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# Evaluation of the Salivary levels of TNF-α and IL35 in Iraqi patients with Rheumatoid Arthritis

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**Abstract:**

The study examines the impact of TNF-α and IL35 levels in saliva on rheumatoid arthritis. It compares responder and non-responder patients, including 30 who responded to treatment and 30 who did not. The study found that rheumatoid arthritis incidence is three times higher in females than males. The age difference between RA patients and healthy controls was significant. TNF-α and IL35 levels were found to be highly significant in two patient groups compared to the control group. The study suggests that salivary TNF-α and IL35 levels can be used to study methotrexate effectiveness and disease activity.

**Keywords**: TNF-α, IL-35, rheumatoid arthritis, methotrexate, Saliva

## 1. Introduction:

Rheumatoid arthritis (RA) is a prevalent immune-mediated illness. Inflammatory arthritis is the most prevalent symptom, and it is marked by symmetrical, polyarticular swelling and pain, generally affecting the tiny joints of the feet and hands (Gravallese and Firestein, 2023). Eventually, the heart, skin, lungs, kidneys and eyes, will be affected. Cartilage and joint bone are frequently destroyed, and ligaments and tendons become weakly (Lee et al., 2017). All of this joint deterioration leads to abnormalities and bone erosion, both of which are brutally painful for the individual who suffers. Stiffness in the morning of the affected joints that lasts more than 30 minutes, weariness, fever, weight loss, pain, heated and swelling joints, and rheumatoid nodules beneath the skin represent all prominent signs of RA (Taibanguay et al., 2019). Because saliva contains serum-derived components, systemic inflammatory diseases may alter the levels of some salivary indicators (Kaczyński et al., 2019). Tumor necrosis factor (TNF-α) is a powerful macrophage-derived cytokine seen in inflamed synovial membranes (Husby, G., & Williams, 1988). TNF-α is a pro-inflammatory cytokine produced during inflammation by cells that include monocytes and macrophages (Idriss and Naismith, 2000).

TNF-α initiates a normal immune response. However, at high levels, it can trigger uncontrolled inflammatory reactions, an increase in osteoclast precursors, and osteoclast development, culminating in bone resorption (Cessak et al., 2014; de Vries et al., 2019). TNF-α inhibitors are used in clinical settings to counteract the elevated TNF-α levels that cause joint inflammation, hence avoiding TNF-α tissue damage in RA. TNF-α inhibitors were initially available in 1998 for the treatment of inflammatory illnesses such as rheumatoid arthritis (RA) (Zamri and de Vries, 2019). IL-35 is a recently found cytokine in the IL-12 family. In 2007, Collison et al. and Niedbala et al. discovered IL-35, a new kind of cytokine (Collison et al., 2007; Niedbala et al., 2007). IL-35 enhances T-reg proliferation while inhibiting Th17 cell differentiation (Liu et al., 2019). A variety of immune cells, especially Tregs and Th17 cells, infiltrate the joint and play a role in synovial inflammation and joint deterioration (Wang and Lei, 2021). In collagen-induced arthritis (CIA) in mice, IL-35 was discovered to reduce the expression of VEGF and its associated receptors, indicating that IL-35 could affect the pathological mechanism of RA (Wu et al., 2016). Bone damage is also a key aspect of RA pathogenesis. It is widely accepted that RANKL receptors (nuclear factor B ligand) activation is required to create and develop osteoclasts (Tanaka et al., 2018). Therefore, this study aimed to evaluate the level of salivary pro-inflammatory cytokine TNF-α and anti-inflammatory IL35 in responder and non-responder rheumatoid patients to treatment.

## 2. Material and methods:

### 2.1 Patients group:

Sixty patients attended the Rheumatology unit of the Baghdad teaching hospital with rheumatoid arthritis diagnosed according to the criteria established by the (ACR/ EULAR 2010 criteria) (Taylor, 2020), during the period from December 2022 and March 2023. This study was approved by the institutional research ethics committee (protocol 714822). The group of patients was divided into two groups (G1 and G2). Thirty patients (G1) who have taken methotrexate (MTX) with a dose ≥ 7.5 mg per week for at least six months and responded to the treatment, and 30 patients (G2) who did not respond to methotrexate. Both groups were enrolled in this study. There was a special questionnaire sheet was given to all patients to get information about name, age, sex, family history, disease duration, morning stiffness, clinical parameters, and therapy side effects.

### 2.2 Control group:

The control group consisted of 28 apparently healthy subjects with no history of any systemic autoimmune diseases.

