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Investigating of correlation between microRNA-146a gene expression and TNF-α Levels among rheumatoid arthritis patients in Babylon province

Zainab N. Nabat[1] and Mahdi H. Alammar[2]

[1]Technical Institute of Babylon, Al-Furat Al-Awsat Technical University (ATU), Iraq.

[2]Faculty of Science, Department of Biology, University of Kufa, Iraq.

Corresponding author: Zainab N. Nabat, Technical Institute of Babylon, Al-Furat Al-Awsat Technical University (ATU), Iraq.
Email: Zainab.nabat@atu.edu.iq

Abstract

Rheumatoid arthritis (RA) is a persistent, progressive autoimmune disorder driven by the complex interplay between immune cells and proinflammatory cytokines, leading to reduced lifespans and increased mortality rates. The principal goal of this case-control study was to explore the potential of microRNA-146a (miRNA-146a) and tumor necrosis factor-alpha (TNF-α) as prognostic markers in RA patients. The study involved 50 RA patients taken from the Rheumatology unit at Marjan Hospital (Babylon) and 50 normal controls. Blood was collected from all patients and controls. Serum levels of TNF-α were detected using an enzyme-linked immunosorbent assay (ELISA). Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed to detect the miRNA-146a. The study found that women have a higher incidence of RA than men. The incidence was highest among patient's aged from15–60 years, with the greatest proportion being patients with disease duration of 1–3 years. Serum TNF-α level in the RA patients were significantly higher than in the control group (p<0.0001). Importantly, patients with stage IV rheumatoid arthritis showed a considerable high TNF-α level (p<0.0001). In conclusion, the results indicated that TNF-α plays a central role in RA chronic inflammation and miRNA-146a has potential as an indicator of disease progression and a novel therapeutic target for treating cytokine-mediated inflammation in RA.

Keywords: TNF-α, miRNA-146a, RA, RT-PCR.

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Introduction

Rheumatoid arthritis (RA) is classified as a chronic autoimmune disease marked by the presence of local and systemic inflammation resulting from the close interaction between immune cells and soluble mediators. RA also causes symmetrical joint stiffness, swelling, and pain due to synovitis, which, if not treated promptly, can lead to permanent damage to cartilage and bone. Globally there are 24.5 million patients and with adding 1.2 million new cases, RA has emerged as a major cause of dysfunction.[1] These data suggested that streptococcus bacteria provide complex adjuvant functions that enhance autoimmunity by elevated antistreptolysin O levels among patients who have acute RA and other streptococcal infections. These findings represent that the *Streptococcus pyogenes* may have an important role in the development of

RA.[2] The connection between the etiology of RA and microbiomes is yet unknown. However, the microbial colonies residing in the human body have a significant impact one the immune system ;consequently, they are implicated in numerous autoimmune diseases.[3] Tumour necrosis factor alpha (TNF-α) stimulates the production of other pro-inflammatory cytokines and chemokines, which are inducers, leading to an increased inflammatory response.[4] TNF and other pro-inflammatory mediators, facilitate the activation of synovial, fibroblasts, chondrocytes, and osteoclasts, leading to the production of tissue-destroying enzymes, the matrix metalloproteinase[5.] TNF-*α* intensifies the activation and differentiation of osteoclasts, which plays a substantial role in local joint injury and systemic bone loss by promoting osteoclast-mediated bone re-sorption.[6] MicroRNAs (miRNAs) are a group of small non-coding ribonucleic acids (RNAs) that regulate gene expression at the RNA level. MicroRNAs have positive regulatory effects on protein translation processes and often induce their performance by binding to the 3'- UTR (un-translated) mRNA region.[7]

MiRNA-146a is differently expressed in several human disorders, including rheumatoid arthritis, and has a significant function in negative control of inflammatory innate immune responses.[8] MiRNA-146a has shown a strong anti-inflammatory effect via preventing tumor necrosis factor receptor-associated factor 6 (TRAF6), which can inhibit osteoclast formation and is specially produced by several of clinical diseases, including RA.[9] Other miRNAs can enhance diagnostic accuracy, differentiate Alzheimer’s disease from mild cognitive impairment, and provide insights into disease progression and severity.[10] The main goal of this study was to assess the potential of miRNA-146a and TNF-α as diagnostic markers in RA patients.

