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Investigate role of miRNA146a and IL-17 level in progressive rheumatoid arthritis disease

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Abstract

Rheumatoid Arthritis (RA) is a chronic, progressive autoimmune disease, involves an intimate relationship between immune cells and cytokines and results in decreased lifespans and higher mortality rates. The goal of the current study was to investigate the impact of MicroRNA (miRNA)146a and interleukin-17 (IL-17) as prognosis markers in RA patients. This case-control study included 120 RA patients who visited the Rheumatology unit at Al-Saddar Medical City in the governorate of Najaf, and 30 normal controls. Venous blood samples were collected from both patients and controls. Blood samples were used for measuring IL-17 levels using an enzyme linked immunosorbent assay (ELISA) testing, and miRNA146a by the **r**everse transcription polymerase chain reaction (RT-PCR). The results showed higher frequency of RA in women than in men with elevate incidence in patients aged 40-59 years and 1-2 years RA disease duration of. The level of IL-17 was significantly higher in serum of RA patients compared with the control group (p<0.0001). IL-17 level was significantly increased among the patients in RA stage 4 (p<0.0001). IL-17 level was significantly increased in patients without treatment compared with treated patients. The expression of miRNA-146a was significantly higher in the patients' group than control group. In conclusion, IL-17 may play critical role in chronic inflammation and can be used as diagnostic biomarker for RA. miRNA-146a is overexpressed in RA patient relative to healthy individuals and it acts as a negative regulator for IL-17.

Keywords: Rheumatoid arthritis (RA), interleukin-17 (IL-17), miRNA-146a

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Introduction

Rheumatoid Arthritis (RA) is a chronic autoimmune illness characterized by both systemic and local inflammation, which leads to increased mortality, decreased longevity, and increasing disability.[1] About 0.5–1% of the global population is estimated to have RA, with 40 out of 100,000 occurrences recorded each year.[2] In Iraq, the prevalence of cases was 1 %.[3] Interleukin-17 (IL-17) is a proinflammatory cytokine, involved in chronic inflammation and triggered by allergies, cancer, and autoimmune diseases. This cytokine is crucial to the development of inflammation or immunity to infection in the intracellular or extracellular space.[4] Increased IL-17 levels and unchecked T

helper 17 (Th17) cell generation may also play a role in the onset of autoimmune disorders.[5] IL-17 is involved in the early stages of autoimmune disease as well as the late stages of chronic disease.[6] MicroRNA (miRNA) is an epigenetic factor that plays a crucial role in regulating several processes, such as immune cell maturation and functions, organ development, cell proliferation, differentiation, death, and signal transmission.[7] One of the few miRNAs, the microRNA-146a, whose expression is substantially elevated after prolonged expression has been associated with immunological tolerance, suggesting that it functions to prevent the inflammatory response from being overstimulated.[8] miRNA-146a is produced differently in a variety of human disorders and crucial in the negative control of inflammatory innate immune responses.[9] This research study aimed to investigate the impact of miRNA-146a and IL-17 as prognostic markers in RA patients.

Subjects and Methods

This case-control study involved 150 participants, of whom 120 were patients with rheumatoid arthritis, and 30 controls. Patients' age ranged from 20 to 79 years and were of both sexes. The patients were recruited from the Rheumatology Unit at Al-Sadder Teaching Hospital in AL-Najaf Al-Ashraf province. Their diagnoses were made based on clinical, radiological, and serological parameters in accordance with the 2010 ACR/EULAR criteria between October 2022 and the end of September 2023. The age range of the control group was 20- 65 years. RA patients suffering from chronic disease, co-morbid autoimmune disease or patients with other type of arthritis types were excluded from this study.

Sample collection

A venous blood sample (4 ml) was collected from each patient and study control subject. Of this, an aliquot blood sample (3 ml) was placed into a gel tube for serum separation, used for measuring IL-17 level. Assessment of IL-17 level was performed by enzyme linked immunosorbent assay (ELISA) kits according to (CAT. NO: EKHU-0082, MELSIN, China). A second aliquot blood sample (0.5 ml) was transferred into an Eppendorf tube, contains 0.5 ml triazole and immediately stored at -٨0 C◦ until used for assessment of miRNA-146a by RT-PCR.

Total RNA was extracted from whole blood using commercial kits (TransZol™ miRNA, Trans, China), following the manufacturer's protocol. Primers for miR-146a and U6 calibrators were designed by a biotechnology company (Macrogen Inc., Korea). The sequence of the miR-146a primers was Forward TGAGAACTGAATTCCATGGGT- Reverse GCAGGGTCCGAGGTATTC. The expression of U6, as an internal control, was used for the normalization of miRNA expression. The first Step of the reverse transcription quantitative polymerase chain reaction (RT-qPCR) reaction was prepared according to Promega company and the thermocycling conditions are show in Table 1.

