Alterations in Immune Parameters ADP, ANA, IL-1, IL-6, IL-8, STAT-4, and TNF Alpha in Patients with Rheumatoid Arthritis and Osteoarthritis

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KEYWORDS
Rheumatoid arthritis; Osteoarthritis, ADP, ANA, IL-1, IL-6, IL-8, STAT-4, and TNF Alpha; pro-inflammation.

ABSTRACT
Background: Rheumatoid arthritis (RA) and osteoarthritis (OA) are chronic joint diseases with distinct pathophysiological mechanisms, but both involve alterations in immune parameters. This research investigates the alterations in several immune parameters, specifically adiponectin (ADP), antinuclear antibodies (ANA), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), signal transducer and activator of transcription 4 (STAT-4), and tumor necrosis factor-alpha (TNF Alpha), in patients with rheumatoid arthritis (RA) and osteoarthritis (OA). Also, the current study aims to identify the changes occurring in the physiological and immune parameters of people suffering from rheumatic diseases, as Evaluation of Immunological Inflammatory biomarkers levels ADP, ANA, IL1, IL6, IL8, STAT-4 and TNF-α.
Material & Methods: A cross-sectional laboratory-based study with 90 patient's participant was conducted. Using ELISA kits and laboratory tests, various biomarkers (Immunological Inflammatory biomarkers levels ADP, ANA, IL1, IL6, IL8, STAT-4 and TNF-α were measured among rheumatoid arthritis and Osteoarthritis patients.
Results: The findings revealed elevated levels of IL-1, IL-6, IL-8, STAT-4, and TNF Alpha in RA patients, reflecting a heightened inflammatory state. In contrast, OA patients exhibited a more moderate increase in these parameters, suggesting differing immune activation pathways. ADP and ANA levels showed distinct variations between the two patient groups, indicating their potential roles in the pathogenesis and progression of RA and OA.
Conclusion: These results contribute to a better understanding of the immune mechanisms underlying RA and OA, highlighting the importance of targeted therapeutic strategies to manage these conditions effectively. Further research is recommended to explore the clinical implications of these findings and develop personalized treatment approaches.

1. Introduction

Rheumatoid arthritis (RA) is a systemic chronic inflammatory autoimmune disease that affects the joints, leading to progressive damage and joint dysfunction. A key feature of RA is the persistent inflammatory infiltration of the synovial membrane, which is the primary site of inflammation in this condition. (Szumilas, K., et al., 2020).

Autoantibodies that recognize citrullinated proteins (anti-citrullinated protein antibodies (ACPAs)) or the Fc region of immunoglobulin G (rheumatoid factor (RF)) are found in most RA cases and define seropositivity. RA is one of the most common immune-mediated diseases, affecting about 0.5-1% of the global population, with a prevalence two to three times higher in women than in men. (Gravallese, E. M., & Firestein, G. S., 2023).

Osteoarthritis (OA) is a debilitating and heterogeneous condition characterized by varying degrees of articular cartilage degradation, osteophyte formation, and synovial inflammation. Evidence suggests that synovitis may appear early in the disease's development and is associated with disease severity and pain, making it a significant therapeutic target. (Boutet, M. A., et al., 2024).

Osteoarthritis (OA) is the most prevalent form of arthritis, impacting 1 in 3 individuals and affecting women more frequently than men. The prevalence of OA is increasing, partly due to the rising occurrence of OA risk factors such as obesity, physical inactivity, and joint injuries. OA is a significant contributor to global disability, particularly affecting the knee joint. Studies have shown a correlation between lipid metabolism disorders and bone disorders in OA. (Hawker, G. A., 2019).

Adiponectin is a protein secreted by adipocytes that plays a significant role in pathological processes

by modulating immune and inflammatory responses. Its pro-inflammatory effect is particularly evident in rheumatoid arthritis (RA). (Łączna, M., et al., 2022).

As is well known, antinuclear antibodies (ANA) are important biomarkers for various systemic autoimmune diseases, including systemic lupus erythematosus (SLE), Sjögren's syndrome (SS), scleroderma (SSc), polymyositis (PM), and mixed connective tissue disease (MCTD). However, the precise clinical implications of ANA in rheumatoid arthritis (RA) and osteoarthritis (OA), and their relationship with other serological markers, have remained unclear. This study aimed to elucidate the correlation between serum ANA and cases of RA and OA. (Liu, F., et al., 2024).

Adipose tissue is a primary source of pro-inflammatory cytokines. In overweight and obese adults, plasma levels of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are significantly elevated. The abnormally high levels of TNF-α, IL-6, and interleukin-1 (IL-1) in OA patients are considered significant contributors to cartilage loss. Furthermore, IL-1, TNF-α, and IL-6 may indirectly contribute to OA by regulating the release of adiponectin and leptin from adipocytes. (Wang, T., & He, C., 2018).

IL-8 mainly promotes inflammation and joint damage by recruiting and activating neutrophils. It plays a more significant role in Rheumatoid Arthritis (RA) but also contributes to the progression of Osteoarthritis (OA). (Sanchez-Lopez, E., et al., 2022).

STAT4 is a transcription factor involved in various cytokine signalling pathways and the differentiation of T-cell subsets. Several studies have linked STAT4 gene polymorphism with various autoimmune diseases, including rheumatoid arthritis. (Ebrahimiyan, H., et al., 2019).

2. Methodology

This study was conducted in Erbil International Hospital and Nawroz Hospital, Erbil, Iraq, using a cross-sectional approach on a study population aged 11 to 77 years. A total of 90 patients attending clinics specialized in Rheumatology and Osteoarthritis at Erbil International Hospital and Newroz Hospital were included and compared with healthy individuals from whom control samples were taken. All participants underwent immunological tests which included ADP, ANA, IL-1, IL-6, IL-8, STAT4, and TNF-α. Based on disease condition and sex, the patients were divided into seven groups as shown in Table 1.

Table (1): - A Table showing the division of patients and healthy into different groups depending on the disease condition and sex

No.		Disease Condition	Sex	Abbreviation Name	Total No. for the specific condition
1		Rheumatoid Arthritis	Male	MRA	10
2		Rheumatoid Arthritis	Female	FRA	25
3		Osteoarthritis	Male	MOA	14
4		Osteoarthritis	Female	FOA	22
5		Rheumatoid Arthritis-Osteoarthritis	Female	FRAOA	9
6		Healthy Control	Male	MCT	5
7		Healthy Control	Female	FCT	5

Samples Collection

Ten milliliters of venous blood was drawn from patients and healthy individuals participating in the current study while they were fasting, using medical syringes (5 ml) twice. After sterilizing the brachial vein area with 76% ethyl alcohol, blood was collected into two gel (yellow) tubes, each containing 3 ml (totaling 6 ml). The blood samples were then allowed to sit for 20 minutes before being centrifuged at 4000 rpm for 12 minutes to obtain serum. The serum was used to conduct immunological tests using the Automatic ELISA 96 Mindray.)