### 2.3 Collection of saliva:

5 ml non-stimulated saliva was drawn from each subject. Then, Saliva was transferred to a sterile plain tube and centrifuged at 3000 rpm for 5 minutes. Next, it was distributed into Eppendorf tubes and kept at -20ºC until it was used for analysis (Navazesh, 1993; Mohammed et al., 2021).

### 2.4 Diagnosis of RA:

The patients have been recognized as having rheumatoid arthritis based on the clinical examination by (rheumatologists) in the rheumatology clinic based on the duration of administration of the methotrexate, routine blood test including erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and clinical disease activity index (CDAI). (CDAI) scored by rheumatologists depends on tender Joint count (TJC) in 28joints and swollen joint count (SJC) in 28 joints according to ACR criteria scored by rheumatologists who did not have access to laboratory data, and the stage of the disease was determined by these criteria based on the duration of the disease and laboratory tests (ESR-CRP). (CDAI) scored in RA patients was classified into four groups based on this score which are: [remission < 2.8], [low disease activity <10], [moderate disease activity 10 - 22], and [a high level of disease activity >22] (Salaffi et al., 2015).




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**2.5 Assessment of salivary TNF-α and IL35:**

The level of TNF-α and IL35 in the saliva of RA patients and healthy control by employing sandwich ELISA kits from (USCN) Cloud clone crop (SEA133Hu) for TNF-α and FineTest (EH3273) for IL35. These kits had been developed to provide quantitative measurements of (TNF-α and IL35) in humans.

**2.6 Statistical analysis:**

SPSS 24 was used for statistical analysis, and Excel program. results are presented as number (%), median, interquartile, mean ± SD. The ANOVA-T test measures the difference between two or more means and the F-test.

**3. Results and Discussion**

A total of 60 RA patients were studied and according to the results of this study, the prevalence of this disease in women was higher than in men (93.3% vs. 6.66% respectively). Table 1 showed that there were extremely significant (P<0.001) differences in age among RA patients and the control group with no arthritis, (mean± standard deviation) of age in Two distinct groups (G1&G2) of RA. G1(50.90±9.98) and G2(48.40±13.04) and control group (31.42±5.74) Table 2.

Table 1: Descriptive of sex in RA patient groups and control group

| | Control | | G1 | | G2 | |
|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | |
| Female | 21 | 75 | 23 | 76.67 | 28 | 93.33 |
| Male | 7 | 25 | 7 | 23.33 | 2 | 6.667 |
| Total | 28 | 100 | 30 | 100 | 30 | 100 |

Table (2) Descriptive of age in RA patient groups and control group

| groups | | No. | Mean | SD | SE | Minimum | Maximum | F-test | P-value | Sig |
|---|---|---|---|---|---|---|---|---|---|---|
| age | Control | 28 | 31.4286 | 5.74410 | 1.08553 | 24.00 | 44.00 | 31.362 | 0.000 | HS |
| | G1 | 30 | 50.9000 | 9.98741 | 1.82344 | 30.00 | 70.00 | | | |
| | G2 | 30 | 48.4000 | 13.04264 | 2.38125 | 26.00 | 69.00 | | | |

Rheumatoid arthritis, also referred to as a systemic inflammatory illness marked by an increase of persistent inflammatory cells. The patient initially complains of inflammatory episodes and pain within the synovium is commonly damaging (Brennan and McInnes, 2008). Alkazzaz, (2013) proved that the rheumatoid arthritis was from the group that was diagnosed in the province of Babylon the number of patients was 1039 females numbers reached 853 [82.09%] and numbers males 186 [17.9%] (Also, this study is consistent with a study conducted in Iraq by Mohammed (2021), which appeared in A higher proportion of women than men (75 to 25) in patients with rheumatoid arthritis.

Omran et al. (2022) found a higher prevalence of rheumatoid arthritis in females compared to males (87.5% vs. 12.5%). Women are about three times more likely than men to be suffering from this disease, and the disease's effect varies between males and females which may be due to physiological nature variations among sexes, such as differences in hormonal content, differences in behavior, the role of genes and heredity, age groupings in the reproductive and premenopausal stages (Nourisson et al., 2017; Romo-García et al., 2019). Because RA is of unknown cause, many studies found a relationship between viral infections such as EBV and CMV and with incidence of Ram (Jassim et al., 2015; Fadhil, 2019).