Subjects and Methods

The study included 50 RA patients and 50 normal controls and was performed during the period from November 2024 to September 2025. Participants, included both sexes and aged 20 to 79 years, were taken from the Rheumatology Unit at Marjan Medical City Babil governorate Iraq. Clinical, radiological, and serological criteria were used to confirm the rheumatoid arthritis diagnosis, in accordance with the /2010 American College of Cardiology (ACR) and European Association of Cardiology (EULAR) classification criteria[11] Patients with any other chronic disease, autoimmune disorder, or any other form of arthritis were excluded from the study.

Sample Collection and Preparation

From each patient and control subject a blood sample (4 ml) was collected. Serum was extracted and used to determine TNF-α level by Enzyme-Linked Immunosorbent Assay (ELISA) kits (CAT. NO: EKHU-0082, MELSIN, China), according to the manufacturer’s instructions. A blood aliquot of 0.5 ml was placed in an Eppendorf tube with 0.5 ml of Triazole and used to determine miRNA-146a by a reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from whole blood the using a commercial kit (TransZol™ miRNA, Trans, China), in strict accordance with the manufacturer’s protocol.

Custom primers were created for the U6 calibrator (internal control) and MIR-146 (Macrogen Inc., Korea). The miR-146a primers sequence was: Forward (F)-TGAGAACTGAATTCCATGG and Reverse (R)-GCAGGGTCCGAGGTATTC. U6 was the expression of the internal control for the normalization of miRNA expression level. Real-Time quantitative polymerase chain reaction PCR (RT-qPCR) was prepared according to the protocol specified by the Promega, company. The thermos-cycling conditions were: one cycle of reverse transcription (RT) at 3,7 ^{0}C for 15 min, one cycle for RT inactivation at 95 0C for 10 min and 40 cycles for denaturation at 95 0C for 10 sec, annealing at 58 0C for 30 sec and extension at72 0C for 30 sec (12).

Calculating expression level (fold change)

The expression of miRNA-for patients' blood samples was normalized to (RNU6-2) reference genes and compared with those in relatively normal controls. Finally, the fold-change between the control and patient was calculated

by using the 2-ΔΔct method described by Mansor and Alammar, 2024.[12] First, the average CT (cycle threshold), value for each triplicated sample was obtained using a real-time PCR devices. Next the ΔCT value for each sample was calculated as follows:

ΔCT= CT (gene of interest)-(CT) ((Reference gene)

The method for calculating the ΔΔCT value is as follows:

ΔΔCT= Δ CT (Treated sample) – ΔCT (untreated sample (control) following the computation of, ΔΔCT for each sample, the gene expression (fold change) is determined using the as following final equation.

RQ = 2-(ΔΔCT) is the fold gene expression

Statistical Analysis

The Statically Package for the Social Sciences (SPSS) was used to analyze all data. The information are presented as means ± standard deviation (SD). One-way Analysis of Variance (ANOVA) and the Independent Samples. T-test were performed to assess diagnostic utility, The- Receiver. Operating- Characteristic (ROC) curve analysis was performed to compare patients and control data. A *p*-value ($p<0.05$) was considered statically significant.

Results

Demographics characteristics of RA patients and control subjects

Demographic data analysis showed a good age and sex match between the RA patients and the control group (Table1), fulfilling one of the key requirements for the comparative study design. The mean ages of the RA patients (47.94 years, 8.21 years) did not differ significantly from those of the control group (44.60 years, 7.95 years; p=0.196). However, the 51–60-year age group had the highest prevalence of RA, followed by the 41–50-year age group. The sex distribution was statistically even, with females comprising 78.0% (n=39 patients) and males 22.0% (n=11 patients), which closely resembles the control group (70.0% female, 30.0% male) (p = 0.362).

