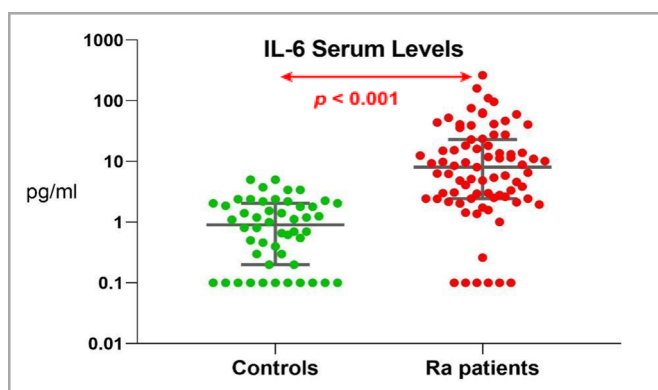
garding disease characteristics, comparisons were carried out using the Kruskal-Wallis test with adjustments for multiple testing. A two-tailed P value of <0.05 was considered significant.

## RESULTS

IL-6 levels were detectable in 73 (92.4%) RA patients and 39 (78%) of the controls.

The serum levels of IL-6 in female RA patients and healthy women are presented in Figure 1.

**Fig. 1.** Serum IL-6 levels in RA patients and Healthy controls. The line denotes the median values, whiskers – minimum and maximum. The log scale is used for the Y-axis.

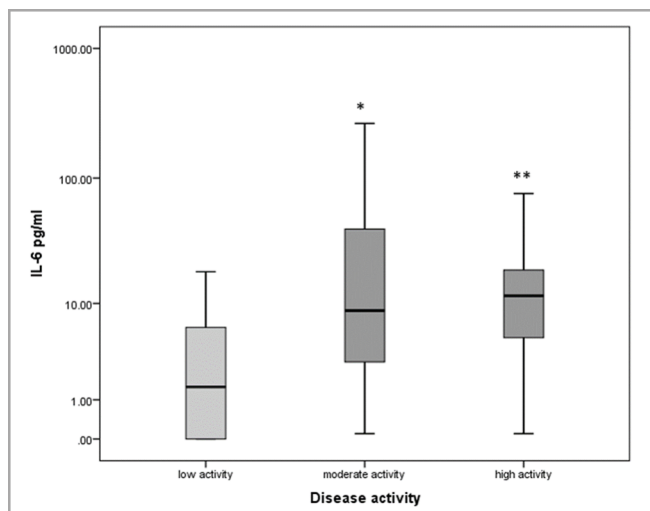


The median (IQR) IL-6 levels in RA patients were significantly higher than those in controls [8.09 (20.59) pg/ml vs 0.91 (1.85) pg/ml;  $p < 0.001$ , Mann-Whitney U test].

Subsequently, we carried out a comparative analysis of IL-6 levels across different patient subgroups based on disease status and clinical characteristics.

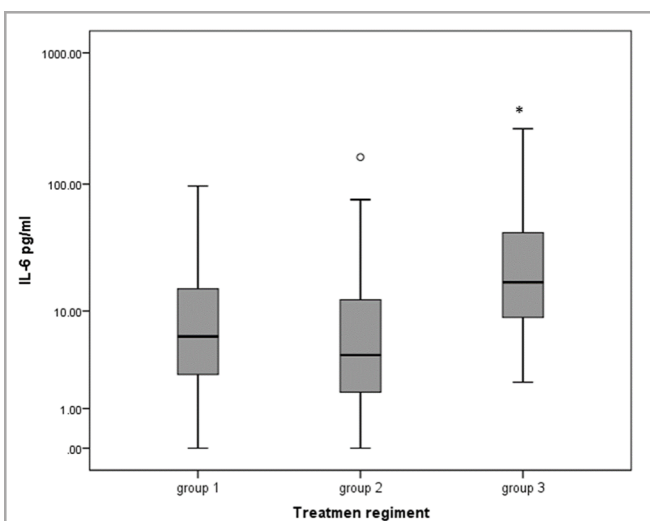
The median (IQR) IL-6 levels increased with disease activity, as follows: low activity – 1.51 (9.7) pg/mL; moderate activity – 8.69 (37.43) pg/mL; high activity – 11.6 (18.18) pg/mL. The IL-6 levels were significantly higher in patients with active disease (DAS28-CRP > 3.2) compared to those with low disease activity (DAS28-CRP ≤ 3.2) ( $p = 0.006$ ; Kruskal-Wallis test). Data are displayed in Figure 2.

**Fig. 2.** Box plot IL-6 serum levels among RA patients with low disease activity (DAS28-CRP ≤ 3.2;  $n=12$ ), moderate disease activity (DAS28-CRP > 3.2 and < 5.1;  $n=48$ ), and high disease activity (DAS28-CRP ≥ 5.1;  $n=19$ ). The line denotes the median values, boxes – the lower (Q1) and upper (Q3) quartiles, whiskers – the minimum and maximum. The log scale is used for the Y-axis. The pairwise comparison showed a significant difference between the moderate vs. low activity groups, \*  $p = 0.008$  (adjusted), and the high vs. low activity groups, \*\*  $p = 0.011$  (adjusted).



