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Original article

EXPRESSION LEVELS OF ENDOSOMAL TOLL-LIKE RECEPTORS (TLRs) IN RHEUMATOID ARTHRITIS: A LINK BETWEEN TLR7, TLR9, AND INFLAMMATORY BIOMARKERS WITH DISEASE ACTIVITY SCORE

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovial inflammation, joint damage, and functional impairment. Recent studies highlighted the role of Toll-like receptors (TLRs), particularly TLR7 and TLR9, in RA pathogenesis through innate immune activation and interferon-alpha (IFN-α) production. This case-control study assessed TLR7 and TLR9 expression in 60 RA patients and 30 healthy controls, alongside serum IFN-α levels and disease activity (DAS28). Blood samples were processed for RNA extraction and cDNA synthesis. Gene expression was quantified via RT-qPCR, and IFN-α levels measured by ELISA. RA patients showed significantly increased TLR7 (1.99-fold, $p = 0.005$) and TLR9 (1.42-fold, $p = 0.024$) expression compared to healthy controls, as determined by the Mann–Whitney U test. A strong positive correlation was observed between their expression levels ($\rho = 0.893$, $p \leq 0.001$), indicating shared co-regulatory mechanisms. IFN-α levels were also significantly elevated in RA patients ($p \leq 0.001$). However, correlations between TLR7 expression and IFN-α ($\rho = -0.047$, $p = 0.804$) or DAS28 ($\rho = 0.148$, $p = 0.435$) were weak and not statistically significant. Similarly, TLR9 expression showed weak, non-significant correlations with IFN-α ($\rho = 0.007$, $p = 0.969$) and DAS28 ($\rho = 0.161$, $p = 0.396$). Likewise, no significant link was found between age and disease activity. These results suggest that while TLR7, TLR9, and IFN-α are elevated in RA, their direct association with disease activity may be limited, highlighting the complexity of immune regulatory networks in RA progression.

KEYWORDS: Rheumatoid Arthritis, Toll-Like Receptors, Interferon-alpha, Disease Activity Score-28, Gene Expression

1. INTRODUCTION

Rheumatoid arthritis (RA) is a predominant inflammatory arthropathy worldwide. A substantial body of evidence encompassing genetic studies, tissue analyses, experimental models, and clinical research, indicates that its pathogenesis is primarily immune-mediated. This immune dysfunction is intricately associated with abnormalities in stromal tissue regulation, which collectively drive persistent inflammation and subsequent degradation of joint structures (Firestein & McInnes, 2017). Synovitis is the main symptom of RA in which there is an incidence rate of 1 in 150, it mostly affects women in their 30s to 50s. Additionally, it brings along a collection of symptoms which include inflammation, stiffness, and pain that may affect multiple organs (Okada *et al*., 2019).

Toll-like receptors (TLRs) represent a distinct subgroup of pattern recognition receptors (PRRs), predominantly expressed on antigen-presenting cells. In humans, the TLR family comprises ten known members. These receptors play a pivotal role in the innate immune defense by recognizing specific molecular structures associated with microbial pathogens. (Kawasaki & Kawai, 2014). Numerous studies have demonstrated a substantial correlation between TLR gene expression patterns and disease state. A rise in TLR expression in blood cells occurs before symptoms appear. RA patients' synovial tissue had greater levels of TLR7 expression, other studies also pointed to TLR9 and endogenous DNA as having a pathogenic function in RA (Radakovics *et al*., 2022). Current studies suggest that the innate immunity plays a key role in the initiation and progression of RA. TLRs are involved in the activation of B cells and T cells and the generation of adaptive

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immunity in RA. However, Substantial research focus has been directed toward understanding the role of TLRs in sustaining RA pathogenesis and joint degradation, mediated through persistent production of inflammatory mediators including TNF-α, IL-1β, IL-6, and IL-8 (Edilova *et al*., 2021). The onset of RA may occur when the common epitope has a high affinity for Cyclic Citrullinated Peptides (CCPs), and this triggers an immune response that produces anti-CCP antibodies. These antibodies may then deposit inside the joint. On the other hand, TNF-α, IL-1, and IL-6 that have been targeted in RA patients are still expressed pathogenically while the immune response and innate immunity that is characteristic of these patients is not being reduced (Kalliolias *et al.,* 2024). The DAS28 was developed as a standardized measure to evaluate and track RA disease activity in diverse settings, such as clinical practice, trials, and observational studies. It is a long-term and holistic appraisal of RA disease activity based on information from such parameters as the number and size of the swollen and tender joints, acute phase reactants, and the patient's overall assessment of his/her well-being (Renskers & Van Riel, 2016). Moreover, chronic infections have been proposed as additional contributors to systemic inflammation and immune dysregulation in RA. Studies have indicated that Helicobacter pylori infection is associated with increased inflammatory and coagulation activity, as reflected by elevated C-reactive protein levels, higher leukocyte counts, greater fibrinogen concentrations, and significant alterations in lipid metabolism (Zefenkey, 2022). These infection-driven pathways may overlap with the immune mechanisms underlying RA progression, providing further insight into the multifactorial nature of disease activity. The study examined in detail how TLR7 and TLR9 play a role in the immunopathogenesis of rheumatoid arthritis. In addition, it looks at the role of these receptors in disease severity which in turn gives attention to their mechanical role in inflammation and immune dysregulation related to RA.

