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ORIGINAL ARTICLE

Exploring the Roles of Pro-Inflammatory TNF-α and Anti-Inflammatory IL-35 Cytokines in the Pathogenesis of Rheumatoid Arthritis among Patients in Baghdad, Iraq

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ABSTRACT

Background: *Rheumatoid arthritis (RA) is a chronic, immune-mediated inflammatory disease characterized by synovitis, particularly affecting the small joints of the hands and feet. Tumor necrosis factor-alpha (TNF-α), a pro-inflammatory cytokine, plays a critical role in the etiology and pathogenesis of RA.* ***Objectives:*** *To evaluate the levels of TNF-α and IL-35, along with various clinical indicators, to assess their potential in predicting disease outcomes in Iraqi patients with RA.* ***Methodology:*** *A total of 125 participants were enrolled in this study, including 100 patients diagnosed with RA and 25 healthy controls. The study was conducted at the Rheumatology Consultation Clinic, Baghdad Teaching Hospital, Medical City, between September 2024 and March 2025. Participants ranged in age from 15 to 70 years. Serum levels of the pro-inflammatory cytokine TNF-α and the anti-inflammatory cytokine IL-35 were measured using the ELISA technique.* ***Results:*** *The study found no statistically significant differences in TNF-α concentration among RA patients based on disease duration or family history of RA. However, TNF-α levels were significantly higher in RA patients compared to healthy controls ($p=0.0055$ and $p<0.0001$, respectively). Furthermore, female patients exhibited significantly higher TNF-α levels than males ($p = 0.0256$), whereas the difference in males was not significant ($p=0.6200$). TNF-α concentration were also significantly higher in older patients compared to younger individuals. Regarding IL-35, its concentration was significantly decreased in both male and female RA patients compared to healthy individuals ($p < 0.0001$).* ***Conclusion:*** *The findings suggest a clear distinction between the roles of pro- and anti-inflammatory cytokines in RA. The decreased levels of IL-35 in RA patients indicate its potential involvement in joint inflammation and damage. IL-35 may contribute to disease progression by modulating immune responses affecting bone and joint health.*

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, immune-mediated inflammatory disease characterized by synovitis, particularly in the small joints of the hands and feet. RA is among the most common immune-mediated diseases, with a global prevalence of approximately 0.5–1%. It occurs two to three times more frequently in females than in males. Prevalence varies across ethnic and geographic populations, reaching 5–6% among Indigenous North Americans and being higher in northern Europe compared to southern Europe[1].

A combination of genetic and non-genetic risk factors contributes to increased susceptibility to RA. These factors may collectively cross a pathogenic threshold, initiating autoimmune processes that result in synovial inflammation, hyperplasia, infiltration by immune cells, cartilage and bone erosion, and angiogenesis. Disruption in the balance between pro- and anti-inflammatory cytokine activity plays a crucial role in disease progression, although the precise regulatory network of these cytokines remains unclear[2].

RA most commonly affects individuals between the ages of 35 and 55. The interaction between mesenchyme stem cells and T cells, key regulators of the cellular immune response, is a notable area of investigation in understanding RA pathogenesis. RA can lead to significant joint damage and long-term disability. In recent years, substantial progress has been made in understanding the pathophysiology of RA, identifying effective outcome measures, developing targeted therapies, and emphasizing the importance of early diagnosis and intervention[3].

RA prevalence is generally higher in industrialized countries. This may be attributed to greater environmental exposures, genetic predispositions, demographic differences, and underreporting in some

regions. Improved understanding of the natural history of RA and the risk factors influencing its development in specific populations may facilitate the design of targeted preventive strategies for this debilitating disease[4].

Epidemiological perceptions of rheumatoid arthritis (RA) have evolved in recent years. Global estimates of RA incidence vary depending on the methodology used, population size, and geographic location. Significant regional differences in RA incidence have been observed, and recent data indicate a global increase in RA cases. However, there has been a concurrent decline in disability-adjusted life years (DALYs), possibly due to improved management and early diagnosis[5].

The prevalence of RA is generally higher in industrialized countries. This may be attributed to greater exposure to environmental risk factors, genetic predisposition, demographic variations, and potential underreporting in less developed regions. A deeper understanding of the natural history of RA and the factors contributing to its development in specific populations could support the development of targeted preventive strategies for this debilitating condition[6].