Determination of Human adiponectin (ADP) conc.

Principle

This ELISA kit employs the Sandwich-ELISA technique as its methodology. The Microelisa stripplate included in the kit comes pre-coated with an antibody that specifically targets ADP, ANA, IL-1, IL-6, IL-8, STAT4 and TNF-α. To conduct the test, either ADP, ANA, IL-1, IL-6, IL-8, STAT4 and TNF-α standards or samples are introduced into the designated wells of the Micro ELISA stripplate, where they interact with the specific antibody. Subsequently, a Horseradish Peroxidase (HRP)-linked antibody that is specific to ADP, ANA, IL-1, IL-6, IL-8, STAT4 and TNF-α is introduced into each well of the Micro ELISA stripplate and left to incubate. Any unbound components are then removed through a washing process. Next, a TMB substrate solution is added to every well. Only those wells containing ADP, ANA, IL-1, IL-6, IL-8, STAT4 and TNF-α and the HRP-conjugated ADP, ANA, IL-1, IL-6, IL-8, STAT4 and TNF-α antibody will exhibit a blue coloration, which will subsequently turn yellow after the stop solution is added. The optical density (OD) is quantitatively measured through spectrophotometry at a wavelength of 450 nm. The OD value is directly proportional to the concentration of ADP, ANA, IL-1, IL-6, IL-8, STAT4 and TNF-α present in the samples. To determine the concentration of ADP, ANA, IL-1, IL-6, IL-8, STAT4 and TNF-α in the samples, this can be achieved by comparing the OD readings of the samples with a standard curve.

Materials provided with the kit

No.	Materials provided with the kit	kit 96 determinations	Storage
1	User manual	1	R.T.
2	Closure plate membrane	2	R.T.
3	Sealed bags	1	R.T.
4	Microelisa stripplate	1	2-8℃
5	Standard：18 ng/ml	0.5ml×1 bottle	2-8℃
6	Standard diluent	1.5ml×1 bottle	2-8℃
7	HRP-Conjugate reagent	6ml×1 bottle	2-8℃
8	Sample diluent	6ml×1 bottle	2-8℃
9	Chromogen Solution A	6ml×1 bottle	2-8℃
10	Chromogen Solution B	6ml×1 bottle	2-8℃
11	Stop Solution	6ml×1 bottle	2-8℃
12	wash solution	20ml (30X) ×1bottle	2-8℃

Sample preparation (Serum preparation)

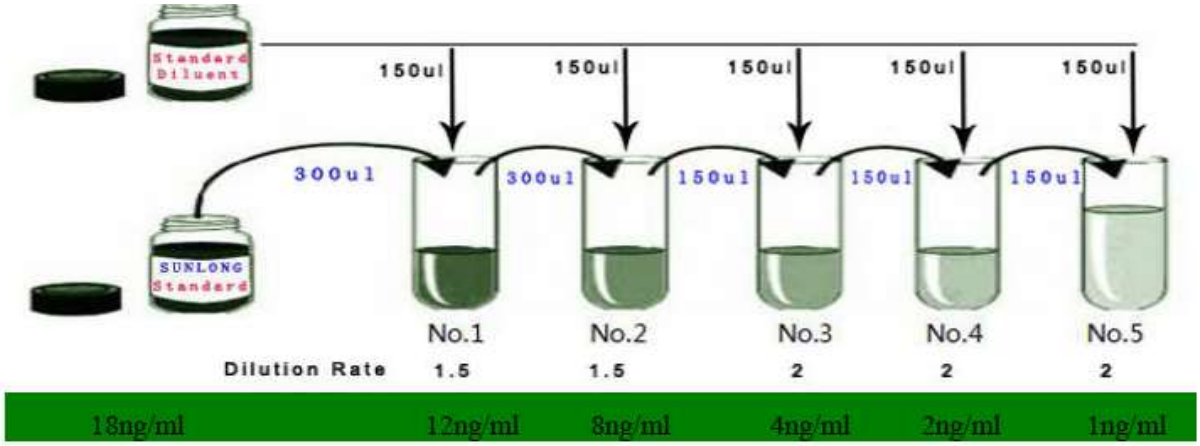
After collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature. This usually takes 10-20 minutes. Remove the clot by centrifuging at 2,000-3,000 rpm for 20 minutes. If precipitates appear during reservation, the sample should be centrifugated again.

Procedure

1. Dilution of Standards, Dilute the standard by small tubes first, then pipette the volume of 50ul from each tube to microplate well, each tube uses two wells, total ten wells.

12ng/ml	**Standard No.1**	**300µl Original Standard + 150µl Standard diluents**
8ng/ml	Standard No.2	300µl Standard No.1 + 150µl Standard diluents
4ng/ml	Standard No.3	150µl Standard No.2 + 150µl Standard diluent
2ng/ml	Standard No.4	150µl Standard No.3 + 150µl Standard diluent

1ng/ml	Standard No.5	150µl Standard No.4 + 150µl Standard diluent



2. In the Microelisa stripplate, leave a well empty as blank control. In sample wells, 40µl Sample dilution buffer and 10µl sample are added (dilution factor is 5). Samples should be loaded onto the bottom without touching the well wall. Mix well with gentle shaking.

3. Incubation: incubate 30 min at 37℃ after sealed with Closure plate membrane.

4. Dilution: dilute the concentrated washing buffer with distilled water (30 times for 96T and 20 times for 48T).

5. Washing: carefully peel off Closure plate membrane, aspirate and refill with the wash solution. Discard the wash solution after resting for 30 seconds. Repeat the washing procedure for 5 times.

6. Add 50 µl HRP-Conjugate reagent to each well except the blank control well.

7. Incubation as described in Step 3.

8. Washing as described in Step 5.

9. Coloring: Add 50 µl Chromogen Solution A and 50 µl Chromogen Solution B to each well, mix with gently shaking and incubate at 37℃ for 15 minutes. Please avoid light during coloring.

10. Termination: add 50 µl stop solution to each well to terminate the reaction. The color in the well should change from blue to yellow.

11. Read absorbance O.D. at 450nm using a Microtiter Plate Reader. The OD value of the blank control well is set as zero. Assay should be carried out within 15 minutes after adding stop solution.

Notes:

1. Store the kit at 4 ° C upon receipt. The kit should be equilibrated to room temperature before the assay. Remove any unneeded strips from Human ADP, ANA, IL-1, IL-6, IL-8, STAT4 and TNF-α Antibody-Coated plate, reseal them in zip-lock foil and keep at 4°C.
2. Precipitates may appear in concentrated washing buffer. Please heat the buffer to dissolve all the precipitates, which will not affect the results.
3. Accurate pipette should be used to avoid experimental error. Samples should be added to the Microplate in less than 5 minutes. If a large number of samples are included, multiple channel pipette is recommended.
4. Standard curve should be included in every assay. Replicate wells are recommended. If the OD value of the sample is greater than the first well of standards, please dilute the sample (n times)

before test. When calculating the original ADP concentration, please multiply the total dilution factor (XnX5).