2. MATERIALS AND METHODS

Study design:

The present study was of a case-control design, and it was conducted in Duhok, Kurdistan Region of Iraq, from October 2024 to February 2025. A total of 60 individuals diagnosed with rheumatoid arthritis were included in the study, along with 30 healthy control participants carefully matched to the patient group by age and body weight to minimize potential confounding factors. All participants were recruited from the Rheumatology Center in Duhok City. A rheumatologist at the Rheumatology Center did in-depth clinical assessments to determine the diagnosis for all patients who were supported with lab confirmation of RA. At the study's onset, patients completed a designed questionnaire, which was used to obtain information regarding their age, sex, disease duration, family history, treatment plan, weight, height, and any complications they had experienced. The participants were assessed for joint pain, swelling, and tenderness to facilitate the calculation of the Disease Activity Score 28 (DAS28) (Michielsens *et al*., 2024). Upon completion of patient recruitment, control subjects were enrolled based on their close matching to the patients in terms of age and body weight. The selection process was conducted at the Rheumatology Center and involved individuals who were either accompanying patients or present at the facility for non-medical reasons. Each candidate underwent a concise medical history assessment to exclude the presence of any underlying health conditions. Subsequently, blood samples were collected, and both rheumatoid factor (RF) and C-reactive protein (CRP) assays were performed to ensure the absence of rheumatoid arthritis and other inflammatory disorders.

Blood Sample:

For each patient, 5 mL of blood was drawn by venipuncture using sterile, single-use syringes. Two milliliters of whole blood were transferred to an Ethylenediamine tetraacetic acid (EDTA) tube. A volume of 250 microliters of EDTA-treated blood was carefully transferred using a pipette into an Eppendorf tube containing 500 microliters of TRIzol reagent to facilitate mRNA extraction. The Eppendorf tube was subsequently stored in a freezer at a temperature of -80°C to ensure optimal preservation. Each tube was assigned a distinct code. The first part, which involved the molecular technique, was performed at Vin Private Hospital in Duhok City.

RNA isolation by TRIzol®:

Total RNA was isolated from all blood samples using TRIzol® LS reagent according to the manufacturer's recommended protocol (Thermo Fisher Scientific, USA). The concentration and purity of RNA were evaluated using the Qubit® 4 Fluorometer, which offers accurate quantification within a dynamic range of 10 pg/µL to 100 ng/µL. Although the reagent used was capable of detecting small RNAs such as miRNA, in this experiment, it served the purpose of quantifying total RNA only, which was intended for messenger RNA expression analysis. All measurements were conducted at room temperature (20–25°C), with consistent signal stability for up to two hours post-assay initiation. The Qubit system ensures reliable performance even in the presence of potentially interfering substances such as proteins, salts, solvents, and other biological contaminants (Thermo Fisher Scientific, Cat# Q32851)

The synthesis of complementary DNA (cDNA):

For downstream analysis, total RNA was reverse-transcribed into cDNA using the ProtoScript® First Strand cDNA Synthesis Kit (New England BioLabs, Cat# E6300S), strictly adhering to the manufacturer's specifications. Each 20 µL reaction mixture contained 5 µL of RNA, 10 µL of reaction buffer, 2 µL of enzyme mix, 2 µL of oligo(dT) primers, and 1 µL of nuclease-free water. The reaction was incubated at 42°C for 1 hour to allow reverse transcription, followed by heating to 80°C to deactivate the enzyme. The resulting cDNA was then stored at –20°C until use.

Real-time-quantitative PCR (RT-qPCR):

Quantitative real-time PCR was employed to evaluate the relative mRNA expression levels of TLR7 and TLR9. Specific primer pairs were designed and utilized for each gene target. The primers were selected to yield amplicons suitable for detection using SYBR Green chemistry. GAPDH was employed as the reference gene for normalization purposes. To ensure its suitability, its cycle threshold (Ct) values were compared to RA patients and healthy controls. The analysis revealed no statistically significant differences, thereby confirming its

expression stability and validating its use as an appropriate endogenous control in this study (Table 1).

Table 1: Primer sequences used for real-time qPCR analysis of TLR7, TLR9, and GAPDH genes, including sequence orientation and nucleotide length.

Gene	Primer Type	Sequence (5' → 3')	Purpose	Length (bp)
TLR7	Forward	CACCTGTGATGCTGTGTGGTT	Amplification of TLR7 cDNA	21
	Reverse	CCAGGGAGATCACACTTTGGC		21
TLR9	Forward	CAGACTGGGTGTACAACGAGC	Amplification of TLR9 cDNA	21
	Reverse	GCCCACAGGTTCTCAAAGAGG		21
GAPDH	Forward	GTCTCCTCTGACTTCAA	Normalization of gene expression data	17
	Reverse	ACCACCCTGTTGCTGTA		17