Genetic studies have identified several candidate gene variants associated with RA, but their individual effects tend to be small and often statistically weak. These studies typically focus on known genetic variants, rather than conducting comprehensive genome-wide analyses[7]. Both biological sex and gender significantly influence the onset and progression of rheumatic and autoimmune diseases. Differences in immune responses between males and females are linked to genetic and hormonal factors. Furthermore, behavioral and social factors related to gender can affect exposure to environmental pathogens and access to healthcare[8].

Tumor necrosis factor-alpha (TNF-α), a key pro-inflammatory cytokine, plays a central role in RA pathogenesis. Elevated TNF-α levels are believed to promote the production of reactive oxygen species (ROS), contributing to tissue inflammation and damage in RA patients. Genetic factors, including TNF-α gene expression, are important in determining disease onset and severity[9].

Interleukin-35 (IL-35) is a recently identified anti-inflammatory cytokine belonging to the IL-12 family. It is a heterodimeric protein composed of two subunits: IL-12p35 and Epstein-Barr virus-induced gene 3 (EBI3). IL-35 is constitutively secreted by regulatory T cells (Tregs) as a p35/EBI3 dimer[10]. Given the pivotal role of Tregs in maintaining immune tolerance and controlling inflammation, IL-35 is thought to contribute to the regulation of autoimmune responses, including those involved in RA development[11].

RA is associated with alterations in both pro- and anti-inflammatory cytokine levels. The present study aims to investigate this hypothesis by measuring the concentrations of TNF-α and IL-35 in Iraqi patients with RA. Specifically, the study seeks to:

Evaluate the significance of TNF-α and IL-35 levels in the progression of RA; Examine the association between cytokine levels and patient parameters such as age, disease duration, family history, and gender; Explore the regulatory relationship between pro-inflammatory TNF-α and anti-inflammatory IL-35 in the context of RA.

METHODOLOGY

Study groups (Patients and controls)

Blood samples were collected from a total of 125 subjects, including 100 patients diagnosed with rheumatoid arthritis (RA) (38 males and 62 females) who were referred to the Rheumatology Consultation Clinic at Baghdad Teaching Hospital, Medical City, and 25 healthy controls. The study was conducted over a period from September 2024 to March 2025. The RA patients had varying disease durations. Participants ranged in age from 15 to 70 years. Informed consent was obtained from all participants, and relevant clinical data were recorded.

Reagents (Chemicals and Solution)

Enzyme-linked immunosorbent assay (ELISA) kits for the detection of human IL-35 and human TNF-α were obtained from Biotech Ltd., China, and used according to the manufacturer's instructions.

Clinical Samples

Sample Collection and Preparation

Venous blood samples were collected using 5 mL disposable syringes via venipuncture. The blood was transferred into 10 mL sterile serum separator tubes (gel tubes), allowed to clot, and then centrifuged to separate the serum. The resulting serum was divided into several 0.5 mL aliquots and immediately stored at -20°C until used for the detection of IL-35 and TNF-α using the ELISA technique.

Cytokine Detection by ELISA

ELISA kits for the detection of human IL-35 and TNF-α (Biotech Ltd., China) were used according to the manufacturer’s protocols.

Assay Principle

The detection of pro-inflammatory cytokine TNF-α and anti-inflammatory cytokine IL-35 was based on the sandwich ELISA principle. The microplates provided in the kits were pre-coated with monoclonal antibodies specific to human TNF-α or IL-35. Standards or serum samples were added to the wells, allowing the target cytokines to bind to the immobilized antibodies.

Following incubation, a biotin-labeled detection antibody specific to TNF-α or IL-35 was added, followed by the addition of avidin–horseradish peroxidase (HRP) conjugate. After further incubation and washing to remove unbound substances, a substrate

solution was added. The wells containing cytokine-antibody-enzyme complexes developed a blue color due to the enzymatic reaction. The color intensity, which correlates with cytokine concentration, was measured spectrophotometrically at 450 nm.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 9.2 (GraphPad Software Inc., La Jolla, CA, USA). Differences between groups were evaluated using Student's *t*-test and two-way ANOVA with Sidak's multiple comparisons test to assess significance. The Pearson correlation coefficient (*r*) was used to analyze associations between continuous variables. The chi-square test was applied for comparisons of categorical data.

