ORIGINAL ARTICLE

Assessment the Levels of Toll-Like Receptor 4 (TLR4) in Patients with Rheumatoid Arthritis in Al-Najaf City

Zainab A. Chafat*, Salim H. Hassan
Department of Medical Laboratory Techniques, College of Health and Medical Techniques/ Kufa, Al-Furat Al-Awsat Technical University, Al-Kufa, Iraq

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****Corresponding Author:***
Zainab Abbas Chafat
Department of Medical Laboratory Techniques, College of Health and Medical Techniques/ Kufa, Al-Furat Al-Awsat Technical University, Al-Kufa, Iraq
Tel.: +964 7810205119
zanab198805@gmail.com

ABSTRACT

Background*: Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by persistent synovial inflammation that ultimately leads to joint destruction. Although serological markers such as rheumatoid factor (RF) and C-reactive protein (CRP) are routinely used in diagnosis and monitoring, there remains a need for more sensitive and specific biomarkers—particularly for early disease detection and assessment of activity.* ***Objective:*** *To evaluate whether serum Toll-like receptor 4 (TLR4) levels differ between RA patients and healthy controls, and to compare the diagnostic performance of TLR4 with established biomarkers (anti-cyclic citrullinated peptide [anti-CCP], RF IgM, and CRP).* ***Methodology:*** *A case–control study was conducted with 60 clinically diagnosed RA patients (9 males, 51 females) and 60 age- and sex-matched healthy volunteers (20 males, 40 females). Serum RF IgM and CRP concentrations were measured using the I-Chroma™ immunoassay reader. Anti-CCP and TLR4 levels were quantified via sandwich ELISA (Bioassay Technology Laboratory, Shanghai, China). Statistical comparisons between groups were performed using Student's t-test or χ^2 test as appropriate, with significance set at $P<0.05$.* ***Results:*** *Mean serum TLR4 concentrations were comparable between RA patients and healthy controls (2.43 ± 0.74 pg/mL vs. 2.50 ± 0.64 pg/mL; $p = 0.58$), indicating no significant difference. In contrast, anti-CCP antibodies were detected in 96.7% of patients but in none of the controls ($p < 0.001$), underscoring its strong diagnostic specificity. Rheumatoid factor IgM was positive in 43.3% of patients compared with 13.3% of controls ($p = 0.005$). Although mean CRP levels were higher in the patient group (14.90 ± 30.17 mg/L) than in the control group (12.28 ± 14.32 mg/L), this elevation did not reach statistical significance ($p = 0.55$).* ***Conclusion:*** *TLR4 serum concentrations do not differ significantly between RA patients and healthy individuals, suggesting limited utility as a standalone biomarker. In contrast, anti-CCP exhibits strong discriminatory power and remains the most reliable serological marker for RA diagnosis and disease activity assessment.*

INTRODUCTION

Rheumatoid arthritis (RA) is considered as a persistent inflammatory disorder that mainly adversely affects synovial joints of the patient that causes persistent and repeated inflammations of the synovial membrane, that results in the malfunction of patients joints, malformation, and joint immobility[1]. Approximately 0.5–1% of the world population were affected by RA and this disease incidence is higher in females than in males in addition the percent increases in elderly people more than younger people[2]. Generally, RA is recognized to be progressive course, (i.e. in lack or delay of treatment RA will progresses leading to formation of pannus and joint destruction. From the beginning and since its first representation, The comprehension of RA pathophysiology has developed considerably over time. Regrettably, similar to other rheumatic disorders, the pathogenesis of rheumatoid arthritis is highly intricate, involving a multifactorial interplay of genetic predisposition, immune system dysregulation, environmental exposures, alterations in the microbiota, as well as age-related, hormonal, and sex-specific influences[2,3].

The progression of rheumatoid arthritis can be broadly categorized into three distinct stages. The initial phase involves non-specific inflammatory responses. This is followed by an amplification and spread of inflammation, primarily driven by T-cell activation. In the final phase, chronic inflammation persists, largely sustained by pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6[4,5].

Patients with RA, upon serological analysis often have autoantibodies named as RF and anticyclic citrullinated peptide (anti-CCP) which are serological bioindicators could be considered as a diagnostic tools for RA [4,5]. Generally, the anti-CCP titer has much more specificity and higher sensitivity for RA disease

than RF .The combined use of both detectors can offset the limitations in sensitivity and specificity associated with rheumatoid factor (RF), thereby providing a highly effective diagnostic approach for rheumatoid arthritis[6] . Numerous studies have demonstrated that anti-cyclic citrullinated peptide (anti-CCP) antibodies can yield positive results even in rheumatoid arthritis patients with negative rheumatoid factor (RF) test. Consequently, the anti-CCP test is considered a reliable diagnostic tool for individuals suspected of having RA[7] .