Table 1. Demographic Characteristics of patients with rheumatoid arthritis (RA) and control subjects.

Characteristic	RA Patients *n* = 50	Controls *n* = 50	*p value*
Age (years)			
Mean ±SD	47.94 ± 8.21	44.60 ± 7.95	NS
Range	20–70 years	20– 70 years	
20-30 years, *n* (%)	5(10.0 %)	8 (16.0%)	NS
31-40 years, *n* (%)	7 (14.0%)	13 (26.0%)	
41-50 years, *n* (%)	15 (30.0%)	11 (22.0%)	
51-60 years, *n* (%)	20 (40.0%)	10 (20.0%)	
61-70 years, *n* (%)	3(6.0%)	8 (16.0%)	
Gender			
Male, *n* (%)	11 (22.0 %)	15 (30.0 %)	NS
Female, *n* (%)	39 (78.0%)	35 (70.0%)	
M:F ratio	1:3.54	1:2.33	

$p > 0.05$ is not significant (NS).

Estimation of the TNF-α level in patients and controls

TNF-α levels were compared between RA patients and control subjects as showed in Figure 1. The mean levels of TNF-α were 65.70 ±11.46 and 34.99±0.9, in RA patients and controls respectively; the difference between the two groups was statically significant (*p*< 0.001). The appropriate cut-off value of TNF-α was 43.8 pg/ml, which had 100% sensitivity, 94% specificity, at an area under the curve (AUC) of 0.99 (95% CI= 0.98–1.00) as shown in Figure 2.

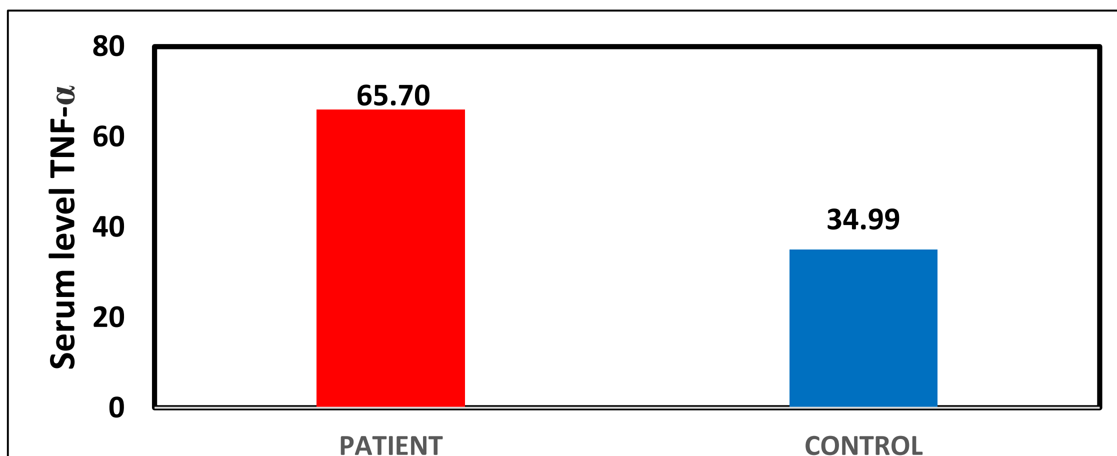

Figure 1. Comparison of the mean tumor necrosis factor- alpha (TNF-α) levels in rheumatoid arthritis patients and controls