Each PCR reaction had a total volume of 20 µL, comprising 10 µL of Luna Universal qPCR Master Mix (New England Biolabs, UK), 1 µL each of forward and reverse primers (10 µM), 5 µL of synthesized cDNA, and 3 µL of nuclease-free water. Prior to amplification, reaction mixtures were briefly centrifuged at 2000xg for 1 minute to purge air bubbles and ensure the complete collection of the reaction components at the bottom of the tubes, and subsequently subjected to amplification using a RT-qPCR system. The RT-qPCR program was designed to ensure efficient denaturation, annealing, and extension of the target sequences, followed by a melt curve analysis to verify the specificity of the amplification. The thermal protocol initiated with a denaturation step at 95°C for 1 minute, followed by 40 amplification cycles consisting of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 30 seconds. To ensure amplification specificity, Following the completion of amplification, a melt curve analysis was conducted by gradually increasing the temperature from 65°C to 95°C in 0.5°C increments at a ramp rate of 0.2°C per second. The relative expression levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ method, also known as the Livak method (Livak & Schmittgen, 2001), to quantify the relative expression levels of the target TLR genes in comparison to GAPDH.

Each RT-qPCR reaction was carried out in duplicate to enhance the precision and dependability of the gene expression analysis. Performing technical replicates enabled confirmation of Ct values and helped reduce potential errors arising from pipetting or equipment inconsistencies. All obtained samples underwent complete processing and analysis. Assessments of RNA concentration, purity, and PCR performance were conducted, and none of the samples were excluded from the study.

Serum Sample:

The remaining three milliliters of the blood sample were aliquoted into a gel tube for serum separation. The gel tubes were centrifuged at 3,000 rpm for 10 minutes after an initial coagulation period of 20 minutes at ambient temperature. The serum samples were aliquoted into two equal portions in Eppendorf tubes and immediately stored at -80ºC to prevent cross-contamination and minimize the effects of repeated freeze-thaw cycles. Each sample set was assigned a distinct identification code to ensure traceability of the individual serum specimens.

The second part of the work concerning the rapid test, including Rheumatoid Factor (RF) and C-Reactive Protein (CRP), was measured using RF and CRP kits following the manufacturer's protocol by latex agglutination test at the College of Health Science, Duhok City, with Catalogue numbers (LXRF0100), (LXCRP100), respectively. The concentration of interferon-alpha (IFN-α) in serum samples was determined using a human IFN-α ELISA kit (Catalogue No. SL0957Hu, SunLong Biotech, China) at Mazi Private Laboratory, Duhok City. The kit provides a detection range of 1.6-100 pg/mL and a sensitivity of 0.5 pg/mL, ensuring precise quantification of IFN-α levels in the analyzed specimens. These techniques, including ELISA, CRP, and RF were performed in duplicate to enhance the reliability and precision of the result.

Calculation of Disease Activity Score 28 (DAS28):

Assess Tender Joint Count (TJC28) and Swollen Joint Count (SJC28): The tenderness and swelling of the 28 specific joints should be conducted and recorded 10 metacarpophalangeal (MCP) joints, 10 proximal interphalangeal (PIP) joints, 2 wrists, 2 elbows, 2 shoulders, and 2 knees.

- Measure Inflammation Level:

Obtain C-reactive protein (CRP).

DAS28-CRP Formula:

$$DAS28\text{-}CRP = 0.56\sqrt{TJC} + 0.28\sqrt{SJC} + 0.36 \log (CRP + 1) + 0.014 \times PGA + 0.96$$

When the logarithm (log) is the natural logarithm (In). TJC and SJC are counts betwwen 0 and 28. CRP is in mg/L. Patient Global Assessment (PGA) is on a scale of 0-10.

The DAS28 score is derived using specific formulas that incorporate TJC, SJC, CRP, and visual analog scale (VAS) scores. Patients are categorized into various states based on standard thresholds: remission is indicated by scores of 2.6 or lower, low disease activity is represented by scores between 2.6 and 3.2, and moderate disease activity is defined as scores between 3.2 and 5.

Statistical analysis:

The Statistical Package for the Social Sciences (SPSS, version 27, IBM®) was used for data analysis. Data were presented as mean ± SD for continuous variables and as frequencies and percentages for categorical variables. The type of statistical test used for data analysis was determined based on

the data types and the aims of the study. The level of significance was set as $p \leq 0.05$.

3. RESULTS

The current study involved 60 patients diagnosed rheumatoid arthritis and 30 controls whose descriptive data showed that the males consisted of 20 participants divided equally between the patients and control groups, the mean age of the male patients was 45.20±18.43 years, their control counterparts mean age was 38.70±6.63 years; furthermore, the mean weight and height of the male patients and male controls were 72.9±11.53 Kg, 75.4±6.74 Kg; 172.5±9.81 cm, 173.2±4.49 cm, respectively. Nevertheless, the mean Body mass index (BMI) of the

male patients were 24.44±2.98 kg/m^2 and of the controls 25.14±2.19 kg/m^2. A total of seventy female subjects were included in the present study, comprising 50 individuals diagnosed with RA and twenty healthy controls, the mean age of the female patients was 49.30±12.19 years, their control counterparts mean age was 44.20±14.58 years; furthermore, the mean weight and height of the female patients and female controls were 76.52±12.4 Kg, 69.15±11.74 Kg; 156.68±6.3 cm, 157.55±5.26 cm, respectively. Yet, the mean BMI of the female patients was 31.34±5.81 kg/m^2 and of the controls 27.98±5.49 kg/m^2 respectively (Figure 1 and 2).