IL-6 levels varied by treatment regimen: 6.06 (12.74) pg/ml in patients on symptomatic treatment (group 1); 4.09 (11.62) pg/ml in patients on csDMARDs (group 2); and 17.2 (34.67) pg/ml in patients on TCZ (group 3). Patients undergoing TCZ treatment showed significantly elevated levels of IL-6 compared to the other patient subgroups ( $p = 0.015$ , Kruskal-Wallis test). The data are presented in Figure 3.

**Fig. 3.** Box plot IL-6 serum levels among RA patients receiving a low dose of systemic glucocorticoids (group 1;  $n=32$ ), csDMARDs (group 2;  $n=27$ ), and TCZ (group 3;  $n=20$ ). The line denotes the median values, boxes – the lower (Q1) and upper (Q3) quartiles, whiskers – the minimum and maximum, and a single point – outliers. The log scale is used for the Y-axis. The pairwise comparison showed a significant difference between group 3 and group 2 groups, \*  $p = 0.014$  (adjusted).



No significant differences in IL-6 levels were observed based on autoantibody status. The median (IQR) IL-6 level in RF (+) patients was 8.5 (15.85), while in

RF (-) patients it was 6.75 (24.2) pg/mL ( $p = 0.691$ ; Mann-Whitney U test). The median (IQR) IL-6 level in anti-CCP (+) patients was 6.35 (15.78) pg/ml, whereas in anti-CCP (-) patients it was 9.74 (33.38) pg/ml ( $p = 0.326$ ; Mann-Whitney U test).

## DISCUSSION

In the current study, we analysed the serum levels of IL-6 in female RA patients and compared them to those in healthy women. Consistent with previous studies, we found significantly elevated levels of IL-6 in RA patients compared to controls [12, 13] with a skewed distribution, which was anticipated due to the heterogeneity of the RA cohort assessed. A key finding of our study is a significant association between higher IL-6 levels and increased disease activity, as shown in Figure 2. Indeed, RA patients with high and moderate disease activity, as measured by DAS28-CRP, exhibited significantly elevated serum IL-6 levels compared to those with low activity. This finding aligns with the results of earlier studies [13, 14].

The reasons for the ongoing induction of IL-6 synthesis in RA remain unclear. The transcription of mRNA for IL-6 and other pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ , in monocytes and macrophages is stimulated by the activation of cell surface and intracellular TLRs. [14]. Signaling from TNF- $\alpha$  or IL-1 $\beta$  also induces IL-6 mRNA transcription [15].

IL-6 expression is controlled at both transcriptional and post-transcriptional levels. One of the post-transcriptional regulation mechanisms involves mRNA stability, which is controlled primarily by RNA-binding proteins and microRNAs (miRNAs). The RNA-binding protein AT-rich interactive domain-containing 5a (Arid5a) enhances cytokine production by positively regulating the mRNA half-lives of pro-inflammatory molecules, such as IL-6, in activated macrophages and T cells. [16]. Indeed, in RA patients, markedly higher Arid5a levels have been found compared to healthy controls [17].

A possible explanation for the continuous production of IL-6 in RA could be autocrine stimulation of IL-6 synthesis. This may happen through intracellular signaling when IL-6-producing cells express both IL-6R and gp130. In addition to the classical IL-6 signaling cascade, extracellular IL-6 can be taken up by the IL-6R and transported to the endosomal compartment via endocytosis, which may contribute to sustained chronic inflammation in RA [18].

An important finding in our study is the significantly higher serum IL-6 levels in RA patients receiv-

ing TCZ compared to those on other therapeutic regimens. TCZ binds to both soluble and membrane-bound IL-6Rs, inhibiting cis- and trans-signaling pathways and preventing IL-6-mediated inflammation in RA [8], which indirectly indicates that abnormal IL-6 production underpins the disease's pathogenesis. Although inhibition of IL-6 signaling is therapeutically effective, TCZ treatment does not lead to a reduction in serum IL-6 levels, as also noted by other investigators [19, 20]. Nishimoto et al. reported increased serum IL-6 and sIL-6R after TCZ administration in RA patients, without an increase in IL-6 production as measured by IL-6 mRNA expression in peripheral blood cells from RA patients before and after TCZ administration [20]. They suggest that the increase in IL-6 and sIL-6R may be due to the occupation of IL-6R by TCZ, which inhibits IL-6R-mediated clearance and extends the half-life of sIL-6R by forming TCZ/sIL-6R immune complexes. Importantly, elevated serum IL-6 levels during TCZ treatment should not be interpreted as a sign of treatment failure. The therapy may remain clinically effective, even in the presence of increased circulating IL-6.

Alternatively, the elevated IL-6 levels in the sera of TCZ-treated patients could be explained by disease activity and severity, necessitating treatment with biological DMARDs. However, in a case-control study, IL-6 level was measured at a fixed point of time during the disease and treatment without knowing the baseline cytokine level. This is one of the study's limitations.

In **conclusion**, our results demonstrate increased IL-6 levels in women with active rheumatoid arthritis, supporting the role of IL-6 as a key cytokine in the pathophysiology of this chronic inflammatory disease. Furthermore, the increased IL-6 levels observed during TCZ treatment might reflect ongoing endogenous IL-6 production and persistent disease activity.

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