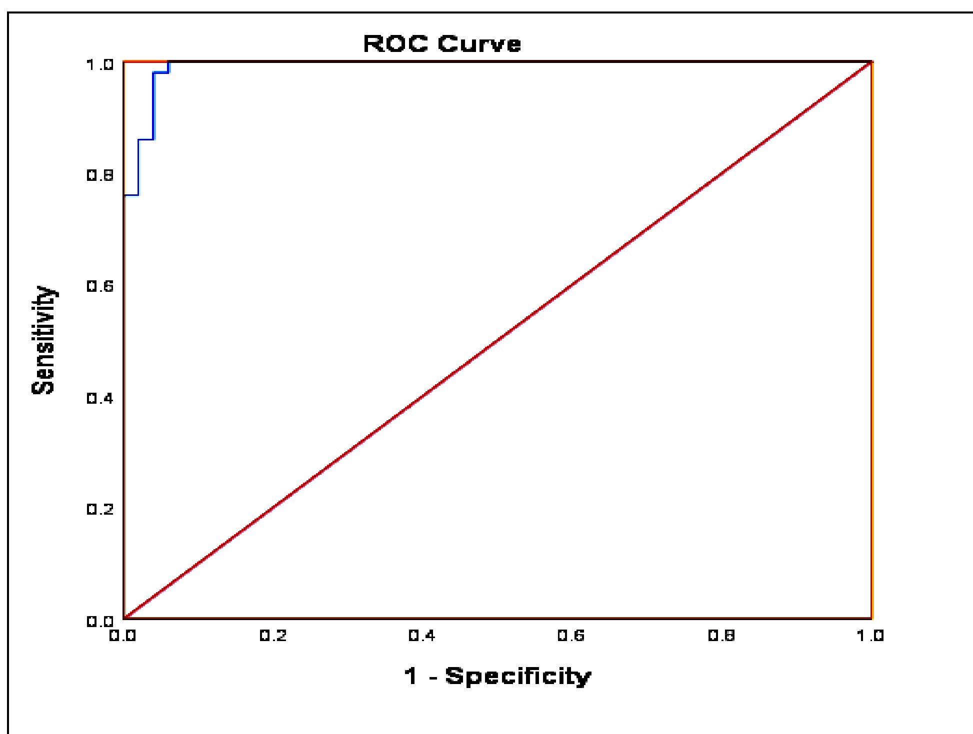

Figure 2. Receiver operating characteristic (ROC) curve analysis of tumor necrosis factor alpha (TNF-α) in rheumatoid arthritis (RA) patients versus controls.

Distribution of TNF-α according to stages of RA disease

The current study showed that TNF-α serum level was increased among RA patients in stage 4 (50.62±3.37) while less increased in patients within stage 1 (50.62± 3.37) Figure 3.

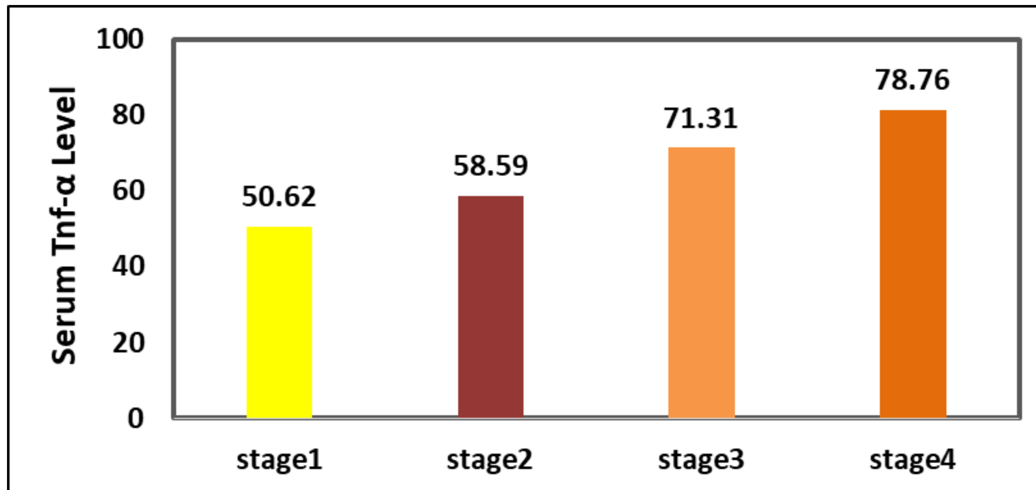

Figure 3. Tumor necrosis factor -alpha (TNF-α) level in rheumatoid arthritis (RA) patients according to stages of the disease.