Receiver operating characteristic (ROC) curve analysis was conducted to determine the area under the curve (AUC) and to identify the optimal cut-off values for predictive markers. Quantitative parametric data were tested for normality using the Shapiro–Wilk test and are presented as mean ± standard deviation (SD). Statistical significance was defined as $p < 0.05$ (*), and $p < 0.01$ (**).

RESULTS

(Figure 1) shows the concentration of TNF-α in RA patients and healthy controls. The current study found a highly statistically significant increase in TNF-α levels in RA patients compared to healthy controls. Additionally, (Figure 1) illustrates a marked reduction in IL-35 concentration among RA patients relative to healthy controls. This difference was also highly statistically significant (p-value < 0.0001).

The results of the study showed elevated concentrations of TNF-α in rheumatoid arthritis patients compared with healthy controls. A highly significant difference was observed in the 20–30 age group ($p = 0.0093^{1}$), while significant differences were also found in the 31–40 and ≥51 age groups ($p = 0.0151^{2}$ and $p = 0.0140^{3}$, respectively). However, no significant difference was detected in the 41–50 age group ($p = 0.9201^{4}$), as shown in (Figure 2).

Regarding IL-35 concentrations, the findings indicate generally lower levels in RA patients compared to healthy controls, except in the 41–50 age group, where no statistically significant difference was observed (Figure 2).

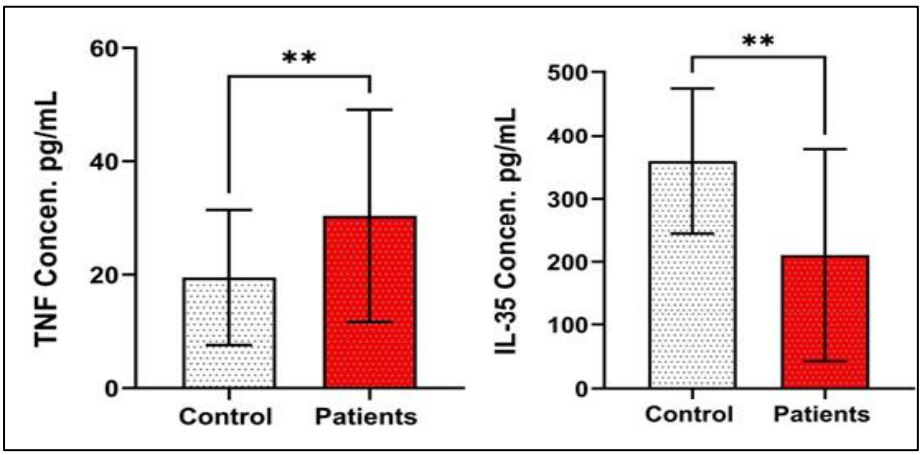

Fig. 1: Concentrations of TNF-α and IL-35 in RA patient and control

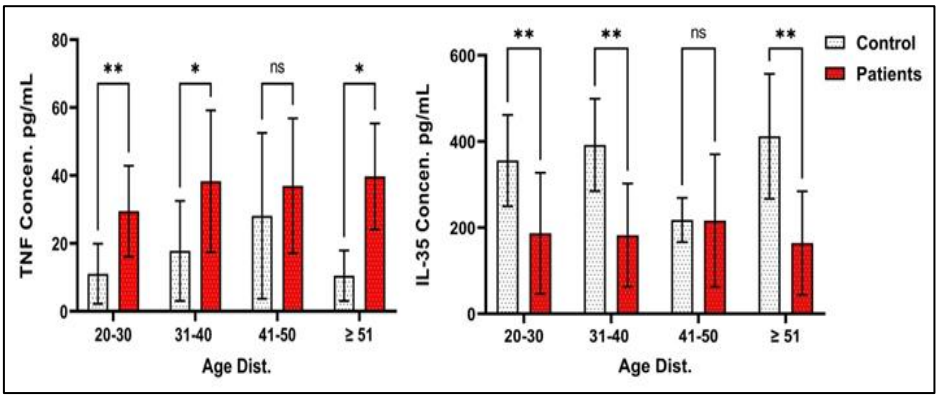

Fig. 2: Relationship between TNF-α and) IL- 35 concentrations with Age,
Note: Significant (S) (*) High significant HS (**). No significant (ns)

Regarding the concentration of TNF-α in males and females, the present study showed no statistically significant difference in males (p = 0.6200). In contrast, females exhibited a statistically significant increase in TNF-α compared with healthy controls (p = 0.0256), as shown in (Figure 2). Additionally, the current study revealed a significant decrease in IL-35 concentration in both male and female RA patients compared to healthy controls (p < 0.0001), as illustrated in (Figure 3).