5. In order to avoid cross-contamination, Microplate sealers are for one-time use only.
6. Please keep Substrate away from light.
7. All the operation should be accordance with the manufacturer's instructions strictly. The results determined by the Microtiter Plate Reader.
8. All the samples, washing buffer and wastes should be treated as infectious agents.
9. Reagents from different lots should not be mixed.

Calculation of Results

Known concentrations of Human ADP, ANA, IL-1, IL-6, IL-8, STAT4 and TNF-α Standard and its corresponding reading OD is plotted on the log scale (x-axis) and the log scale (y-axis) respectively.

The concentration of Human ADP, ANA, IL-1, IL-6, IL-8, STAT4 and TNF-α in sample is determined by plotting the sample's O.D. on the Y-axis. The original concentration is calculated by multiplying the dilution factor.

3. Results and discussion

Descriptive Characteristics:

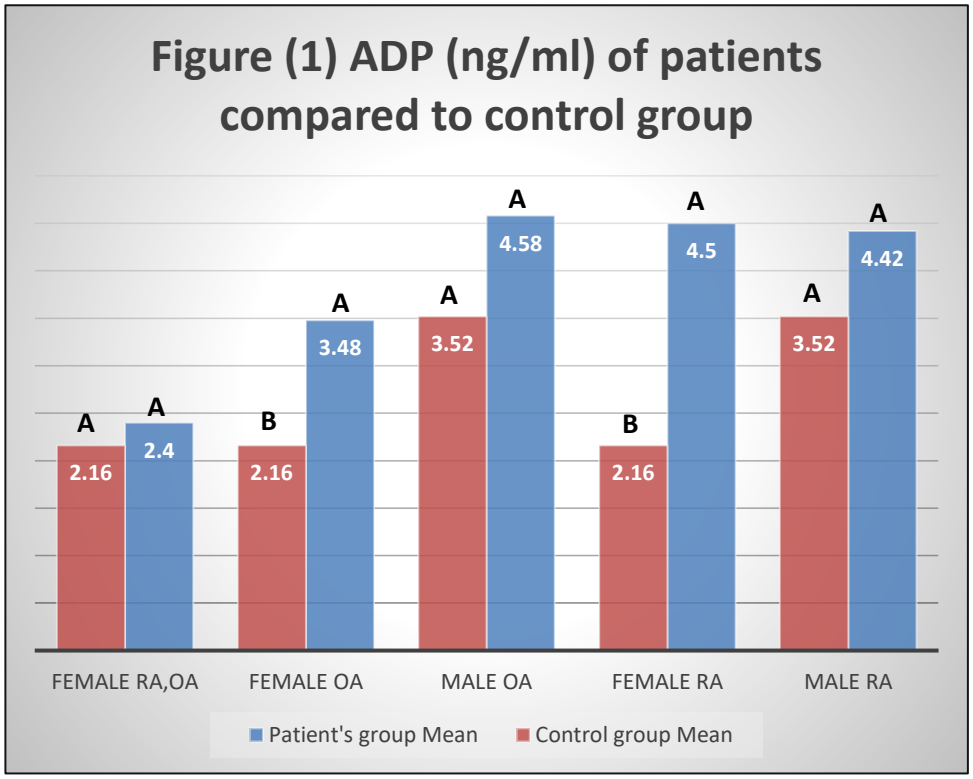
A total of ninety patients, both males and females, were included in the present study. They were divided into five groups: Male RA, Female RA, Male OA, Female OA, and Female RA-OA, with ten healthy individuals serving as a control group. The findings of this research indicate that despite significant advancements in managing rheumatoid arthritis (RA), the disease continues to have a considerable impact on many patients. Notably, specific factors, including certain parameter levels, are strongly associated with various symptoms such as fatigue, pain, morning stiffness, and changes in functional ability. These factors continue to significantly affect the daily lives of individuals with rheumatoid arthritis and osteoarthritis.

ADP of patients compared to control group

Table (1), which included ADP of patients (ng/ml) compared with the ADP of control group, shows that there are significant differences appeared in the **Female RA** (**4.5)** and **Female OA (3.48)** groups, while no significant differences in all other groups when compared with the control groups (Male control= **3.52**, Female control= **2.16**). **At P ≤ 0.05** as shown in table (1) and figure (1).

Table (1) ADP (ng/ml) of patients compared to control group

Case of study	Male RA	Female RA	Male OA	Female OA	Female RA, OA
Patient's group **Mean ± SD**	**4.42 A ± 1.5**	**4.5 A ± 1.59**	**4.58 A ± 1.42**	**3.48 A ± 1.41**	**2.4 A ± 1.38**
Control group **Mean ± SD**	**3.52 A ± 1.47**	**2.16 B ± 1.44**	**3.52 A ± 1.47**	**2.16 B ± 1.44**	**2.16 A ± 1.44**
T test	**1.14**	**3.66**	**1.49**	**2.81**	**0.53**
P value	**0.13**	**0.00051**	**0.076**	**0.0046**	**0.30**



The findings of our present study were consistent with those reported by Baker and colleagues in 2024, demonstrating a significant elevation in adiponectin levels, particularly evident in the female rheumatoid arthritis group (FRA) and the female osteoarthritis group (FOA). Conversely, the remaining study groups exhibited a negligible increase in adiponectin levels compared to the control group. (Baker, J. F., et al., 2024).

In addition, the findings of the present study align with those of Szumilas, K., et al., 2020. Their research demonstrated that individuals with rheumatoid arthritis (RA) exhibit elevated levels of adiponectin in both serum and synovial fluid, particularly among the female rheumatoid arthritis (FRA) and female osteoarthritis (FOA) groups examined in our study. (Szumilas, K., et al., 2020).

The findings of our current study align closely with those reported by Shyaa and colleagues, indicating a significant and noticeable increase in serum adiponectin levels among RA patient groups. In comparison to the control group, the levels of adiponectin in RA patients' serum were notably higher, especially prominent in the female rheumatoid arthritis group (FRA) and the female osteoarthritis group (FOA). Conversely, the remaining study groups demonstrated only a minimal increase in adiponectin levels when compared to the control group. (Shyaa, S. S., et al., 2022).

Adiponectin, a protein secreted by fat cells, has a significant role in pathological processes by influencing the regulation of immune and inflammatory responses. Its involvement in modulating these responses is particularly notable in conditions such as rheumatoid arthritis (RA), where it demonstrates pro-inflammatory effects. (Łączna, M., et al., 2022).

Increased concentrations of adiponectin, a protein hormone primarily secreted by adipose tissue, can arise from various factors, which include:

Inflammatory Conditions: Although adiponectin is typically recognized as an anti-inflammatory adipokine, its levels may paradoxically escalate in certain inflammatory conditions like rheumatoid arthritis and inflammatory bowel disease due to dysfunction in adipose tissue and systemic inflammation.