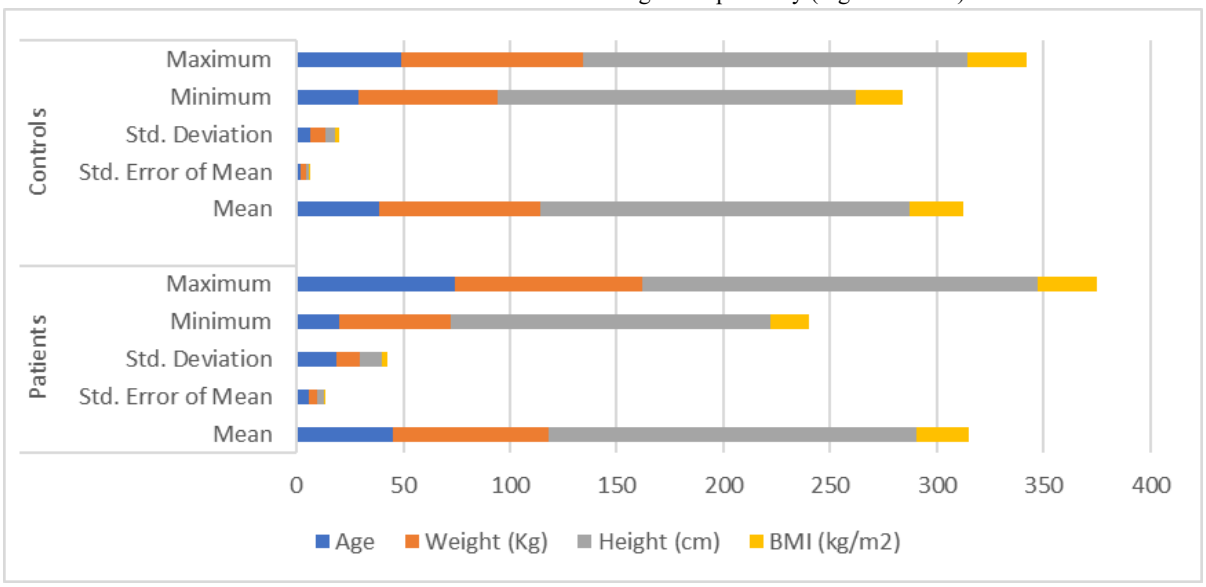

Figure 1: Comparative Analysis of Baseline Characteristics Among Male Participants (10 Patients vs. 10 Controls). Statistical differences between the two groups were assessed using the independent samples t-test.

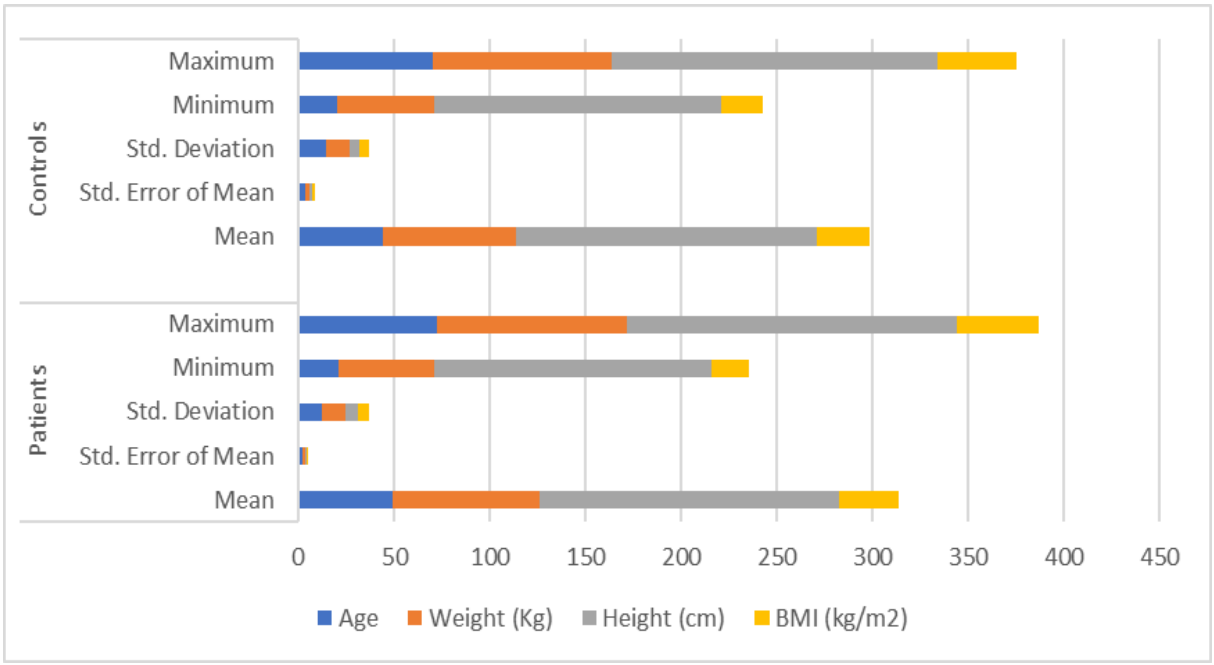

Figure 2: Comparative Analysis of Baseline Characteristics Among Female Participants (50 Patients vs. 20 Controls). Statistical differences between the two groups were assessed using an independent-samples t-test.