Regarding the period of disease, the current study found no statistically significant difference between patients with a recent onset and those with a longer disease duration, as shown in (Figure 3). The statistical analysis indicated no significant difference between new and old rheumatoid arthritis cases (p = 0.6080), as illustrated in (Figure 4).

The present study showed no statistically significant difference in TNF-α and IL-35 concentrations between RA patients with a hereditary history and those without, as shown in (Figure 4). The results indicate no significant association between hereditary history and RA status (p = 0.9576), as illustrated in (Figure 5).

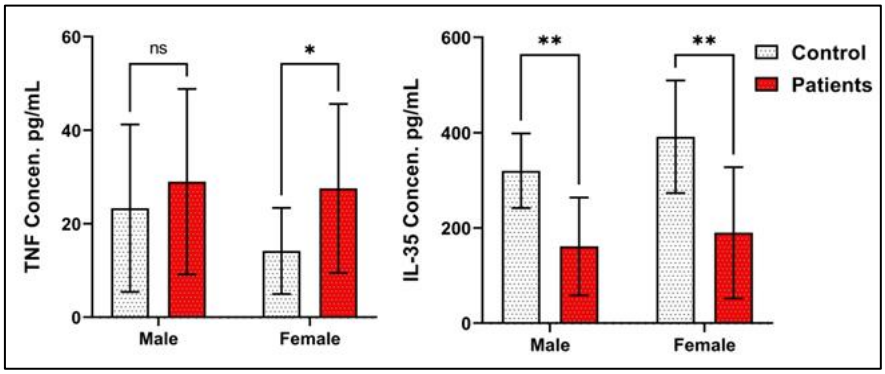

Figure 3: Correlation of TNF-α and IL-35 concentrations according to gender.

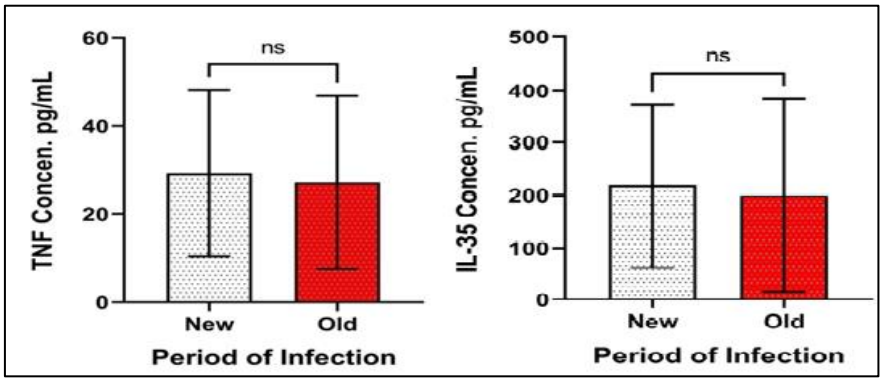

Fig. 4: Relationship between TNF-α and IL-35 concentrations and the period of disease in RA patients.

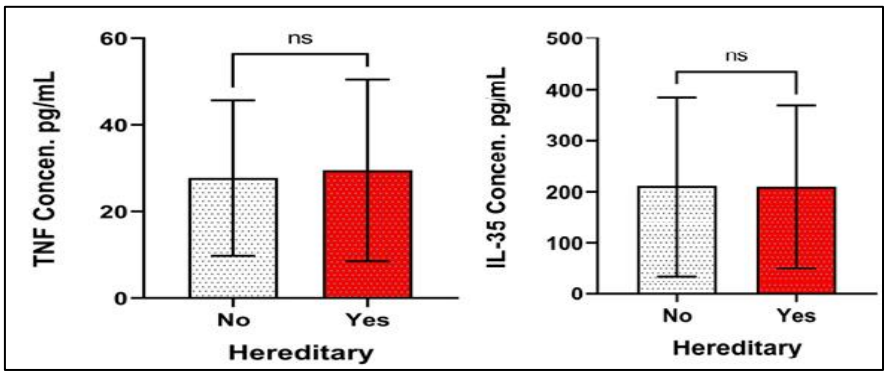

Fig. 5: Relationship between TNF-α and IL-35 concentrations and hereditary history in RA patients.