Table 1. One step RT-qPCR programs.

Step	Temperature	Duration	No of Cycles
Reverse transcription	37℃	15 min.	1
RT inactivation/ Hot-start activation	95℃	10 min.	1
Denaturation	95℃	10 sec	50
Annealing	58℃	30 sec.	
Extension and data collection	72℃	30 sec.	

Calculations

Gene expression or gene fold (Relative quantification) value calculated according to the method described by [10] Relative quantification (RQ) = $2^{-(\Delta\Delta CT)}$

First, determine the gene fold for each triplicated sample by obtaining the CT (cycle threshold) average value from the real-time PCR equipment. Next, compute the ΔCT value for each sample in the following manner:

Δ CT = CT (tested miRNA146) – CT (reference gene U6)

ΔΔ CT = Δ CT (tested sample) – Δ CT (reference gene)

Fold gene expression RQ = $2^{-(\Delta\Delta CT)}$

Statistical Analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 20. Data are expressed as means ± standard deviation (SD). Statistical analyses were performed through an independent T-test, one-way ANOVA, the receiver operating characteristic (ROC) curve analysis. A *p*-value of <0.05 was considered significant.

Results

Demographical Distribution of Rheumatoid Arthritis patients and control subjects

The results showed that the arthritis patients included more females 109 (90.0%) than male 11 (9.2%). In the 30 normal controls, females were 26 (86.6 %) and male 4 (13.3) as shown in Table 2. The RA patients' mean age was 47.75±14.20 years while the control subjects recorded 35.9±8.36 years. The mean duration of RA disease was 5.95±7.88 years.

Table 2. Distribution of study subjects according to gender, age and duration of disease

Variable	Rheumatoid arthritis patients n (%)	control group n (%)
Sex		
Male	11 (9.2)	4 (13.3)
Female	109 (90.8)	26 (86.6)
Age		
Age years (mean)	47.75±14.20	35.9±8.36
Age years (range)	20-79	20- 65
< 40	30 (25%)	15 (50%)
40-59	63 (52.5%)	12 (40%)
> 60	27(22.5%)	3 (10%)
Total	120 (100%)	30 (100%)
Duration of Disease		
Duration of Disease (mean)	5.95±7.88	
Duration of Disease (range)	< 1- > 10	

Estimation the level of IL-17 in RA patients and controls

The results indicated that level of IL-17 was significantly increased in the serum of RA patients to 41.36±9.64 pg/ml in compared with 20.7±1.04 pg/ml in the control (p<0.0001) as shown in Figure 1. The appropriate cut-off value of IL-17 was 26.86 pg/ml, which had 97.0 % sensitivity, 90.0 % specificity, area under the curve (AUC) of 0.99 (95%CI= 0.98–1.00) as shown in Table 3 and Figure 2.

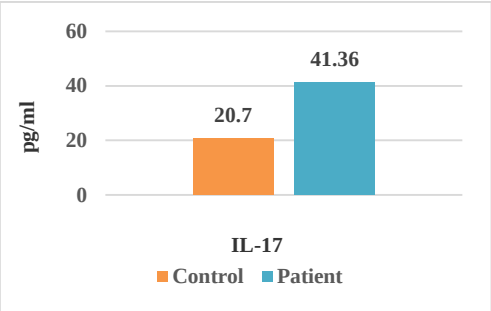

Figure 1. Interleukin 17 (IL-17) Level in rheumatoid arthritis (RA) patients and controls.

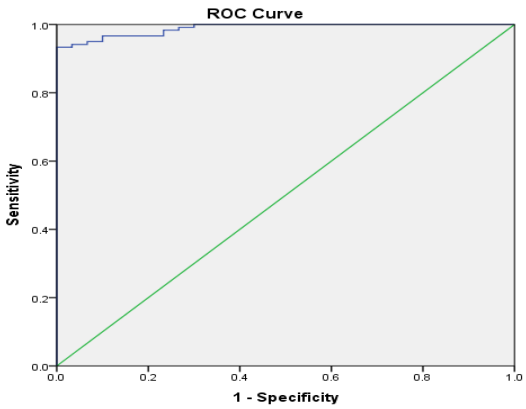

Figure 2. Receiver operating characteristic (ROC) curve analysis of interleukin 17 (IL-17) in rheumatoid arthritis (RA) patients versus controls.