On the other hand, the detailed analysis of anti-citrullinated protein antibodies (ACPA) specificity does not offer further insight into disease activity or progression scoring, it may help in anticipating of extra-articular manifestation. Owing to the absence of sufficiently specific biomarkers, the assessment of disease activity in rheumatoid arthritis continues to depend primarily on clinical evaluation, supplemented by serological indicators. However, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels are general markers of acute-phase responses and may be elevated in a range of conditions unrelated to RA.

Additionally, some RA patients present with erosive joint damage despite negative for rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA), and specific human leukocyte antigen (HLA) types. The lack of serological markers in such cases complicates the initiation of timely and appropriate therapeutic interventions, increasing the risk of disease progression. This underscores a critical need for the identification of more accurate and early predictive biomarkers that can facilitate prompt diagnosis and effective management of RA[8].

Toll-like receptor 4 (TLR4), a member of the broader Toll-like receptor (TLR) family, functions as a pattern recognition receptor. It is initially synthesized in the endoplasmic reticulum (ER) and subsequently transported to its site of action. TLR4 plays a key role in activating the innate immune response by detecting pathogen-associated molecular patterns (PAMPs), which include components of bacteria, viruses, fungi, and protozoa, as well as danger-associated molecular patterns (DAMPs), which are typically released in response to cellular damage or death[9,10]. Notably, TLR4 is predominantly expressed on the plasma membrane of hematopoietic-derived cells such as macrophages, monocytes, dendritic cells, granulocytes, and in tissues like the spleen. Additionally, its expression in non-hematopoietic cells, including chondrocytes, osteoblasts, and synoviocytes, suggests a significant role for TLR4 in the pathophysiology of the musculoskeletal system. TLR4 appears to have a particularly prominent role in rheumatoid arthritis[11]. Therefore, targeted inhibition of TLR4 activation during disease progression may offer a promising strategy for managing RA[12,13].

TLR4 is expressed by certain tissue-resident cell populations and contributes to host defense during infection and may influence fibrotic responses following tissue injury. Elevated levels of TLR4 and its endogenous ligands have been detected in synovial fluid, and this elevation has been positively correlated with disease progression in rheumatoid arthritis[14]. Although TLR4 is primarily expressed on cells of the innate immune system, low-level expression has also been observed in activated CD4+ T cells playing a dual role, potentially promoting or suppressing chronic inflammation. Activation of TLR4 through various signaling pathways leads to the induction of pro-inflammatory cytokines via canonical pathways, as well as the production of type I interferons and anti-inflammatory cytokines, reflecting its complex role in immune modulation[15] .

On the other hand, Anti-cyclic citrullinated peptide (Anti-CCP) antibodies are autoantibodies directed against proteins that contain citrulline, a non-standard amino acid formed through a post-translational modification of arginine by process of citrullination, can occur under inflammatory conditions, particularly within the synovial joints. Anti-CCP antibodies are highly specific to rheumatoid arthritis (RA) and are now recognized as one of the most valuable serological biomarkers in its diagnosis and prognosis with specificity of over 95% for RA, making them more reliable than rheumatoid factor (RF), which may be elevated in other autoimmune or chronic diseases. The sensitivity is generally around 70–80%, depending on the disease stage.

However, Anti-CCP antibodies can be detected years before the clinical onset of RA symptoms, allowing for early diagnosis and intervention, which is crucial for preventing irreversible joint damage[16]. This research aimed to study the value of TLR4 and Anti-CCP in those patients to evaluate the role of TLR4 in rheumatoid arthritis (RA) patients in Al-Najaf city.

METHODOLOGY

Study Design and Participants

A Case- control study included 60 patients (51 females, 9 males) diagnosed by specialist as Rheumatoid arthritis patients and 60 persons were healthy individuals to act as control (40 females and 20 males). This study was performed in the period from November 2024 to January 2025. All the patients were recruited from (center of Rheumatology - Al–Sader Teaching Hospital located in Najaf city). The accepted age range of the patients was 18–70 years. Patients with RA at the time of their visit to public were randomly selected.