Rheumatoid arthritis was diagnosed in all cases according to (ACR/ EULAR 2010 criteria) and clinical disease activity index (CDAI) scored in two groups of RA. There were high significant differences P<0.001 in comparison to the two groups of patients who responded to methotrexate (7.70±2.19) and those who did not respond to methotrexate (24.53±3.11), see Figure (1). While ESR (26.86 ±22.45, 9.37±7.67) and CRP (64.25±32.24 & 39.66±34.34) respectively were highly Significant variations among the two groups of patients who responded to methotrexate and




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alongside those who did not respond to methotrexate and no significant difference when comparing CRP to control (2.26±0.76) as can be seen in Figure 2.

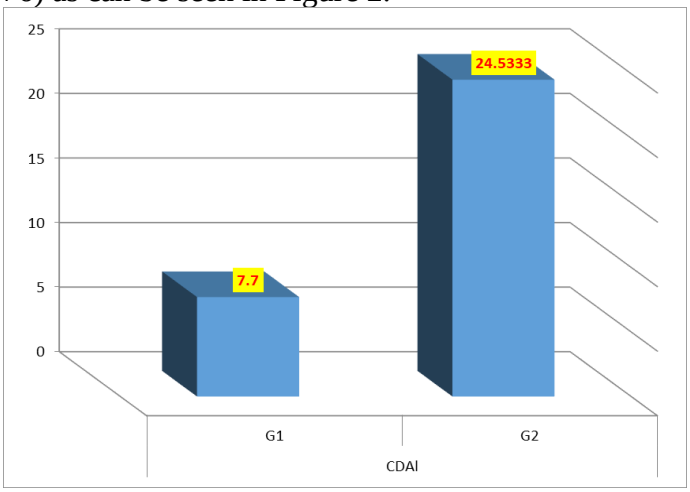


Figure 1: Descriptive of CDAl scored in two groups of RA

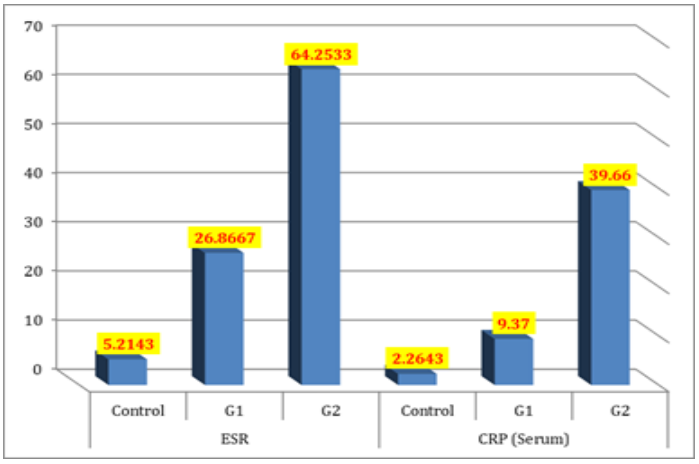


**Figure 2: Descriptive of ESR and CRP in RA patients and control group**

The main results of this research showed the salivary level of TNF-α and IL35. The TNF-α and IL35 levels showed high significance in two groups of patients against the control group, while the mean concentration of pro-inflammatory TNF-α level (256.36) increased in non-responders to MTX compared with responder RA patients. The mean of IL35 (106.35) decreased in non-responders to MTX compared with responder RA patients as can be shown in Table 3.

**Table 3. Determination of salivary TNF-α and IL35 levels in patient groups and control group**

| | | No. | Mean | SD | SE | Minimum | Maximum | F-test | P-value | Sig |
|---|---|---|---|---|---|---|---|---|---|---|
| TNF | Control | 28 | 47.7500 | 5.24154 | 0.99056 | 40.60 | 57.80 | 553.616 | 0.000 | HS |
| | G1 | 30 | 190.9133 | 30.89140 | 5.63997 | 60.80 | 217.90 | | | |
| | G2 | 30 | 256.3633 | 27.45329 | 5.01226 | 206.70 | 308.10 | | | |
| IL35 | Control | 28 | 93.8679 | 6.44659 | 1.21829 | 78.30 | 100.00 | 71.554 | 0.000 | HS |
| | G1 | 30 | 175.7667 | 41.83056 | 7.63718 | 92.60 | 271.10 | | | |
| | G2 | 30 | 106.3500 | 23.72006 | 4.33067 | 75.10 | 181.30 | | | |




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Fadhil, (2019) were found to have greater CMV and RA occurrences. ESR and CRP were routine examinations in the rheumatology clinic for disease activity. In this study, it was seen that the level of CRP increased in did not respond to treatment, and the level decreased when responding to treatment. CRP stimulates the synthesis of proinflammatory cytokines, resulting in inflammation (Newling et al., 2019).