Table 3. Sensitivity and specificity of interleukin 17 (IL-17) between rheumatoid arthritis (RA) patients and controls.

Area under the curve	*p* value	Asymptomatic 95% Confidence interval		Cutoff	Sensitivity	Specificity
		Lower bound	Upper bound			
0.99	0.0001	0.977	1000	26.8595	0.97	0.90

*$p \leq 0.05$ is significant.

Evaluation the IL-17 level in RA patients according to stages of disease

The present study showed that IL-17 serum level was significantly increased among the patients in stage 4 (59.38± 8.12 pg/ml) while the increase was less in patients within stage 1 (27.44± 3.35 pg/ml) ($p \leq 0.0001$) as shown in Figure 3.

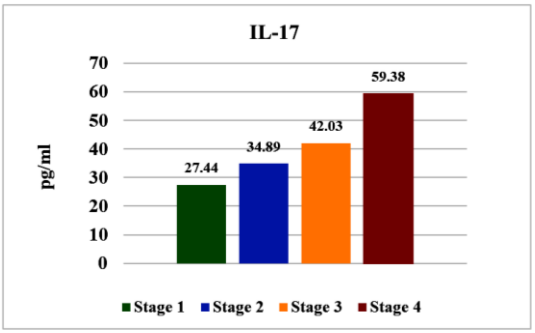

Figure 3. Interleukin 17 (IL-17) level in rheumatoid arthritis (RA) patients according to stages of disease.

Evaluation the IL-17 level in RA patients according to types of treatment

The result indicated that IL-17 was significantly increased in patients without treatment (57.64 ± 10.21 pg/ml) in comparison with patients treated by chemical, biological and combination therapy which were about (40.76±5.62 pg/ml, 31.56±6.12 pg/ml, 29.98±10.21 pg/ml, respectively), ($p \leq 0.0001$) as shown in Figure 4.

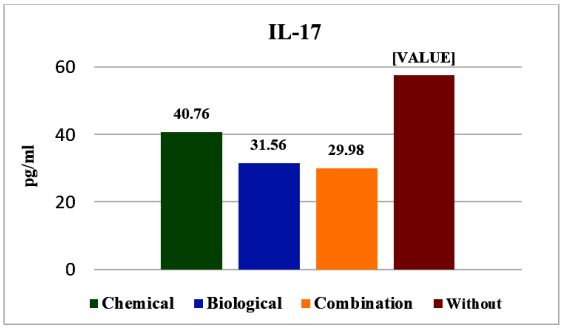

Figure 4. Interleukin 17 (IL-17) level in rheumatoid arthritis (RA) patients according to types of treatment.

miRNA-146a expression in rheumatoid arthritis patients and controls

The level of expression of miRNA-146a was significantly higher in the patients' group (5.31 ± 1.79), while in control group was (1.7 ± 0.45) (p< 0.0001) as shown in Figure 5 and Figure 6. The appropriate cut-off value of miRNA-146a was 2.095, which had 100 % sensitivity, 100 % specificity, AUC= 1.000, and was *P*-value at (*P*=0.000) as shown in Table 4 and Figure 7.

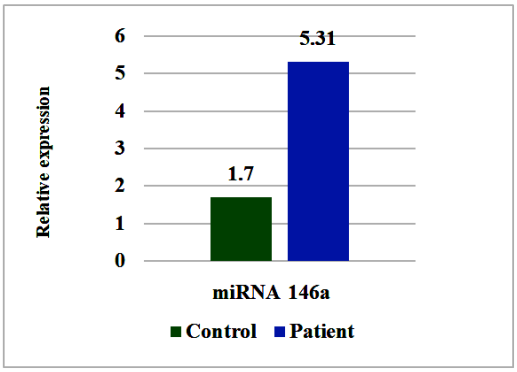

Figure 5. miRNA-146a expression level in rheumatoid arthritis (RA) patients and controls.

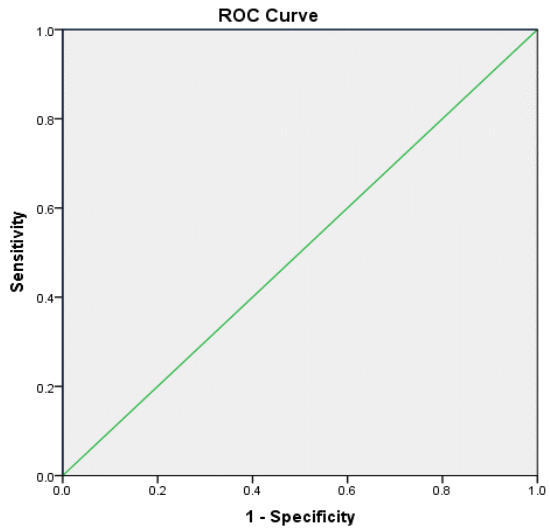

Figure 6. Receiver operating characteristic (ROC) curve analysis of miRNA-146a in rheumatoid arthritis (RA) patients versus control.