Expression of miRNA-146 gene in RA patients and controls

Comparison of miRNA-146 gene expression between RA patients and control subjects was carried out, and the results are demonstrated in Figure 4 and Figure 5. The mean miRNA146 gene expression was 4.06 ± 1.4 in RA patients and significantly higher than the controls (1.00 ± 0.2, $p < 0.001$).

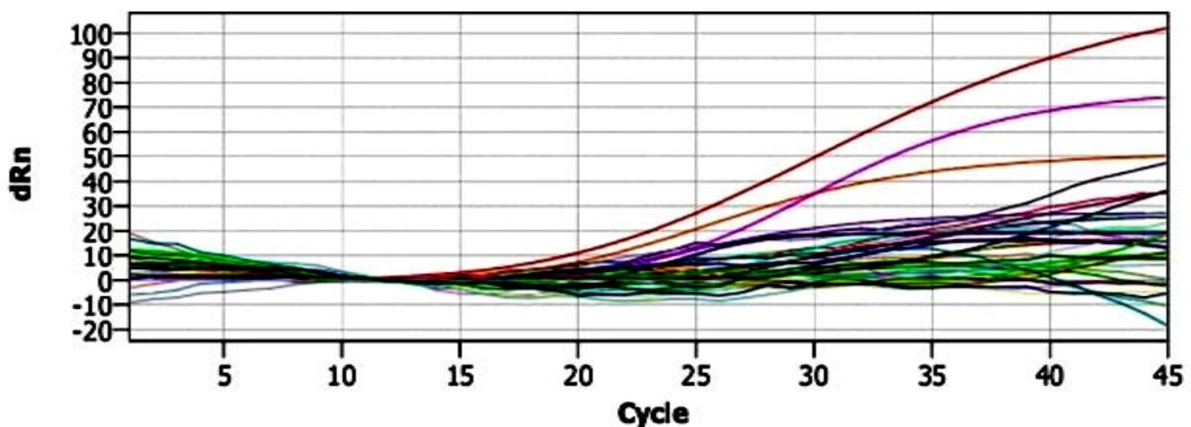

Figure 4. Image of cycle threshold (CT value) of Real-Time Polymerase Chain Reaction (PCR) for *miRNA146 gene expression*.

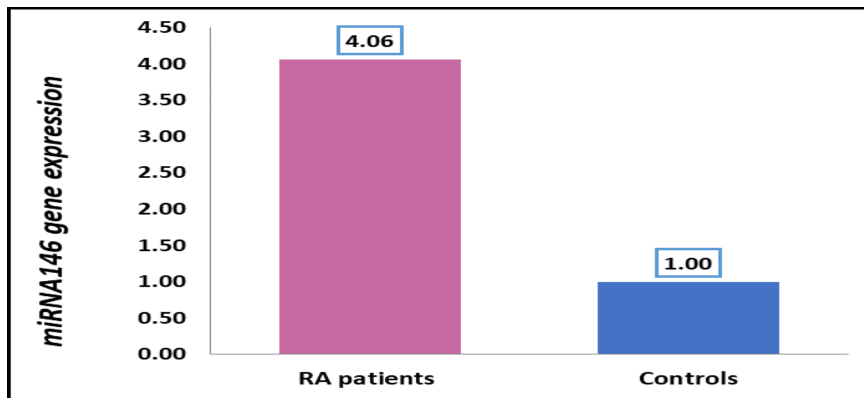

Figure 5. The mean miRNA146 gene expression in patients and control groups.