Hormonal Regulation: Adiponectin concentrations can be impacted by hormonal factors such as sex

hormones and growth hormone. For instance, estrogen has been demonstrated to elevate adiponectin levels, contributing in part to the higher levels observed in females compared to males.

Insulin Sensitivity: Adiponectin levels exhibit an inverse relationship with insulin resistance. Therefore, circumstances characterized by enhanced insulin sensitivity, such as weight loss, physical activity, and specific medications like thiazolidinediones, can lead to heightened adiponectin levels.

Medications: Certain medications, including various antidiabetic drugs (e.g., metformin), lipid-lowering agents (e.g., statins), and antihypertensive medications (e.g., angiotensin receptor blockers), have been demonstrated to elevate adiponectin levels.

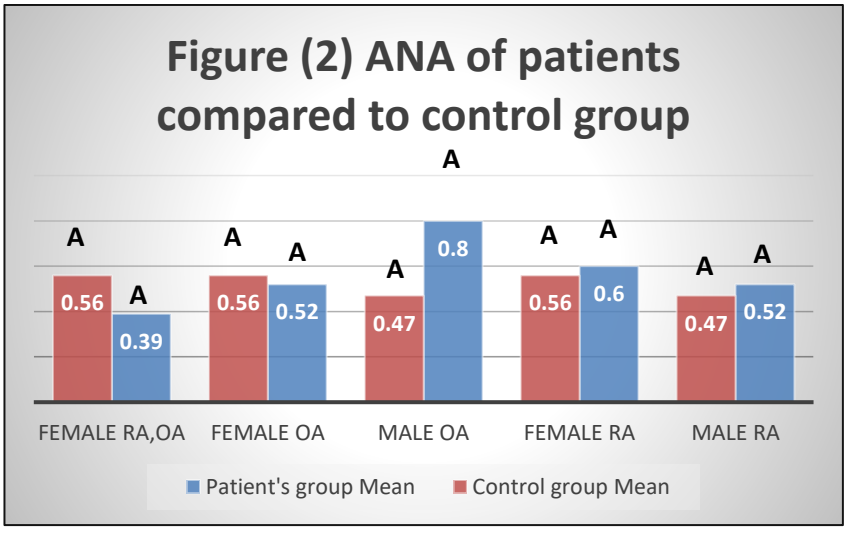
Diet: Certain dietary constituents, such as omega-3 fatty acids, fiber-rich foods, and foods with a low glycemic index, have been linked to increased adiponectin levels. Conversely, diets abundant in saturated fats and refined carbohydrates may diminish adiponectin concentrations.

ANA of patients compared to control group

Table (2), which included ANA of patients compared with ANA of control group, shows that there are no significant differences in all groups when compared with the control groups. (According to the cutoff value ≥2.861= +ve, ≤0.609= -ve). **At P ≤ 0.05** as shown in table (2) and figure (2).

Table (2) ANA of patients compared to control group

Case of study	Male RA	Female RA	Male OA	Female OA	Female RA, OA
Patient's group Mean ± SD	**0.52 ^{A} ± 0.17**	**0.6 ^{A} ± 0.18**	**0.8 ^{A} ± 0.21**	**0.52 ^{A} ± 0.40**	**0.39 ^{A} ± 0.402**
Control group Mean ± SD	**0.47 ^{A} ± 0.380**	**0.56 ^{A} ± 0.388**	**0.47 ^{A} ± 0.380**	**0.56 ^{A} ± 0.388**	**0.56 ^{A} ± 0.388**
T test	**0.38**	**0.35**	**1.05**	**0.22**	**1.6**
P value	**0.35**	**0.36**	**0.15**	**0.41**	**0.066**



The findings of our present study were consistent with those reported by Alyami and colleagues in their 2024 study, wherein negative or weak test results for antinuclear antibodies (ANA) were observed. This concurred with the results of our current study, as no significant increase in ANA levels was noted across all patient groups compared to the control groups. (Alyami, M. B., et al., 2024).

While, the findings of our current study were incongruent with those of d'Apuzzo and colleagues in

their 2023 study, as no notable disparities were observed in the levels of antinuclear antibodies (ANA) compared to the control groups. (d'Apuzzo, F., et al., 2023).

Furthermore, the findings of the present study were not consistent with those of Odewusi and colleagues in their 2022 study, as there was no discernible elevation in the level of antinuclear antibodies (ANA) compared to the control samples. (ODEWUSI, O. A., et al., 2022).

The lack of a noticeable increase in the level of antinuclear antibodies (ANA) in patients with rheumatoid arthritis (RA) and osteoarthritis (OA) can be attributed to several factors:

Disease Pathophysiology: ANA are autoantibodies that target components of the cell nucleus. While ANA are commonly associated with systemic autoimmune diseases such as systemic lupus erythematosus (SLE), they are not typically a hallmark feature of RA or OA. Instead, RA is characterized by autoantibodies such as rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP) antibodies, while OA is primarily a degenerative joint disorder without systemic autoimmune involvement.

Specificity of Autoantibodies: The presence and levels of autoantibodies, including ANA, can vary depending on the specific autoimmune condition. While ANA may be elevated in diseases like SLE, their levels may not be as prominently increased in RA or OA.

Heterogeneity of Patient Population: Patients with RA and OA represent heterogeneous groups with diverse clinical presentations and underlying pathophysiologic mechanisms. Not all individuals with RA or OA may develop detectable levels of ANA, particularly if their disease course does not involve significant autoimmune or inflammatory processes.

Diagnostic Criteria: The diagnosis of RA and OA is primarily based on clinical evaluation, imaging studies, and laboratory tests such as assessment of inflammatory markers and autoantibodies specific to each condition (e.g., RF and anti-CCP antibodies for RA). ANA testing is not routinely performed as part of the diagnostic workup for RA or OA unless there are clinical indications of overlapping autoimmune diseases.

Overlapping Conditions: While RA and OA are distinct conditions, they can coexist with other autoimmune diseases that are associated with elevated ANA levels. In such cases, the lack of a noticeable increase in ANA levels specifically in RA or OA patients may be overshadowed by the presence of other autoimmune conditions.

Overall, the lack of a significant increase in ANA levels in patients with RA and OA reflects the differences in the underlying pathophysiology and clinical manifestations of these conditions compared to systemic autoimmune diseases characterized by prominent ANA elevation.