Table 4. Sensitivity and specificity of Interleukin 17 (IL-17) between rheumatoid arthritis (RA) patients and controls.

Parameter	AUC	*p*-value	Cut off	Sensitivity	Specificity
miRNA-146a	1.000	0.000	2.095	100%	100%

*$p \leq 0.05$ is significant.

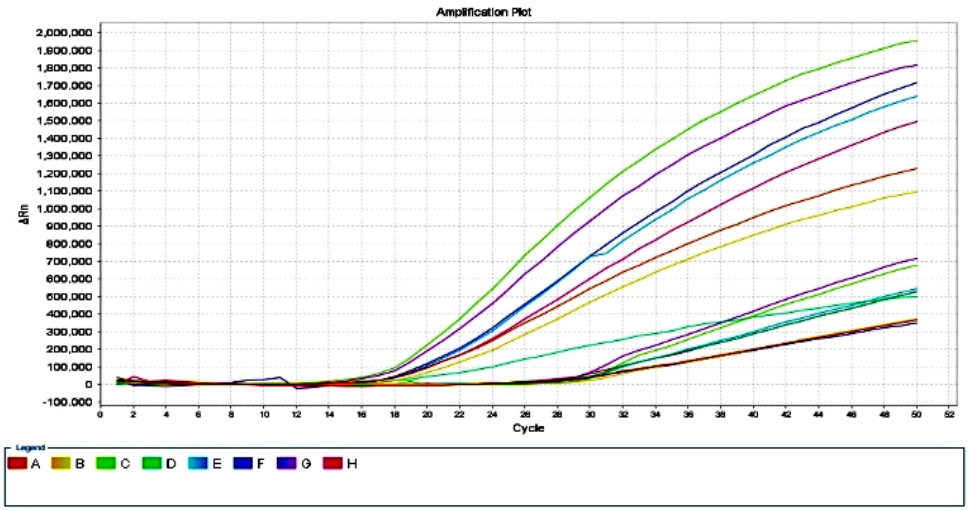

Figure 7. Real time PCR image showing cycle threshold (Ct) values of microRNA-146a gene.

Discussion

The present study aimed to investigate the impact of miRNA-146a and IL-17 as prognostic markers in RA patients. In 2019, a previous study reported that females were more likely than males to have chronic autoimmune inflammatory RA disease, as the female to male ratio was 2:1 to 5:1.[11] According to a prior study conducted in 2021, women were 2-3 times more often than men to get RA, and they also usually fare worse than males in terms of disease activity and disability.[12] A study by Palmowski et al., 2023, showed that women were more often than men to have RA disease, with a ratio of around 3:1.[13] Another study found that while pregnancy was linked to disease remission, nulliparity frequently increases the risk of RA.[14]

A study in the Duhok province (Kurdistan Region of Iraq), reported that patients with RA had a mean age of 47.7±10.9 years (ranging from 20 to 70 years). Of these patients, 90.9% were females and 5.09% males. The patients' disease duration was found to be 7.08±6.96 years, and the progression of the disease (counts of total tender joints (TTJ) and swollen joints (TSJ)) was positively correlated with the length of time the disease had been present (p<0.05).[15] The high prevalence in women may be related to sex hormones, which have complex effects on the immune system.[16] The majority of epidemiological research indicated that women often have RA illness beginning symptoms around middle age or menopause. However, males typically experience symptoms later in life and are more likely to test positive for RF and have higher titers of ACPAs.[14] A previous study, by Baker et al., 2022, reported that the length of the disease had a significant impact on the chance of responding to therapy for RA, as longer disease duration was associated with lower odds of responding to treatment.[17] Another previous study by Vázquez-Del Mercado et al., 2017, pointed to disease duration as a predictor of vascular stiffness in RA patients. Therefore, patients with RA disease duration of ≥10 years exhibited significantly increased carotid-femoral pulse wave velocity, that is considered the gold standard to evaluate arterial stiffness compared with patients with disease duration <2 years and ≥2 to <10 years, respectively.[18] In 2021, another study reported that a longer duration of rheumatoid arthritis is a risk factor of interstitial lung disease.[19]