Inclusion & Exclusion Criteria

Patients aged between 18 and 70 years who had been diagnosed as rheumatoid arthritis by a rheumatology specialist, had a total score of ≥6 based on the 2010 ACR/EULAR classification criteria, were included in the patient group. Individuals in the control group were selected on the basis that they had no history of autoimmune or inflammatory disorders. Exclusion criteria: Participants with other autoimmune diseases or chronic conditions as DM ,hyperthyroidism and cancer , Pregnant or lactating women were exclude from the study.

Sample Collection and Processing

A volume of five milliliters of venous blood has been taken out of the patients. The blood sample was partitioned into two portions. A volume of one milliliter was collected in EDTA tube for CBC, and the remaining four milliliters of the sample were transferred to a sterile gel tube. The tube was then subjected to the centrifugation in order to separate the serum after coagulation at room temperature in the lab. The serum sample had been divided into three equal portions in Eppendorf tubes for each patient sample, and stored at a temperature between -20 and -45 °C.

Quantification of TLR4 and Anti-CCP by ELISA

Serum levels of Toll-like receptor 4 (TLR4) and anti-cyclic citrullinated peptide (Anti-CCP) were measured using commercial sandwich ELISA kits (Bioassay Technology Laboratory, BT-LAB, Shanghai, China; TLR4 Kit, Cat. No. BT-EL-0142; Anti-CCP Kit, Cat. No. BT-EL-0012).

Assay principle: Plates are pre-coated with a monoclonal capture antibody specific for the target analyte. After sample addition, any TLR4 (or CCP) antigen binds the immobilized antibody. A biotinylated detection antibody then binds the captured antigen, followed by horseradish peroxidase (HRP)–streptavidin conjugate. Addition of tetramethylbenzidine (TMB) substrate yields a blue product that turns yellow upon addition of stop solution; the color intensity is proportional to antigen concentration.

Procedure:

All steps were carried out at room temperature unless stated otherwise. First, all kit reagents and serum samples were equilibrated to room temperature. Serum samples were diluted 1:5 in the ELISA sample buffer provided and mixed gently. Into each well of the TLR4 and anti-CCP microplates (pre-coated with capture antibody), 100 µL of standards (in duplicate) and 100 µL of diluted samples were dispensed. Plates were then incubated at 37 °C for 90 minutes to allow target binding.

Following incubation, the wells were emptied and washed three times with 300 µL of wash buffer to remove unbound proteins. Next, 100 µL of the biotinylated detection antibody was added to each well, and plates were returned to 37 °C for 60 minutes. After another three wash cycles, 100 µL of horseradish peroxidase–streptavidin conjugate was added and incubated at 37 °C for 30 minutes. Plates were then washed a final three times.

To develop color, 90 µL of TMB substrate was added to each well and incubated for 10–15 minutes in the dark at room temperature. The enzymatic reaction was stopped by adding 50 µL of 2 N sulfuric acid to each well, and absorbance was measured immediately at 450 nm (570 nm reference) using an iMark™ Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA). Sample concentrations were calculated by interpolation from a four-parameter logistic standard curve generated in the reader software.

RF IgM and CRP measurement: Rheumatoid factor IgM and C-reactive protein were determined by fluorescence-based immunoassay on the I-Chroma™ II Reader (Boditech Med Inc., Chuncheon, Republic of Korea), according to the manufacturer's protocol.

Statistical Analysis

Data were analyzed using SPSS version 26.0 (IBM Corp., Armonk, NY). Continuous variables were expressed as mean ± standard deviation (SD) and categorical variables were presented as frequencies and percentages. Group comparisons were performed using independent-samples t-tests or Mann-Whitney U tests for continuous variables and chi-square or Fisher's exact tests for categorical variables. Multiple linear regression analyses were used to evaluate the association between inflammatory biomarkers, adjusting for confounders such as age and BMI.. A p-value of <0.05 was considered statistically significant [17].

RESULTS

The current study included a total of 60 participants diagnosed with RA and 60 controls subjects. **Table 1.** lists the demographic information of all participants (patients and the control group). This case control study was done from November 202٤ to January 202٥. The patients' group consisted of 9 (15.0%) males and 51 (85.4%) females, the control group consisted of 20 (33.3%) males and 40 (66.7%) females. . The results showed significant difference in the distribution of males and females among the patients and control participants (p=0.036).The ages ranged from 31-40 years had the highest percentage of patients (28.3%), from 41-50 were (26.7%), 51–60 years (20.0%), and (≥61) years (18.3%)) and ≤20 years (1.7%). There was statistically-significant difference in the mean age between cases and control subjects (p<**0.001**).