Orr et al. (2018) demonstrate a substantial association between blood CRP and ESR levels and tissue inflammation scores from knee synovium biopsy samples in RA patients. According to Saptarini et al. (2016), the ESR can be used as a conformity indicator for patients with non-progressive RA.

In RA patients, CRP levels within synovial fluid and serum were found to be substantially linked with IL-6 levels. Sikorska et al. (2016) indicated that CRP concentrations in saliva linked strongly with those in serum and reduced significantly after treatment effectiveness. It was found in this study the levels increased in patients who did not respond to medication. Saliva, like the serum, contains hormones, antibodies, growth factors, enzymes, microorganisms, and their products (Pfaffe et al., 2011). Saliva can be viewed as a reflection of the body's physiological activity. TNF-α) is a pro-inflammatory cytokine produced during inflammation by cells that include monocytes and macrophages (Idriss and Naismith, 2000). In therapeutic settings, TNF- inhibitors are used to counteract the high TNF- levels that promote joint inflammation, hence avoiding TNF-tissue damage in RA. TNF inhibitors have been used to treat inflammatory diseases such as rheumatoid arthritis (RA), ankylosing spondylitis, psoriatic arthritis, ulcerative colitis, psoriasis, and Crohn's disease since 1998. Anti-TNF- reduces the inflammatory response of the body by inhibiting TNF- and preventing it from attaching to its receptor (TNFR1 or TNFR2) (Zamri and de Vries, 2020). There are few studies on the measurement of salivary TNF-α in rheumatoid patients, but there are studies on the relationship between RA with periodontitis, observed elevated TNF-α levels in the periodontitis group (Mutlak et al., 2015; Kaczyński et al., 2019). This study agreed with another study that indicated decreased TNF-α levels in saliva in RA patients receiving anti-TNF-α therapy (Mirrielees et al., 2010). Methotrexate is known to inhibit TNF-alpha production and lower levels of TNF-α have been associated with better response to methotrexate treatment (Olsen et al., 2014). However, Methotrexate acts by inhibiting an enzyme called dihydrofolate reductase, which is involved in the metabolism of folate. By interfering with folate metabolism, methotrexate reduces the production of certain immune cells and decreases inflammation (Hess and Khasawneh, 2015). Differences in folate metabolism and its impact on methotrexate response may contribute to the observed variations in treatment outcomes (van Ede et al., 2002). IL-35 is a recently found cytokine in the IL-12 family. Collison et al. (2007) and Niedbala et al. (2007) discovered IL-35, a new kind of cytokine. This study indicated that the concentration of IL35 was high in those who responded to the MTX, compared to its low concentration in the non-responders to MTX. A previous study of Iraqi patients proved IL-35 levels were somewhat higher in rheumatoid arthritis patients, but not by a significant amount ($p = 0.055$) (Omran et al., 2022). Because IL-35 is an anti-inflammatory and immunosuppressive cytokine that is mostly released by Tregs. It has the ability to stimulate Treg proliferation while inhibiting Th17 cell differentiation (Teymouri et al., 2018). By regulating the balance of Tregs and Th17 cells, IL-35 plays a key function in the pathogenic phase of RA (Akl et al., 2019). This suggests that IL-35 may play protective and damaging roles in osteoclast development. IL-35 may additionally inhibit MMP secretion in chondrocytes and synovial fibroblasts, additionally, aggrecanases and collagenases are activated, boosting the degradation of cartilage proteoglycan and collagen and enhancing osteoclast destruction (Shui et al., 2018; Liu et al., 2019; Sun et al., 2019). Treatment with IL-35 enhanced regulatory function by decreasing inflammatory cytokines such as interferon- γ and IL-17 as well as the cellular development of effector T-cells triggered by conjugation with CD2, CD3, and CD28, showing that IL-35 may have several targets for therapy in RA (Nakano et al., 2015). Wu et al. (2018) revealed that IL-35-mediated suppression of angiogenesis and inflammatory mediators in fibroblast-like synoviocytes provides a probable basis for antiangiogenetic benefits identified in RA



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experimental models. These findings showed that IL-35 could be exploited as a therapeutic target for RA and other angiogenesis-related illnesses.

**4. Conclusion:** Salivary of TNF-α and IL-35 were used to study the effectiveness of the drug MTX, and in case of non-response to treatment, patients will be referred to biological therapy. IL-35 might serve as a protective factor in RA as well as a novel therapy target.

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