Disease Activity Score 28 (DAS28)

The patients were divided into four age groups to find a possible association between age categories and the DAS score groups. Participants were grouped by age using descriptive statistics. Numerical values and related proportions were calculated using SPSS. Since this variable was intended solely for descriptive demographic analysis, no inferential statistical tests were applied (Table 2).

Table 2: Age-Stratified Distribution of Patients with Rheumatoid Arthritis (50F,10M)

Age groups (years)	Frequency	Percent (%)
20-30	6	10.0
30-40	11	18.3
40-50	13	21.7
>50	30	50.0
Total	60	100.0

In Table 3, the data are presented as frequencies along with their corresponding percentages. The p-value indicates the overall statistical relationship between the age categories and DAS groups, determined using the Chi-square test

Table 3: Distribution of Disease Activity Score 28 (DAS28) Across Age Groups in Rheumatoid Arthritis Patients (50F,10M)

DAS groups	Age groups Frequency (%)				p-value
	20-30 years	30-40 years	40-50 years	>50 years	
Remission	1 (7.7%)	1 (7.7%)	3 (23.1%)	8 (61.5%)	0.51
Low disease activity	2 (12.5%)	1 (6.3%)	4 (50.0%)	9 (56.3%)	
Moderate disease activity	3 (9.7%)	9 (29.0%)	6 (19.4%)	13 (41.9%)	
Total	6 (10.0%)	11 (18.3%)	13 (21.7%)	30 (50.0%)	60 (100)

Figure 3 displays a clustered bar chart illustrating the percentage distribution of RA disease activity levels, classified according to the DAS, concerning gender. The x-axis represents gender (male and female), while the y-axis indicates the proportion of individuals within each disease activity category. The chart differentiates three DAS-based classifications: remission (depicted in blue), low disease activity (green), and moderate disease activity (maroon). Among male subjects, 3.0% were in remission, 5.0% exhibited low disease showed a distribution of 10.0% in remission, 11.0% with low disease activity, and 29.0% with moderate disease activity. Each bar is labeled with the corresponding percentage value to enhance clarity and enable direct comparison across gender and disease activity levels.

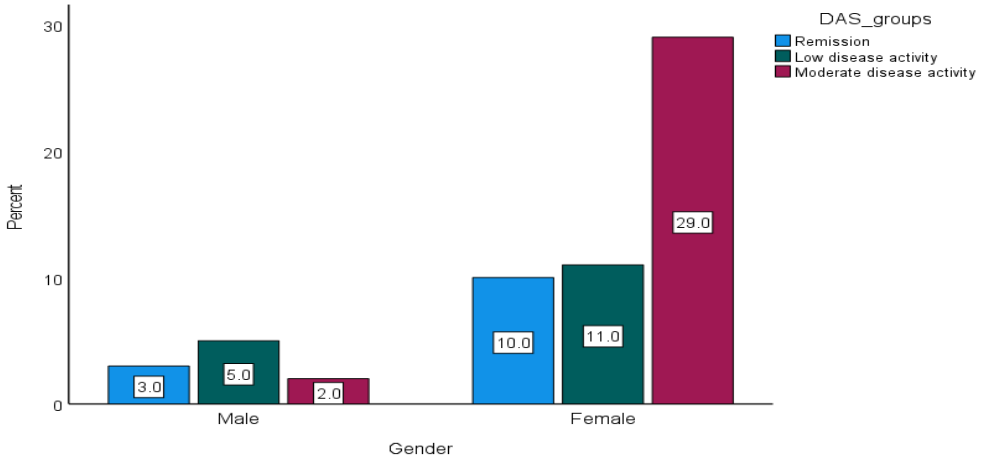

Figure 3: Gender-Based Distribution of Disease Activity Score 28 (DAS28) in Rheumatoid Arthritis Patients (50F, 20M). Statistical differences in distribution were assessed using the Chi-square test of independence

TLR7 and TLR9 gene expression study:

Owing to financial constraints, the initial stage of the study involved a purposive selection of 30 patients and 10 control subjects for the evaluation of Toll-like receptor gene expression through RT-qPCR. The RNA was extracted, reverse transcribed to cDNA. The amount of cDNA template was standardized across all samples to minimize variability, followed by amplification to assess the alteration in gene expression as a function of cycle threshold (Ct) which indicates the point at which the primary amplification signal is detected. GAPDH served as the internal control gene, exhibiting a mean Ct value of 31.43, and was used to normalize target gene expression through the comparative Ct (2^-ΔCt) method. This method calculates the relative gene expression level by determining ΔCt, the difference between the Ct of the target gene and that of the reference gene, thereby quantifying fold changes normalized to the internal control. The results revealed that the expression of the TLR7 gene in the patients was 1.99 times greater than that of the controls (Table 4), and the expression of the TLR9 gene was 1.42 times greater in the patients in comparison to the controls (Table 5).