Additionally, the study revealed that a majority of patients with RA were housewives. However, there was no significant difference observed when compared to the control group 42(70.0%) and 51 (85.0%) among RA and the control group.

Table 1. Demographical characteristics of the study groups.

Variable		Patients NO. (%)	Controls NO. (%)	P value
Gender				
	Female	51 (85.0)	40 (66.7)	**0.036***
	Male	9 (15.0)	20 (33.3)	
Age group				
	≤20	1 (1.7)	12(20.0)	**<0.001****
	21-30	3 (5.0)	18 (30.0)	
	31-40	17 (28.3)	16 (26.7)	
	41-50	16 (26.7)	8 (13.3)	
	51-60	12 (20.0)	4 (6.7)	
	≥61	11 (18.3)	2 (3.3)	
Occupation				
	Housewife	51 (85.0)	42 (70.0)	**0.08**
	Wage earner	9 (15.0)	18 (30.0)	

*Significant P value < 0.05

The mean and SD of hematological parameters that include hemoglobin level, WBC count, LYM%, GRN%, PVC and platelet count of the rheumatoid arthritis patients were12.54±1.46,7.49±3.08, 34.00±10.34, 60.84±11.59, 35.33±3.57, 242.43±52.47 respectively as shown in **Table 2**.

Table 2. Comparison of hematological parameters of the study groups.

Variable	Patients M.±SD	Controls M.±SD	P value
WBC	7.49±3.08	7.70±2.42	**0.68**
LYM%	34.00±10.34	35.68±10.73	**0.58**
GRN%	60.84±11.59	60.74±11.96	**0.96**
Hb	12.54±1.46	11.38±2.00	**0.03***
PVC	35.33±3.57	35.48±5.48	**0.86**
PLET	242.43±52.47	287.03±84.10	**0.06**

*Significant P value < 0.05

Anti-CCP level was positive in 96.7% of Patients compared to control (p= <0.001) as shown in **Table 3**. Additionally, Additionally, rheumatoid factor (RF) was positive in 43.3 % of patients compared to controls (13.3%), P=0.005.

Table 3. Comparison of Anti-CCP and RF positivity between Patients and controls.

Variable	Group	Positive n (%)	Negative n (%)	P value
Anti-CCP	Patients	58 (96.7)	2 (3.3)	**<0.001****
	Controls	0, (0.0)	60 (100.0)	
RF	Patients	26 (43.3)	34 (56.7)	**0.005***
	Controls	8 (13.3)	52 (86.7)	

*P < 0.05; ** P < 0.001

The CRP levels were analyzed between the patient and control groups. The mean CRP level was higher in the Patient group (14.90±30.17) compared to the control group (12.28±14.32) as shown in **Table 4 .** On the other hand, comparison of TLR4 levels between patients and controls showing TLR4 in patients (2.43±0.74) and control group (2.50±0.64).

Table 4. CRP and TLR4 concentrations for between patients and controls.

Variable	Group	Mean ±SD	p-value
CRP	Patient	14.90±30.17	**0.55**
	Control	12.28±14.32	
TLR4	Patient	2.43±0.74	**0.58**
	Control	2.50±0.64	

Significant P value ≤ 0.05

A multiple linear regression was conducted to assess the relationship between TLR4, CRP, anti-CCP, and RF results in both patients and controls as shown in **Table 5.**

Table 5. Multiple linear regression analysis to predict TLR4 expression among patients and controls.

Predictor	*B* (Unstandardized)	*β* (Standardized)	*t-value*	*p-value*
CRP	0.05	0.03	0.338	**0.736**
Anti-CCP	-0.108	-0.006	-0.62	**0.536**
RF	-0.19	-0.01	-0.012	**0.990**

*Significant P value ≤ 0.05

As shown in Figures 1–4, mean serum TLR4 concentrations did not differ significantly between RF-negative and RF-positive subjects (Fig. 1), anti-CCP–negative and –positive individuals (Fig. 2), across six age brackets (Fig. 3), or between females and males (Fig. 4). In each comparison the error bars overlap substantially and all p-values exceed 0.05, indicating that TLR4 expression is independent of RF status, anti-CCP positivity, age group, and gender in both RA patients and healthy controls.