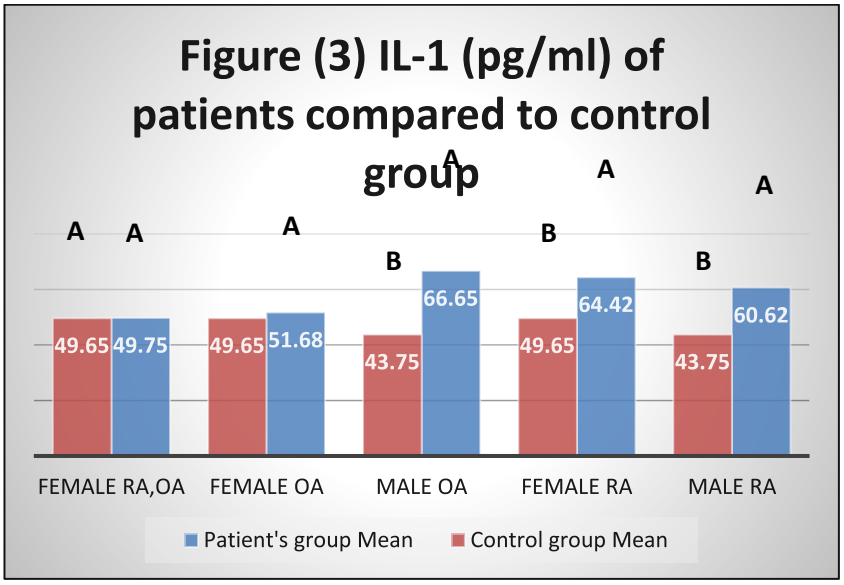
IL-1 of patients compared to control group

Table (3), which included IL-1 of patients (pg/ml) compared with the IL-1 of control group, shows that there are significant differences appeared in the **Male RA (60.62)**, **Female RA (64.42)** and **Male OA (66.65)** groups, while no significant differences in all other groups when compared with the control groups (Male control= **43.75**, Female control= **49.65**). **At P ≤ 0.05** as shown in table (3) and figure (3).

Table (3) IL-1 (pg/ml) of patients compared to control group

Case of study	Male RA	Female RA	Male OA	Female OA	Female RA, OA
Patient's group Mean ± SD	60.62 ^{A} ± 23.42	64.42 ^{A} ± 10.05	66.65 ^{A} ± 7.49	51.68 ^{A} ± 9.89	49.75 ^{A} ± 14.17
Control group Mean ± SD	43.75 ^{B} ± 13.97	49.65 ^{B} ± 14.19	43.75 ^{B} ± 13.97	49.65 ^{A} ± 14.19	49.65 ^{A} 14.19
T test	2.72	5.14	3.09	0.24	0.019

P value	0.008	0.00001	0.0033	0.4	0.49



The findings of the present study were consistent with those of Almeida-Santiago and colleagues in 2022, showing a significant elevation in interleukin 1 levels compared to the control group. This elevation was observed in the male rheumatoid arthritis group (MRA), female rheumatoid arthritis group (FRA), and male osteoarthritis group (MOA). While the other groups included in the study also exhibited an increase in interleukin 1 levels, it was not as pronounced, particularly evident in the female osteoarthritis group (FOA) and the female rheumatoid arthritis group (FRA-OA). (Almeida-Santiago, C., et al., 2022).

These findings indicate that IL-1 might play a role in the onset of Rheumatoid Arthritis (RA) and Osteoarthritis (OA). Particularly, IL-1 could serve as an additional indicator for differentiating individuals with RA and OA from those without these conditions.

Interleukin-1, an inflammatory cytokine, is recognized for its multifaceted physiological roles and pathological implications, crucially influencing both health and disease. Over the past decade, the interleukin-1 family has shown expansion, with mounting evidence emphasizing its pivotal role in connecting innate immunity to various diseases beyond inflammation-related conditions. (Kaneko N., et al., 2019).

The findings of the present study were consistent with those of Sun and colleagues in 2022, indicating a notable rise in interleukin -1 levels among the patient groups, particularly in the male osteoarthritis group (MOA). However, there was no significant increase in interleukin -1 observed in the female osteoarthritis group (FOA) or the rheumatoid arthritis - osteoarthritis female group (FRA-OA). (Sun, Z., et al., 2022).

The fragility of bones in Rheumatoid Arthritis (RA) stems from a blend of factors including systemic inflammation, the circulation of autoantibodies, and the release of proinflammatory cytokines. Inflammatory cytokines like TNF-α, IL-6, IL-1, along with cytokines from immune cells, hinder the formation of osteoblasts while encouraging the generation of osteoclasts. Anti-citrullinated peptide/protein antibody (ACPA) plays a crucial role in bone loss, exerting a direct and separate impact on osteoclasts. This effect might be facilitated through IL-8-dependent activation of osteoclasts, leading to bone loss predominantly around the joints of RA patients who test positive for ACPA. Together, these elements contribute adversely to bone health. (Wu, D., et al., 2022).

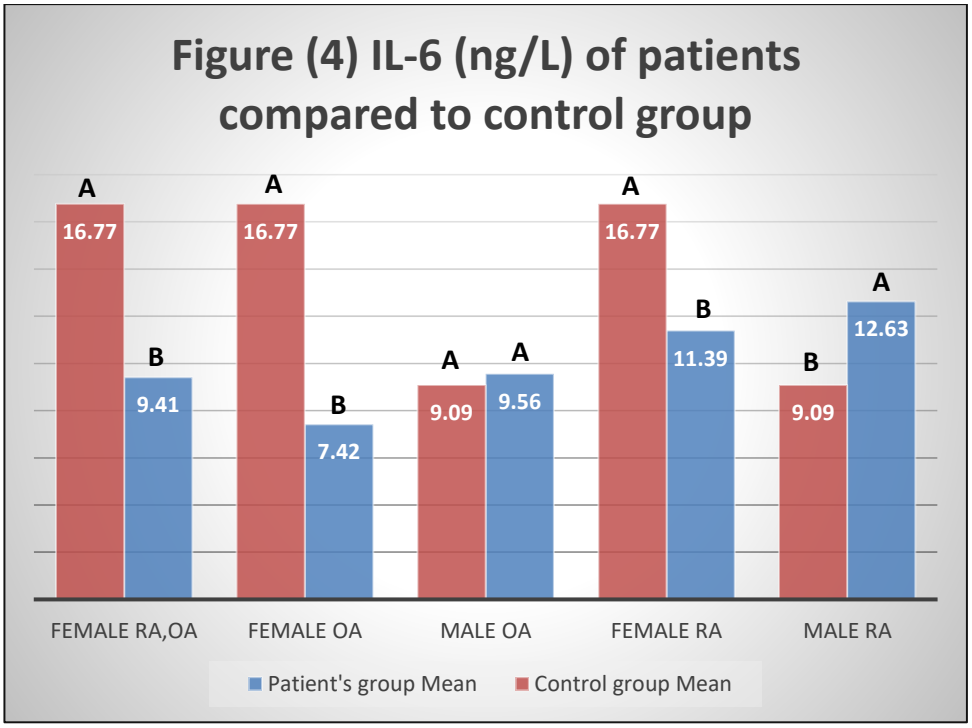
Interleukin 1 is considered a pro-inflammatory cytokine and contributes directly or indirectly to the pathophysiology of osteoarthritis and cartilage degeneration. (Jrad, A. I. S., et al., 2023).