Table 4: Quantitative Assessment of TLR7 Gene Expression in 30 Patients (26F,4M) and 10 Controls (6F,4M)

Groups	Mean Ct TLR7	Means Ct GAPDH	ΔCt (TLR7- GAPDH)	ΔΔCt (vs. Control)	Fold Change $(^{2-\Delta\Delta Ct})$
Patients	22.61	30.87	-8.26	-0.99	1.99
Controls	24.16	31.43	-7.27	0.00	1.00

Table 5: Quantitative Assessment of TLR9 Gene Expression in 30 Patients (26F,4M) and 10 Controls (6F,4M)

Groups	Mean Ct TLR9	Mean Ct GAPDH	ΔCt (TLR9- GAPDH)	ΔΔCt (vs. Control)	Fold Change $(^{2-\Delta\Delta Ct})$
Patients	23.09	30.87	-7.78	0.51	1.42
Controls	24.16	31.43	-7.27	0.00	1.00

Note: Gene expression fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method, where ΔCt = Ct target – Ct GAPDH, and ΔΔCt = ΔCt patients – ΔCt controls. Figure (6) displays the relative fold changes in the expression levels of TLR7 and TLR9 genes in patients with RA compared to healthy individuals, determined by the $2^{-\Delta\Delta Ct}$ quantitative PCR method. Expression values in the control cohort were normalized to 1.00 and used as the baseline reference. In the RA group, TLR7 expression exhibited a 1.99-fold increase relative to controls, reaching statistical significance (p = 0.005), as denoted by the double asterisk (**). Likewise, TLR9 expression was upregulated by 1.42-fold in patients versus controls, also demonstrating statistical significance (p = 0.024), marked by a single asterisk (*). These results indicate a significant elevation of TLR7 and TLR9 expression in RA patients, implying their involvement in the underlying mechanisms of disease development. This difference was statistically significant.

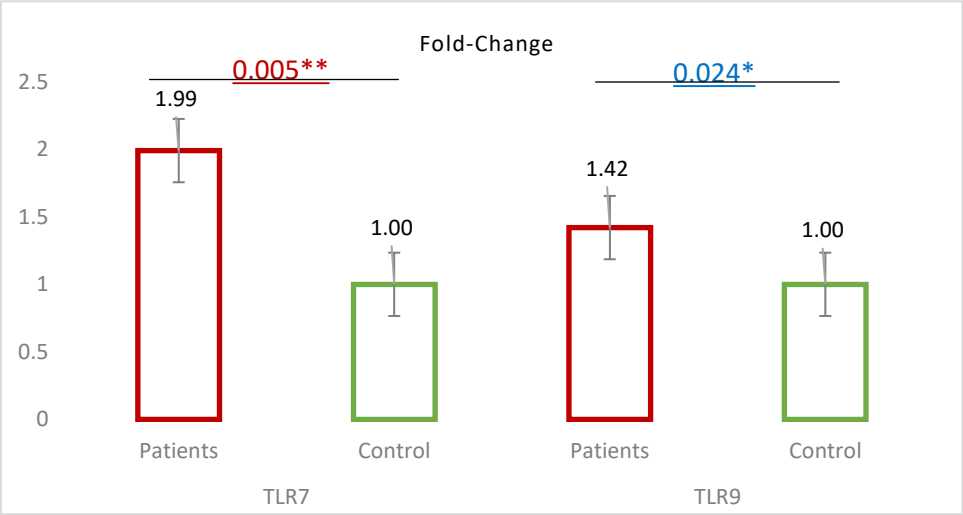

Figure 3: Comparative Fold-Change Expression Analysis of TLR7 and TLR9 Genes in Patients (26F,4M) vs. Controls (6F,4M). Statistical significance was assessed using the Mann–Whitney U test

A correlation study was performed to determine the nature and the strength of relationships between TLR7, TLR9, IFN-□□□, and DAS28 variables measured for the above selected thirty samples. Table 7 summarizes the outcomes of Spearman's rank correlation analysis conducted to evaluate the associations among TLR7 and TLR9 gene expression levels, serum IFN-α concentrations, and DAS28 in individuals diagnosed with RA. There was a strong and statistically significant positive

correlation between TLR7 and TLR9 gene expression levels among RA patients (rho = 0.893, p < 0.01), reflecting a closely associated expression pattern that may imply a coordinated regulatory mechanism. This finding was based on a sample size of 30 participants. In contrast, IFN-α levels demonstrated no significant correlation with either TLR7 (rho = -0.047, p = 0.804) or TLR9 (rho = 0.007, p = 0.969), suggesting no meaningful association between serum cytokine levels and the expression of these endosomal TLRs in the studied cohort. Furthermore, the DAS28 score did not show statistically significant correlations with TLR7 (rho = 0.148, p = 0.435), TLR9 (rho = 0.161, p = 0.396), or IFN-α levels (rho = -0.234, p = 0.213). All correlations were computed based on data from 30 subjects. Notably, the only statistically significant relationship was observed between TLR7 and TLR9 expression, with significance maintained at the 0.01 level (two-tailed).