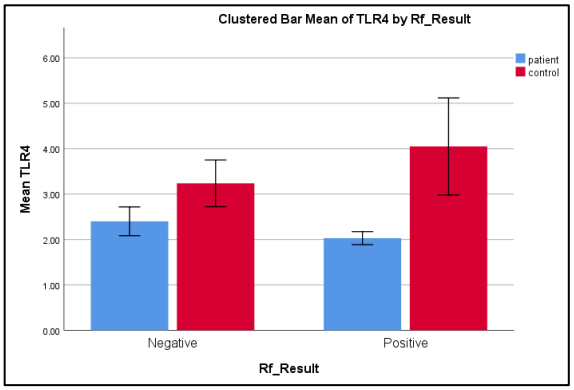

Fig. 1: The relationship between TLR4 and RF.

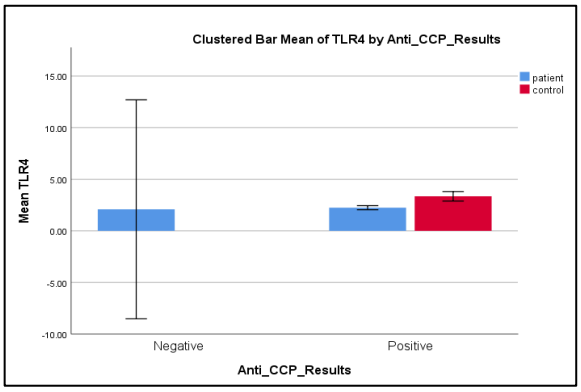

Fig. 2: The relationship between TLR4 and ACCP.

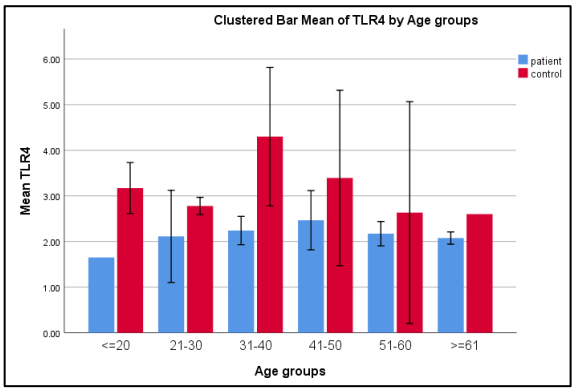

Fig. 3: The relationship between TLR4 and Age groups.

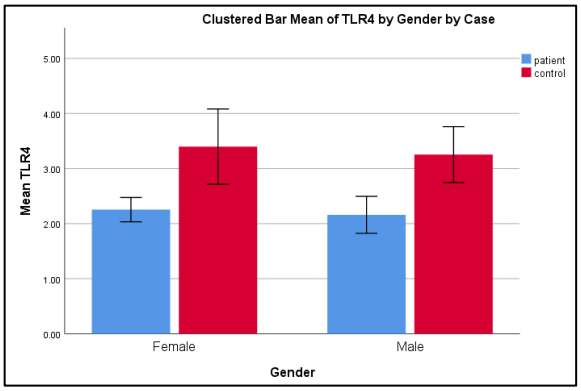

Fig. 4: The relationship between TLR4 and Gender.

DISCUSSION

This study found that females had a higher percent of developing RA. ((85.0%) of patients were females while (15.0%)of patients were males. This result is in agreement with another study conducted by Jwad, *et al.* [18],Lamkhanat, *et al.* [19], which also reported a higher percentage of females in their RA patient populations. Women are more likely to develop RA than men, possibly due to the influence of hormone levels on inflammatory proteins in the blood. Estrogen, in particular, can impact immune cells This finding is in agreement with Favalli 2019 [20].

The age group ranged between 18 to 70 , the highest percentage was in the age group of (31-70) years and the lowest percentage in the age group (18 -30) years. This study found that there was statistically significant difference between patients and control subjects in the mean age (P<**0.001**). This finding is in agreement with Castillo-Cañón, *et al.* [21]. Regarding occupation, the majority of patients were housewives (85.0%). This is comparable to Bajraktari, *et al.* [22] , who found that the largest portion of RA patients were housewives, among other occupations.

The current study found that the mean hemoglobin level for RA patients was (12.54±1.46) g/dl, while the mean level for the control group was (11.38±2.00) g/dl. the patient's hemoglobin levels were significantly lower in comparison to the control individuals (p <0.05). Anemia, characterized by low hemoglobin (Hb) levels, is frequently observed in patients with rheumatoid arthritis and has been independently linked to physical disability. Moreover, reduced Hb concentrations are considered a potential indicator of active clinical or subclinical inflammation[23]. Consequently, hemoglobin levels must be considered by clinicians in their clinical management of RA[24], These results are in line with results obtained by Demir, *et al.* [25]. According to this study, the median WBC count in the Patients with RA was within the normal limits. This is in line with the findings of Ershov, *et al.* [26] and Based on the findings of this study, the parameters LYM, GRN, PVC in the patients with RA were not statistically significant.