IL-6 of patients compared to control group

Table (4), which included IL-6 of patients (ng/L) compared with the IL-6 of control group, shows that there are significant differences appeared in all study groups **Males RA** (**12.63**), **Female RA** (**11.39**), **Female OA** (**7.42**), and the **Female RA-OA** (**9.41**) groups, except **Males OA** (**11.39**) group, when compared with the control groups (Male control= **9.09**, Female control= **16.77**). **At P ≤ 0.05** as shown in table (4) and figure (4).

Table (4) IL-6 (ng/L) of patients compared to control group

Case of study	Male RA	Female RA	Male OA	Female OA	Female RA, OA
Patient's group Mean ± SD	12.63 ^{A} ± 2.83	11.39 ^{B} ± 2.98	9.56 ^{A} ± 4.06	7.42 ^{B} ± 3.81	9.41 ^{B} ± 3.95
Control group Mean ± SD	9.09 ^{B} ± 4.50	16.77 ^{A} ± 3.92	9.09 ^{A} ± 4.50	16.77 ^{A} ± 3.92	16.77 ^{A} ± 3.92
T test	2.05	2.14	0.31	4.44	2.31
P value	0.03	0.02	0.37	0.00007	0.019



The findings of our current research align with Giordano and colleagues' study, indicating a notable rise in interleukin 6 among males with rheumatoid arthritis (MRA), accompanied by a marginal and non-significant increase in males with osteoarthritis (MOA).

The inflammatory pathways associated with IL-6 play a significant role in the onset and advancement of rheumatoid arthritis (RA). (Pandolfi, F., et al., 2020).

The findings of the present study are consistent with those of a study conducted by Gopal and

colleagues in 2019, demonstrating a notable elevation in interleukin 6 levels in a group of males with rheumatoid arthritis (MRA). (Gopal, K., et al., 2019).

However, our results diverge in observing a substantial reduction in interleukin 6 levels among females with rheumatoid arthritis (FRA), females with osteoarthritis (FOA), and females with rheumatoid arthritis-osteoarthritis (FRA-OA), contrasting the trends observed in males. (Giordano, R., et al., 2020).

The reason for the lack of interleukin 6 in women suffering from rheumatoid arthritis and osteoarthritis may be due to several reasons, the most important of which may be hormonal differences, as fluctuations in estrogen levels, especially during menopause, can affect the production of interleukin 6, as low estrogen levels lead to A decrease in the synthesis of interleukin 6 in women, in addition to immune regulation. Dysregulation of the immune system, including disturbances in T-cell function or cytokine balance, can lead to a decrease in the production of interleukin 6. This may appear in women with rheumatoid arthritis and osteoarthritis, which leads to changes. In immune responses, which lead to a decrease in the synthesis of interleukin 6.

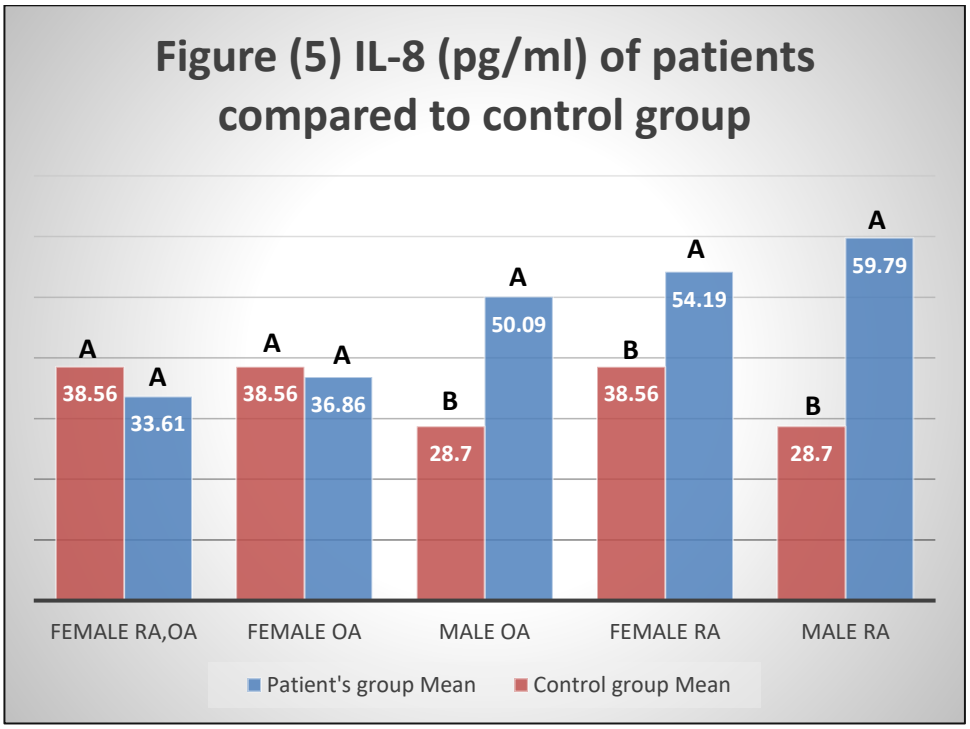
It's important to highlight that the development of Osteoarthritis (OA) results from a disruption in the equilibrium between cartilage breakdown and repair processes, accompanied by inflammatory alterations across the joint, ultimately resulting in joint degeneration and discomfort. (Liao, Y., et al., 2022).

IL-8 of patients compared to control group

Table (5), which included IL-8 of patients (Pg/ml) compared with the IL-8 of control group, shows that there are significant differences appeared in the **Males RA** (**59.79), Female RA** (**54.19)**, and **Males OA** (**50.09)** groups, while no significant differences in **Female OA** and **Female RA-OA** groups, when compared with the control groups (Male control= **28.7**, Female control= **38.56**). **At P ≤ 0.05** as shown in table (5) and figure (5).

Table (5) IL-8 (Pg/ml) of patients compared to control group

Case of study	Male RA	Female RA	Male OA	Female OA	Female RA, OA
Patient's group Mean ± SD	59.79 ^{A} ± 12.85	54.19 ^{A} ± 13.54	50.09 ^{A} ± 10.22	36.86 ^{A} ± 11.17	33.61 ^{A} ± 13.44
Control group Mean ± SD	28.7 ^{B} ± 13.98	38.56 ^{B} ± 13.94	28.7 ^{B} ± 13.98	38.56 ^{A} ± 13.94	38.56 ^{A} ± 13.94
T test	4.57	3.44	3.41	0.32	0.77
P value	0.00025	0.00091	0.0016	0.37	0.22



The findings of the current study were consistent with those of Ruan and colleagues' research in 2019, showing a substantial elevation in interleukin 8 levels within the patient groups, notably in the male rheumatoid arthritis group (MAR), the female rheumatoid arthritis group (FRA), and the male osteoarthritis group (MOA). However, there was no significant increase in interleukin 8 observed in the female osteoarthritis group (FOA) or the female rheumatoid arthritis - osteoarthritis group (FRA-OA). (Ruan, G., et al., 2019).