Evaluation of miRNA146 gene expression

The miRNA146 cutoff value and the prediction of RA disease were evaluated as diagnostic tests using the receiver operator characteristic (ROC) curve. The outcomes are displayed in Table 2 and Figure 6. The miRNA146 cutoff value was >1.73-fold and at an area under the curve of 90.0%, which showed sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), of 90.0%, 92.0%, 91.8%, 90.2%, and 0.910 (0.849-0.971). The present results indicated that miRNA146 can be regarded as an excellent diagnostic marker.

Table 2. Sensitivity and specificity of *miRNA146* (> 1.73-fold) in RA disease.

miRNA146	Patients (n = 50)	Controls (n = 50)
> 1.73	45	4
< 1.73	5	46
Sensitivity %	90.0 %	
Specificity %	92.0%	
PPV %	91.8 %	
NPV %	90.2%	
AUC (95% CI)	0.910 (0.849- 0.971)	

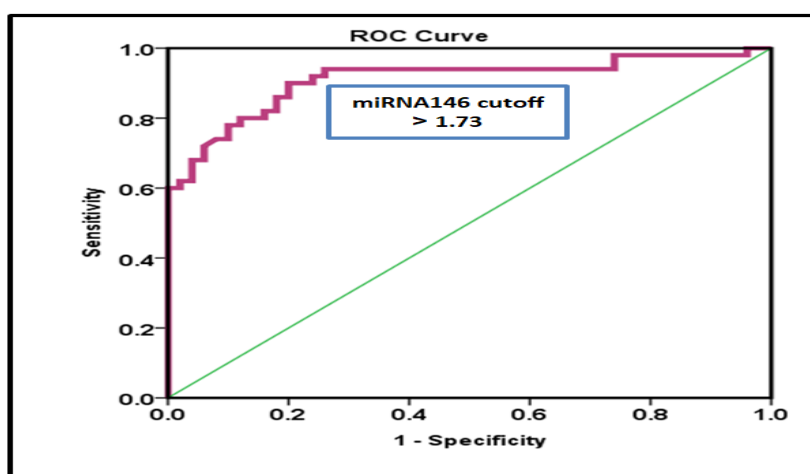

Figure 6. Receiver operator characteristic curve to determine the cutoff value of miRNA146 as a potential diagnostic marker.

Correlation between miRNA146 gene expression and TNF-α in RA patients

The relation between *miRNA146 gene expression* and TNF-αin RA patients are shown in Table 3. The results show significant positive correlation between *miRNA146 gene expression* and TNF-α (r= -0.374, *p*= 0.008) in RA patients.

Table 3. Correlation between *miRNA146 gene expression* and tumor necrosis factor-alpha (TNF-α) in RA patients.

Immunological parameters	*miRNA146 gene expression*	
	r	*p value*
TNF-α	0.374	0.008

r: Pearson correlation. $p \leq 0.05$ is significant.

Discussion

The present study found that the (51-60) age group is the most susceptible to the RA disease. This observation is consistent with previous research by Alhammami & AL-Ammar, 2020.[13] However, there were statistical differences in age between the rheumatoid patients and controls (p>0.05). While another study reported that the highest prevalence (52%) was in patients over 50 years of age.[14] Age was also associated with disease severity; as one report indicated that patients experience a 1.026-fold increase in joint degeneration with each year of advancing age. The fact that RA can occur in both young and elderly individuals is clinically significant. As Palmowski et al., 2023, noted these patient age groups often require different treatments, exhibit diverse laboratory findings, and generally have varying disease outcomes.[15] A previous study by Ibrahem *et a*l., 2019 mentioned that chronic autoimmune inflammatory RA disease affected females, and that they are more prone to disease than males; with females: male ratio 2:1–5:1.[16] Another study illustrated that RA disease is more common in females with ratio of about 3:1.[17] In general, females are twofold to three fold more likely to develop RA than males because fluctuations in hormone may have an impact on the level of proteins in the blood that cause inflammation. The immune response related to B and T cells are influenced by the hormone estrogen, and environmental factors may explain the reverse in the way for females.[18] The result of the current work indicated that the number of patients with RA was more in the last years than previous years which mean that the RA more incidence in the present time than past time. This result is in agreement with Nair et al., 2019, who showed that the incidence of RA disease demonstrated variation within the study period ranging from 33 per 100,000 to 73 per 100,000 while the prevalence of RA increased over time in 2014–2015.[19] In patients receiving active treatment, there were reduced odds of response to treatment with greater duration.