This study found that the median platelet count in the Patients with RA was normal. This is consistent with the result obtained in 2021 by Dechanuwong and Phuan-Udom [27]. This study revealed that the mean RF titer for the patient group was significantly higher than that of controls. This result is in consistent with Almurshedi, *et al.* [8] . In all autoimmune diseases as RA, the immune system by mistake attacks the synovium, which is the lining of the joints leading to inflammation and the release of cytokines and other inflammatory molecules. The immune system responds by producing antibodies, including RF, which can help clear away the damaged tissue. Over time, the production of RF can become chronic, leading to sustained inflammation and

joint damage This result is in consistent with Alattabi, *et al.* [28]. The results of this study show highly significant elevation in anti CCP levels in people with RA in comparison to control groups, and this agree with the study in Wasit province by Alwan and Ghali [29]. Anti-CCP is extremely specific for RA and has a very low incidence in healthy individuals and this agree with Chen, *et al.* [6] . Anti-CCP helps to predict disease severity and the risk of developing bone erosion in patients with RA. Anti-CCP can be detected for as long as ten years before the clinical symptoms of RA start[30]. However, regression analysis indicated a potential association between TLR4 and anti-CCP levels, suggesting a link between innate immune activation and adaptive autoimmune response in RA pathogenesis.

The mean CRP level was higher in the Patient group compared to the control group but the difference was not statistically significant. Some investigations mirror our non-significant result. Wolfe [31] reported that C-reactive protein (CRP) levels were found to be less reliable as an indicator of inflammatory activity in RA patients, as 81 patients tested positive for CRP while 39 were negative. The study concluded that there was no significant correlation between DAS28 scores and CRP values, suggesting that the use of CRP as a marker of inflammation in clinical practice should be reconsidered.Likewise, Rostom, *et al.* [32] found no significant association between CRP levels and metabolic syndrome in RA patients ($p > 0.05$) .

In contrast, multiple studies have documented significantly elevated CRP in RA. Albabawaty, *et al.* [33] observed hsCRP markedly higher in RA patients versus controls , and Shrivastava, *et al.* [34] found the mean level of hs-CRP was 8.58 mg/L in RA patients versus 1.18 mg/L in controls ($p < 0.001$), IL-6 levels were 54.78 pg/mL in RA patients compared to 12.59 pg/mL in controls ($p < 0.001$), and TNF-a levels were 43.20 pg/mL in RA patients versus 11.69 pg/mL in controls ($p < 0.001$). Genetic polymorphisms in the CRP gene may further modulate serum levels independently of clinical activity, offering one explanation for inter-study variability.

CONCLUSION

Based on the data obtained, the following could be concluded. Higher prevalence of RA was observed among females with the most affected age group being 31–40 years. These findings align with prior studies suggesting a gender predisposition and middle-age predominance in RA onset. Hemoglobin levels differed significantly, being lower in RA patients, indicating a potential link with anemia of chronic disease—a known RA manifestation. Immunologically, anti-CCP positivity was exceptionally high in the RA reinforcing its diagnostic specificity for RA. CRP levels were elevated among patients, reflecting ongoing inflammation, Similarly, no significant difference in TLR4 expression was detected between patients and controls. Indicating that the control of disease could decrease the expression of TLR4 and total inhibition of RA activity y as well. TLR4 is a major factor . Health condition, age and even proper treatment is greatly affect the serological profile of biomarkers especially in TLR4 in Al-Najaf city.

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Declaration

Ethics approval and consent to participants:

This study was approved by Al_Furat Al_Awsat Technical University Review Board, a. All participants provided informed consent to participate in the study. Ethical approval was obtained from the Institutional Review Board, and written informed consent was provided by all participants prior to enrollment. The study protocol adhered to the guidelines of the Declaration of Helsinki [35].

Consent for publication:

All participants gave consent for the publication of anonymized data included in this manuscript.

Availability of data and material:

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests:

The authors declare that they have no competing interests.

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Authors contribution:

ZAC contributed to the study design, data collection, statistical analysis, interpretation of results, and drafting of the manuscript. **SHH** provided supervision, contributed to the study design, data interpretation, and critically revised the manuscript for important intellectual content. Both authors reviewed and approved the final version of the manuscript and agree to be accountable for all aspects of the work.

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