Nevertheless, IL-8, regarded as an additional inflammatory soluble mediator, plays a role in promoting pathogenic actions by inducing harmful activation of immune and stromal cells in the synovial membrane, tendons, and extra-articular sites of RA, along with affecting blood vessels and lungs, leading to extra-articular complications. These complications may be mitigated by treatments targeting anti-TNFα and anti-IL6. (Gremese, E., et al., 2023).

Additionally, the results of the present study were consistent with those of Henrotin's research in 2022, showing a notable elevation in interleukin 8 across all patient groups, except for the female osteoarthritis group (FOA) and the female osteoarthritis-rheumatoid arthritis group (FRA-OA) did not exhibit any significant difference compared to the control group. (Henrotin, Y., 2022).

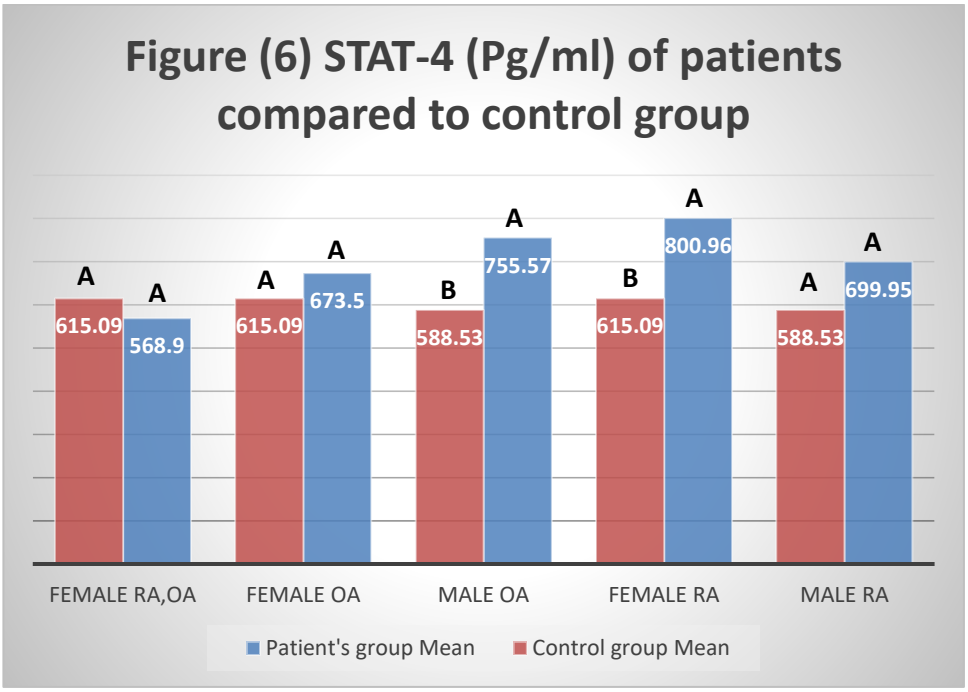
STAT-4 of patients compared to control group

Table (6), which included STAT-4 of patients (Pg/ml) compared with the STAT-4 of control group, shows that there are significant differences appeared in the **Female RA** (**800.96)** and **Males OA** (**755.57)** groups, while no significant differences in all other groups when compared with the control groups (Male control= **588.53**, Female control= **615.09**), **At P ≤ 0.05** as shown in table (6) and figure (6).

Table (6) STAT-4 (pg/ml) of patients compared to control group

Case of study	Male RA	Female RA	Male OA	Female OA	Female RA, OA
Patient's group Mean ± SD	699.95 A ± 22.85	800.96 A ± 152.56	755.57 A ± 134.71	673.5 A ± 129.45	568.9 A ± 150.68
Control group Mean ± SD	588.53 A ± 160.44	615.09 B ± 161.54	588.53 B ± 160.44	615.09 A ± 161.54	615.09 A ± 161.54
T test	1.3	3.23	2.44	0.7	0.53

P value	**0.10**	**0.0015**	**0.019**	**0.24**	**0.30**



The findings of our present research align closely with those of Bravo-Villagra et al. (2024), particularly in relation to the female rheumatoid arthritis (FRA) and male osteoarthritis (MOA) cohorts in our study. These cohorts exhibited a significant elevation in STAT-4 levels compared to the control group. Additionally, although there was a tendency for increased STAT-4 levels in the male rheumatoid arthritis (MRA) and female osteoarthritis (FOA) groups, this trend did not reach statistical significance. Notably, the female rheumatoid arthritis-osteoarthritis group (FRA-OA) did not display any marked or significant elevation in STAT-4 levels compared to the control group. (Bravo-Villagra, K., et al., 2024).

The current study's findings are in line with a previous investigation conducted by Ebrahimiyan et al. in 2019, which demonstrated a correlation between STAT-4 and the susceptibility to rheumatoid arthritis. This earlier research highlighted STAT-4 as a transcription factor crucial in various cytokine signaling pathways and T cell subset differentiation. These findings bolster the current study's results, which revealed a significant elevation in STAT-4 levels, particularly in the female rheumatoid arthritis (FRA) and male osteoarthritis (MOA) cohorts, compared to the control group. As previously noted, the male rheumatoid arthritis (MRA) and female osteoarthritis (FOA) groups exhibited increased STAT-4 levels, albeit not reaching statistical significance.

Moreover, the female rheumatoid arthritis-osteoarthritis group (FRA-OA) did not display a significant increase in STAT-4 levels compared to the control group. (Ebrahimiyan, H., et al., 2019).

It's important to acknowledge that the rise in STAT-4 levels could stem from several significant factors, encompassing the inflammatory response, T-cell differentiation, disease severity, and genetic influences. Regarding the inflammatory response, STAT-4 is recognized for its involvement in mediating inflammatory reactions, particularly in autoimmune disorders like rheumatoid arthritis (RA) and osteoarthritis (OA). The heightened levels of STAT-4 may mirror an augmented inflammatory state characteristic of these conditions. Concerning T-cell differentiation, STAT-4's role in the differentiation of T helper 1 (Th1) cells, crucial in cellular immunity and inflammation, suggests that disruptions in Th1 cell differentiation could contribute to raised STAT-4 levels, especially in autoimmune ailments such as RA. Additionally, disease severity could correlate with increased STAT-4 levels. More severe or advanced stages of RA or OA may prompt heightened