The cytokines are produced by Th2 lymphocytes, mast cells, basophils, and eosinophil, and other cells, including endothelial cells, lymphocytes, and macrophages.[20] TNF-α is often seen at the site of inflammation and in the bloodstream, and it is thought to be the cause of the alterations in systemic inflammation.[21] These results indicated that TNF-α is an essential mediator of inflammation in RA and significantly contributes to the pathogenesis and progression of RA.[22] The results of the present study are consistent with those of Mousa et al., 2025 who showed that TNF-α level had a statistically significant higher value in patients with RA compared to controls.[21]

The elevated levels of TNF-α observed in the more advanced stages suggest its role as a substantial mediator of inflammation in RA, playing a key role in the development and advancement of the disease.[22] The mean serum level of TNF–α was lower in early RA stages because the initiation of RA and the

involvement of autoimmune and immunological cells that become activated and release soluble mediators. This support the pathogenic processes while the level of TNF–α was higher in stage 4 because the disease progress in these stages lead to synovial hyperplasia, cartilage degradation, and bone erosion.[23] These finding demonstrated that TNF-α levels increase with the advancement or severity of the disease.

The results showed that miRNA-146a is overexpressed in the serum of RA patients (p<0.0001). These outcomes agreed with the results of Yousif et al., 2023 and Illahi et al., 2021, which revealed that miRNA-146a expression levels were statistically higher in both synovial tissues and serums in cases than in controls.[23,24] In a local studies in Al-Najaf province it was revealed that the expression efficiency of miRNA-146a was plotted as the efficiency of miR-146 using the receiver operating characteristics (ROC) curve, which demonstrated an AUC of 0.95 (95% CI 0.91-0.99).[25,26] The cutoff value was 1.57, with high sensitivity (91.7%) and high specificity (91.1%). Serum MiR-146 was significantly overexpressed in RA patients (fold change) (2.59±1.18). The ROC curve was used to predict the diagnosis of Graves' ophthalmopathy and showed good sensitivity and specificity. These findings suggested that miRNA-762 is a good diagnostic marker for Graves' ophthalmopathy.[27, 28]

The current study showed a positive correlation between micro146a expression and TNF-α levels in RA patients, which is in agreement with earlier reports that showed that miR-146a is significantly up-regulated in peripheral blood mononuclear cells, synovial fibroblasts, and $CD4^+$ T cells of RA patients, and its expression correlates positively with TNF-α.[29, 30] This finding supports the concept that miR-146a is part of the inflammatory cascade that characterizes RA. Astudy also confirmed these findings in an Iraqi cohort, demonstrating a 4.8-fold increase in miR-146a levels in the blood of RA patients, which was significantly associated with TNF-α.[31] The positive correlation suggests that increased TNF-α in RA drives compensatory up-regulation of miRNA-146a, which may act as a feedback regulator of inflammation (32). In conclusion, the elevated expression of miRNA-146a in RA patients and its potential to act as a positive activator of TNF-α indicates heightened inflammatory activity and may represent a compensatory mechanism to counteract elevated TNF-α level. These findings support the important regulatory role of miRNA-146a in the inflammatory pathway and highlight its potential value as a biomarker of RA disease progression.

Author Contributions

ZNN, MHA; collected the data and wrote the draft of the manuscript. ZNN; proposed the topic of this research and designed the study, and revised draft of the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The Ethics Committee of the College of Medicine at the University of Kufa reviewed, and approved the study protocol (reference no. HK/1068, dated 5/10/2024).

Informed consent

A signed consent form was obtained from each study participant.

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