To evaluate the effectiveness of various pro- and anti-inflammatory cytokines in predicting rheumatoid arthritis among Iraqi patients, Receiver Operating Characteristic (ROC) curve analysis was performed. This method plots the true positive rate (sensitivity) against the false positive rate (1-specificity). The area under the ROC curve (AUC) was calculated as an indicator of the test's accuracy in predicting disease status.

Interpretation of AUC values is as follows: less than 0.600 indicates a failed predictor, 0.600 to 0.700 indicates a sufficient predictor, 0.700 to 0.800 indicates a good predictor, 0.800 to 0.900 indicates a very good predictor, and greater than 0.900 indicates an excellent predictor.

Based on these criteria, serum TNF-α was a sufficient predictor of disease with an AUC of 0.6817, while IL-35 was a very good predictor with an AUC of 0.8077 (Figure 6).

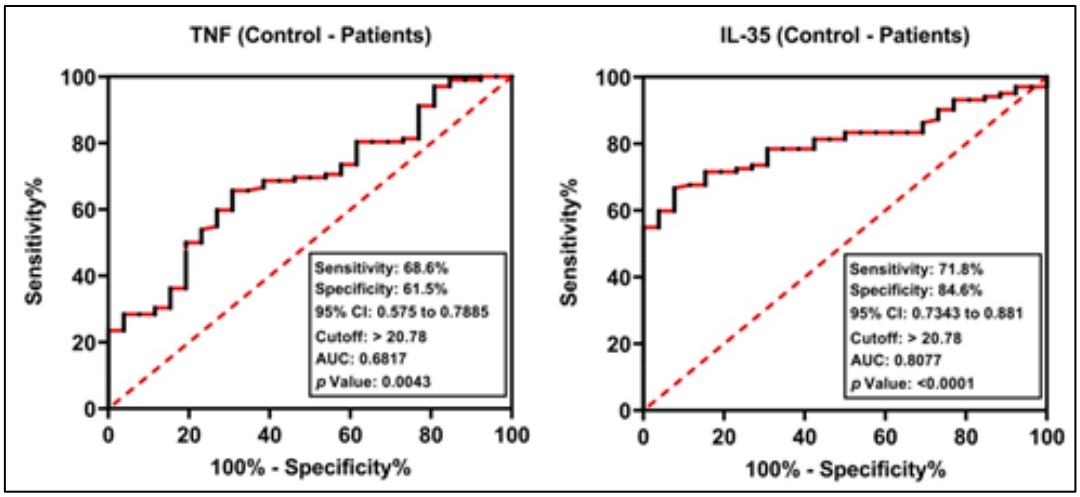

Fig. 6: ROC assesses the validity of pro and anti-inflammatory cytokine in predicting RA disease severity

DISCUSSION

The present study demonstrated an increase in TNF-α concentration in RA patients compared to healthy controls. This finding aligns with Moelants et al.[12], who reported that TNF cytokine levels are significantly higher in RA patients than in healthy individuals. Moreover, TNF-α plays a major role in the pathogenesis of RA. Elevated TNF concentrations are produced by various cells, such as monocytes and T cells, in RA patients. Our results also agree with previous studies that found increased TNF-α levels in RA patients[13].

The study shows that TNF concentration is higher in elderly RA patients compared to healthy controls, consistent with the findings of Targońska et al.[14], who noted that RA generally appears after age 40 but can occur at different ages, including in children. Another study supports this, showing a gradual increase in RA incidence in individuals over 60 year's old[15]. Our results indicate that TNF concentration is higher in female RA patients than in males, although the difference was not statistically significant. However, TNF levels in both genders were elevated compared with healthy controls. These findings are consistent with prior research showing significant differences between RA patients and healthy controls regarding TNF levels by gender[16]. Another study reported that TNF concentrations are higher in women than men[17], this study disagreement with Martinez et al.[18] who found the men have highly level of TNF concentration than women.

Regarding disease duration, the current study found that newly diagnosed RA patients had higher TNF concentrations compared to those with longer disease duration, although the difference was not statistically significant. This agrees with previous studies suggesting that disease duration may predict TNF-α levels, with newly diagnosed cases showing higher concentrations[19].