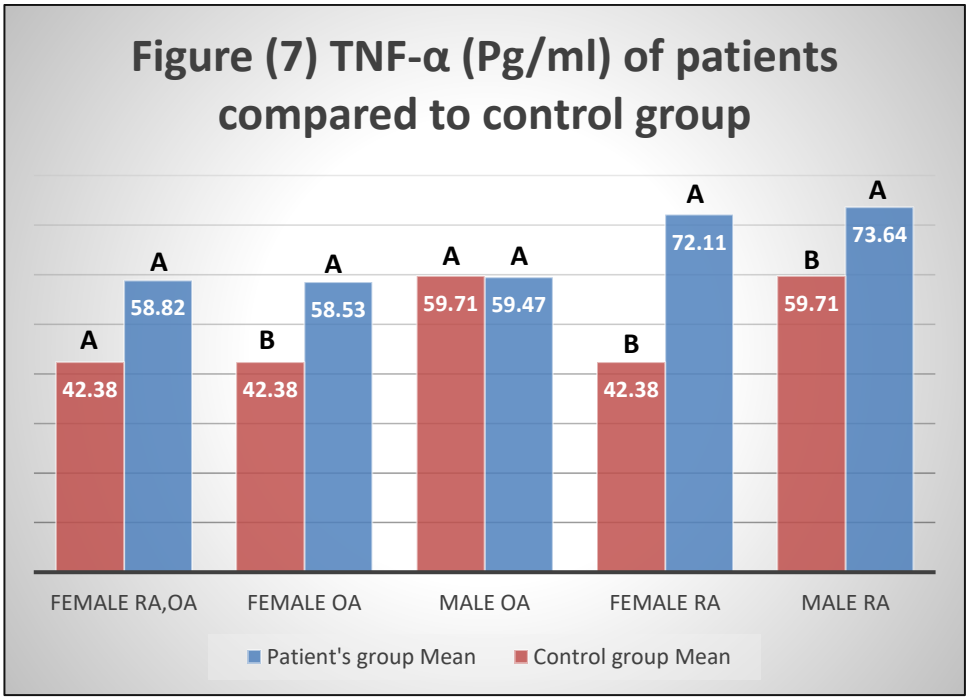
production of inflammatory cytokines and immune activation, thereby elevating STAT-4 expression. Furthermore, genetic factors play a role, with genetic variations or polymorphisms in the STAT-4 gene linked to heightened autoimmune disease risk, including RA. Certain genetic predispositions may lead to overexpression or hyperactivation of STAT-4, culminating in elevated protein levels.

TNF-α of patients compared to control group

Table (7), which included TNF-**α** of patients (Pg/ml) compared with the TNF-**α** of control group, shows that there are significant differences appeared in the **Males RA** (**73.64), Female RA** (**72.11)** and **Female OA (58.53)**, while no significant differences in **Males OA** and **Female RA-OA** groups, when compared with the control groups (Male control= **59.71**, Female control= **42.38**). **At P ≤ 0.05** as shown in table (7) and figure (7).

Table (7) TNF-α (Pg/ml) of patients compared to control group

Case of study	Male RA	Female RA	Male OA	Female OA	Female RA, OA
Patient's group **Mean ± SD**	**73.64** ^{A} **± 5.26**	**72.11** ^{A} **± 7.47**	**59.47** ^{A} **± 10.17**	**58.53** ^{A} **± 13.00**	**58.82** ^{A} **± 14.60**
Control group **Mean ± SD**	**59.71** ^{B} **± 16.05**	**42.38** ^{B} **± 14.67**	**59.71** ^{A} **± 16.05**	**42.38** ^{B} **± 14.67**	**42.38** ^{A} **± 14.67**
T test	**3.17**	**4.42**	**0.03**	**1.89**	**1.61**
P value	**0.0037**	**0.00006**	**0.48**	**0.035**	**0.065**



The findings of the present study aligned with those of the Farrugia and Baron study conducted in 2016, indicating a marked elevation in TNF-alpha levels. Specifically, the current study demonstrated a significant increase in TNF-alpha levels in the male rheumatoid arthritis group (MRA), female rheumatoid arthritis group (FRA), and female osteoarthritis group (FOA) compared to the control samples. However, there was no noticeable increase observed in the male osteoarthritis group (MOA), while the female rheumatoid arthritis-osteoarthritis group (FRA-OA) exhibited a very slight and insignificant increase relative to the control sample. (Farrugia, M., & Baron, B., 2016).

In general, inflammatory cytokines, particularly TNF-A, along with two interleukins, IL-1 and IL-6, play pivotal roles in initiating inflammation and causing joint damage, thus contributing significantly

to the development of RA. (Pandolfi, F., et al., 2020).

The findings of the current study contrasted with those of the 2023 study by Krenytska and colleagues, which demonstrated a significant decrease in TNF levels among patients with rheumatoid arthritis (RA) and osteoarthritis (OA). In contrast, the current study revealed a notable increase in TNF levels in the male rheumatoid arthritis group (MRA), female rheumatoid arthritis group (FRA), and female osteoarthritis group (FOA), with no significant increase or notable decrease observed in the male osteoarthritis group (MOA). Additionally, the female rheumatoid arthritis- osteoarthritis group showed an increase in TNF levels, though not statistically significant compared to control samples.

The divergence in results between the two studies may be attributed to factors such as the patients' medical history, including potential prior infection with the Covid-19 virus. The impact of Covid-19 infection on TNF levels in patients with rheumatoid arthritis and osteoarthritis could have influenced the outcomes of TNF measurement in Krenytska and colleagues' study. (Krenytska, D., et al., 2023).

The increase in TNF (tumor necrosis factor) levels in patients with rheumatoid arthritis (RA) and osteoarthritis (OA) can be attributed to several factors:

Inflammation**:** TNF is a key pro-inflammatory cytokine involved in the pathogenesis of RA and OA. In RA, TNF plays a central role in perpetuating chronic inflammation by promoting the recruitment of immune cells to the synovial joints and stimulating the production of other inflammatory mediators. In OA, TNF contributes to the inflammatory response within affected joints, leading to cartilage degradation and joint damage.

Synovial Activation: In RA, TNF is produced primarily by activated macrophages within the synovial membrane. The inflamed synovium in RA serves as a major source of TNF production, contributing to the perpetuation of inflammation and joint destruction.

Cartilage Degradation: TNF contributes to cartilage degradation in both RA and OA by stimulating the production of matrix metalloproteinases (MMPs) and other proteolytic enzymes that break down the extracellular matrix of cartilage. This process leads to the erosion of cartilage tissue and exacerbates joint damage.

Bone Resorption: TNF promotes bone resorption by stimulating osteoclast activity and inhibiting osteoblast function. In RA, TNF-mediated bone resorption contributes to the characteristic bone erosion and joint deformities observed in affected individuals.

Pain Sensitization: TNF has been implicated in pain sensitization pathways in both RA and OA. It can directly stimulate nerve endings within joints, leading to increased pain perception and hypersensitivity.

Angiogenesis: TNF plays a role in angiogenesis, the formation of new blood vessels, which is a characteristic feature of the synovial inflammation seen in RA. Increased angiogenesis contributes to the perpetuation of inflammation and joint damage in RA.

Autoimmune Response: In RA, TNF contributes to the perpetuation of the autoimmune response against self-antigens, leading to chronic inflammation and tissue damage within the joints.

These factors collectively contribute to the elevation of TNF levels observed in patients with RA and OA.

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