Table 7: Correlation Matrix of TLR7, TLR9, IFN-α, and Disease Activity Score 28 (DAS28)

Variable	Output	TLR7 gene fold	TLR9 gene fold	IFN-□	DAS 28
TLR7 gene fold	Spearman's rho	--			
	P-value	.			
	N	30			
TLR9 gene fold	Spearman's rho	0.893**	--		
	P-value	≤0.001	.		
	N	30	30		
IFN-□ pg/ml	Spearman's rho	-0.047	0.007	--	
	P-value	0.804	0.969	.	
	N	30	30	30	
DAS 28	Spearman's rho	0.148	0.161	-0.234	--
	P-value	0.435	0.396	0.213	.
	N	30	30	30	30

**. The correlation was statistically significant at the 0.01 level (two-tailed)

4. DISCUSSION

The data indicate the demographic and anthropometric characteristics of a cohort of RA patients and a control group, establishing a basis for exploring potential connections between these variables and disease manifestation (Rastogi *et al*., 2015). The study population included a higher proportion of females, consistent with the established epidemiological trend of RA, which shows a greater prevalence in women, with female-to-male ratios generally around 3:1 (Suta *et al*., 2015; Venetsanopoulou *et al*., 2023). This skewed gender distribution could be the result of environmental exposures, genetic predispositions, or hormonal factors that affect women's susceptibility to the disease differently (Kvien *et al*., 2006). It's also worth noting the effect of gender on the phenotype of RA (Smolen *et al*., 2016). The severity of RA may have been impacted by variations in therapy and personal experiences (van Vollenhoven, 2009). In a study that included only 20 male participants equally distributed between the patient and control groups, this may suggest disparities between male and female groups in data trends (Asuzu-Samuel, 2021).

The state in which there is age distribution in the patient and control groups is that RA tends to present in the 4th and 5th decades of life (Jahid *et al*., 2023). Also, there is a mean age of male patients (45.20±18.43 years) and female patients (49.30±12.19 years) at midlife, which in turn supports the common knowledge that rheumatoid arthritis's onset is in middle age (Smolen *et al*., 2016).

Anthropometric data, including weight, height, and BMI, are used to look at the subject's body composition. Average weight and height values in conjunction with BMI offer a more complete picture of the body composition in people with rheumatoid arthritis that may be affected by the disease's activity (Baker *et al*., 2023). The mean body weight of RA patients was 75.92 ± 12.24 kg, notably higher than that of the control group (71.23 ± 10.65 kg). In parallel, the mean BMI was also elevated in RA patients (30.19 ± 6.0 kg/m²) compared to controls (27.04 ± 4.8 kg/m²). These findings are consistent with previous reports indicating that individuals with RA tend to exhibit higher BMI and body weight. Elevated BMI in this population has been linked to increased disease activity and a diminished probability of achieving remission. This may be attributable to the pro-inflammatory role of adipose tissue, which secretes cytokines that contribute to systemic inflammation and may worsen disease severity (Soták *et al*., 2025). In contrast, height values were comparable between the two groups, suggesting that RA does not significantly influence stature in the absence of complicating factors such as osteoporosis (Ashai & Harvey, 2020).

Upon stratification of the data by gender, distinct trends were observed. Among male participants, individuals diagnosed with RA exhibited a mean BMI of 24.44 ± 2.98 kg/m², which was marginally lower than that of the male control group (25.14 ± 2.19 kg/m²), despite comparable averages in body weight and height. This slight reduction in BMI among male RA patients may be attributed to disease-associated reductions in muscle mass, a phenomenon previously documented in the literature (Letarouilly *et al*., 2021). In contrast, female RA patients had a

significantly higher mean BMI (31.34 ± 5.81 kg/m²) than their healthy counterparts (27.98 ± 5.49 kg/m²). This disparity underscores a potentially stronger correlation between obesity and RA in women, which may be influenced by sex-specific hormonal factors and the pro-inflammatory activity of adipose tissue (Mathkhor *et al*., 2022).

According to the study, there is not large enough correlation (p=0.51) in the same data set of rheumatoid arthritis out of age groups as was done out of DAS28 joints, despite the higher percentages of the patients were grouped to the moderate disease activity group and were at the middle or the higher age groups 9.7%, 29%, 19.4%, and 41.9% for the 20-30 years, 30-40 years, 40-50 years, and more than 50 years, respectively. The measures of disease activity inferred and explained by DAS28 may not, in fact, reveal which age category the patient falls into (Greenmyer *et al*., 2020). In addition, other players like genes, lifestyle, and environment may be more critical in terms of what causes the degree of the disease in patients with RA (Matsui *et al*., 2007). While DAS28 is widely utilized, a critical evaluation of its advantages and drawbacks remains essential. It measures 28 joint tenderness and swelling, assesses a patient's health report from head to toe, and evaluates an acute-phase reactant, which can be either ESR or CRP (Wells *et al*., 2009). Furthermore, the way in which these elements are weighted may not fully present the picture of the complex disease activity across different age groups. In addition, the patient's reports may be very subjective and can be affected by factors outside of joint inflammation, which may, in turn, play out differently across age groups. Similarly, the use of ESR in the score may be affected by age-related physical changes. It may therefore not be the best marker for examining the relationship between age and DAS28 score (Son *et al*., 2016). Current tools may lack the sensitivity to detect age-specific variations in RA progression (Hansen *et al*., 2017).