Our study also found that RA patients with a family history of the disease had higher TNF levels than those without, but this difference was not statistically significant. This result aligns with other studies showing that RA is more common among patients with a family history, and smoking is considered a significant etiological factor affecting RA patients[20]. Furthermore, genetic and environmental factors contribute to increased susceptibility in patients with a hereditary history[21]. The TNF-α gene is an important genetic factor in inflammatory and autoimmune diseases[22].

Regarding IL-35 concentration, the current study demonstrated a highly significant difference between RA patients and controls, with IL-35 levels being higher in healthy controls than in patients. These findings are consistent with Choi et al.[23], who reported higher IL-35 serum concentrations in healthy individuals compared to

RA patients. Similarly, other studies have shown that IL-35 expression is higher in healthy controls and is significantly associated with lower disease activity[24]. Elevated endogenous IL-35 may contribute to a negative immune response to inflammatory stimuli, counteracting excessive inflammation and playing a protective role in RA. A previous study in Iraqi patients found elevated IL-35 concentrations in RA patients, though not statistically significant ($p = 0.055$)[25].

IL-35 also inhibits matrix metalloproteinase (MMP) secretion in chondrocytes and synovial fibroblasts and stimulates aggrecans and collagenase, which are involved in cartilage degradation and osteoclast activation, promoting joint destruction[26]. IL-35 treatment enhances regulatory mechanisms by reducing inflammatory cytokines such as interferon-γ and IL-17 and suppresses effector T cell proliferation through interactions with CD2, CD3, and CD28, indicating multiple therapeutic targets in RA[27].

The findings indicate low significance of IL-35 concentration variation with age among RA patients compared to healthy controls. This disagrees with some studies that showed higher IL-35 levels in elderly RA patients (>60 years)[28], while others found decreased IL-35 levels with aging in RA patients consistent with our findings[29].

Regarding gender, the current study revealed a significant decline in IL-35 concentration in both male and female RA patients compared with healthy controls. Muhammad et al.[30] found that RA is more common in females than males. Omran et al.[25] reported higher IL-35 concentrations in females compared to males (87.5% vs. 12.5%). Women are approximately three times more likely to develop RA than men, influenced by physiological differences such as hormonal variation. Our study found no statistical difference in IL-35 concentration between patients with new and old RA disease, although IL-35 levels were higher in new RA patients. This aligns with the 2015 American College of Rheumatology guidelines, which showed no significant difference between disease durations. Singh et al.[31] also reported increased IL-35 serum levels in RA patients related to clinical criteria. However, other studies indicated elevated IL-35 in patients with longer disease duration, suggesting IL-35 may play different roles at various RA stages[32].

Our results showed no statistical difference ($p = 0.9576$) between RA patients with or without a family history of the disease. This is consistent with previous studies[33], although some reports indicate a significant association between family history and RA susceptibility, with patients having a family history being more prone to developing the disease[34].

ROC analysis demonstrated that TNF-α was a sufficient predictor of RA with an AUC of 0.6817, while IL-35 was a very good predictor with an AUC of 0.8077. Previous studies using ROC analysis have confirmed TNF-α as a strong marker for RA and disease severity, reflecting its role as an inflammatory mediator. Biologic therapies targeting TNF-α effectively reduce inflammation in RA patients[12]. Meanwhile, research has shown significantly lower IL-35 levels in RA patients compared to healthy controls, with IL-35 negatively correlating with disease markers and contributing to inflammatory regulation in RA. Preliminary data suggest IL-35 plays a critical role in regulatory T cell function, making it a promising target in autoimmune disease research[35].

CONCLUSION

The pro-inflammatory cytokine TNF-α is found at elevated levels in patients with rheumatoid arthritis (RA) compared to healthy controls. This increase is associated with several important factors, including age, gender, genetic predisposition, and disease duration. In contrast, the anti-inflammatory cytokine IL-35 shows a distinct pattern. Based on these findings, it can be inferred that IL-35 plays a role in modulating the immune response and influences bone joint condition, contributing to joint damage in RA.

Ethical Approval

The study was approved by the Ethics Committee of the Rheumatology Consultation Clinic of Baghdad Teaching Hospital, Medical City, according to protocol number 613 on 3-Sep.-2024, based on verbal consent from all patients.

Conflict of Interest

All authors declare that there are no conflicts of interest in this article.

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