Rheumatoid arthritis shows a higher prevalence among women than men, indicating a gender-related difference that may be attributed to the intricate interaction between hormonal influences and genetic predispositions, which modulate immune system function in a sex-specific manner (Piccinni *et al*., 2016). The presented hypotheses are supported by what is observed in that many autoimmune diseases either appear soon or later and progress in different ways at hormonal transitions like late adolescence and pregnancy, highlighting a potential link between hormonal fluctuations and disease susceptibility. Moreover, there is an increased report of low to moderate disease activity in females in the DAS groups, although the overall association is not significant, which points out that there may be gender-based differences in disease presentation, perception, and treatment response (van Vollenhoven, 2009). In the subjective elements of the DAS, which include patient-reported pain and global assessment, the gender specific factors may be at play, which may be reported in the score despite similar objective measures of inflammation, such as erythrocyte sedimentation rate or C-Reactive protein (Baker *et al*., 2023). Besides, differences in pain perception, coping strategies, and communication approaches between the genders may also contribute to what is observed in DAS results.

The present study demonstrates marked upregulation of TLR7 and TLR9 gene expression in individuals with RA compared with healthy controls. This observation is consistent with the work of Hurst and von Landenberg (2008), who underscored the pathogenic relevance of these endosomal TLRs in autoimmune conditions. Their research demonstrated that aberrant activation of TLR7 and TLR9 by self-nucleic acid complexes can trigger a cascade of immune responses, particularly of inducing type I interferons. This observed pattern suggests a potential co-regulation between TLR7 and TLR9. While direct co-regulation in RA has not been conclusively established, it has been postulated in earlier studies due to their shared endosomal compartment, signaling via the MyD88 adaptor protein, and their involvement in type I interferon production—factors that collectively contribute to the persistence of chronic inflammation. In support of this hypothesis, our findings reveal a 1.99-fold increase in TLR7 expression and a 1.42-fold increase in TLR9 expression among RA patients. These elevated levels may reflect enhanced innate immune sensitivity to endogenous nucleic acids, reinforcing the proposition that TLR7 and TLR9 are key contributors to the dysregulated immune responses characteristic of RA (Duffy & O'Reilly, 2016).This break in immunological homeostasis may be what tips the balance toward RA's main features of autoimmunity and chronic inflammation. In RA, the innate and adaptive immune responses play out in the synovial fluid, creating a very complex environment in which the disease persists (Fattahi & Mirshafiey, 2012). The increased expression of TLR7 and TLR9 in patients with RA also means that these receptors may be players in the disease's pathogenesis, through which they put forth inflammatory signals in the synovium (Ramos-González *et al*., 2022). Similarly, there is the fact that TLRs have a well-known role in the identification of both exogenous pathogens and endogenous danger signals, which in turn leads to activating subsequent signaling pathways that drive the secretion of inflammatory mediators (Swain *et al*., 2022).

In the case of RA, there are ongoing inflammation regression cycles, which are the hallmark of the disease's persistence. Constant activation of TLRs may be what keeps these cycles going via continuous immune system stimulation. Likewise, in the setting of RA, there is constant synovial inflammation, autoantibody production, and progressive joint damage (Weyand & Goronzy, 2020). It is recommended to conduct additional studies involving broader and more demographically varied populations of rheumatoid arthritis patients to confirm the clinical reliability of TLR7, TLR9, and interferon-alpha (IFN-α) as robust biomarkers for diagnostic purposes, prognostic evaluation, and the monitoring of disease activity in RA.

Study Limitation: The correlation analysis in this study was conducted on a limited sample size of 30 individuals, which may reduce the statistical power and affect the strength and reliability of the observed associations. Therefore, these findings should be interpreted with caution, and validation in larger cohorts is necessary to confirm the observed correlations.

CONCLUSION

The findings of the present study offer significant insight into the contributory role of endosomal TLR7 and TLR9 in the pathophysiological processes underlying RA. The marked elevation in their expression levels among patients, alongside increased serum concentrations of IFN-α, highlights their potential involvement in initiating and sustaining innate immune responses that drive systemic inflammation. Although the associations between TLR expression, IFN-α levels, and DAS28

scores did not reach statistical significance, the strong positive correlation observed between TLR7 and TLR9 expression indicates the presence of a shared regulatory axis within the innate immune pathway. These data support the notion that aberrant recognition of self-nucleic acids via TLR-mediated signaling may contribute significantly to the initiation and persistence of autoimmune responses in RA. Given these observations, further investigations incorporating broader patient cohorts and mechanistic studies are recommended to clarify the clinical relevance of TLR7, TLR9, and IFN-α as candidate biomarkers for disease diagnosis, progression, and therapeutic monitoring in RA.

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Ethical statement:

The study was approved by the Research Ethics Committee of Duhok Directorate General of Health in the Kurdistan Region of Iraq (Reference number: 25092024-8-23. Date: 25 September 2024).

Author Contributions:

The authors have reviewed and approved the final version of the manuscript and endorse the integrity and accuracy of the work.

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