The formation and progression of the pathogenicity of RA disease is significantly influenced by IL-17, a powerful pro-inflammatory cytokine with joint-destructive actions that can be used as a target site for biological therapy.[5] The results of the current study demonstrated a significant difference in IL-17 levels between the RA group and the normal control group. These findings were in line with a local study conducted in Erbil, Iraq by Albarzinji & Albustany (2022) that demonstrated the critical role of IL-17 in various autoimmune diseases, particularly the RA disease.[20] These findings may be explained by the notion that IL-17 plays a major role in stimulating other proinflammatory agents such as tumor necrosis factor (TNF-α), neutrophils, and monocytes cause inflammation, which in turn causes the condition to worsen and eventually destroy the joint.[21] This outcome is consistent with that of a research study conducted in 2023 that found that RA patients have higher levels of Th-17 and IL-17 frequency than apparently healthy controls.[22]

IL-17 has a direct role in the early induction and late chronic phases of a number of inflammatory disorders including rheumatoid arthritis, where it causes alterations in the synovium that result in synovitis and sustain local inflammation.[23] Given that IL-17 is known to be extensively generated in chronic inflammatory diseases, our investigation verified that the mean level of IL-17 was greater in stage 4, indicating that the condition has progressed to chronic inflammation. When the immune system's capacity for self-tolerance is reduced in the early stages of RA disease, different autoantibodies trigger the immune system, which causes immune cells to infiltrate the joint's synovium. Numerous cytokines, including TNF-α and interleukins, are involved in this process. Patients with RA had varied degrees of elevated blood levels of TNF-α, IL-17, IL-6, and IL-1β.[5] According to a study by Roşu et

al., 2012, that found the synovium is highly suggestive of an aggressive disease and may express specific therapeutic targets, simultaneous IL-17 assessment of serum and synovium may be helpful in defining activity and predictive patterns during early stages of untreated RA.[24] In Iraq's Kurdistan, researchers found that serum IL-17 levels in RA women were significantly higher than in the control group (p<0.001), and that these levels positively correlated with RA women's age, obesity, and length of illness. The best IL-17 cutoff value was 69.5 pg/ml for predicting the presence of RA, with a 96% sensitivity and a 75% specificity.[25] In Egypt, a study reported that serum IL-17 levels were greater in the RA group (226.6±215.6 pg/ml) compared to the control group (48.17±54.9 pg/ml), with a p-value of <0.001 and sensitivity of 81.2% and specificity of 75%. These findings illustrated strong association with disease activity score in 28 joints (DAS-28), wherefore serum IL-17 level may be a useful indicator of RA disease activity.[26] Additionally, a prior study by Al-Saadany et al., 2016, found strong correlation between the DAS-28, measured blood levels of IL-17 and the disease activity, confirming the significance of IL-17 in the pathophysiology of the destructive and inflammatory pattern typical of RA.[27]

High levels of IL-17 in serum of RA patients without any treatment may indicate progress of disease including synovial inflammation and joint damage. RA patients with chemical therapy recorded increased level of IL-17 in some patients not responding to drugs such as methotrexate (MTX). This is consistent with finding of other previous studies, they found some risk factors that may be responsible for RA patients not responding to MTX such as female gender, the onset of symptoms at a younger age, higher body mass index, smoking, higher baseline disease activity (DAS-28), positive RF status and diabetes. However, approximately one quarter of patients discontinue MTX within 12 months.[28,29] In this study, RA patients who took biological treatment were remarkably responsive than those administered chemical therapy. This is may be the reason that these drugs had made a revolution in the treatment of inflammatory rheumatic disorders resulting in proven to control disease activity and stop or reducing of the disease progression. The findings of this investigation are consistent with those of research conducted in 2021. They found that Iraqi RA patients who start etanercept medication early on experienced better results than those who received other treatment.[30] A local research study, conducted in the province of Baghdad, found greater amounts of IL-17 in the synovial fluid of RA patients and blood levels of this cytokine strongly corresponded with indications of the disease's activity than in the control group.[31] According to a previous research by Lopez-Pedrera et al., 2020, some disease-modifying antirheumatic drugs (DMARDs), including TNFα inhibitors (etanercept), are more effective than others. The majority of these drugs work directly to suppress proinflammatory cytokines, while some work upstream of the inflammatory cascade, preventing either the B cell depletion or the T cell activation.[32] Methotrexate is frequently started as a monotherapy and has the ability to decrease the production of proinflammatory cytokines. It can also be used with other DMARDs, such as hydroxychloroquine, to treat joint inflammation. When this doesn't work, methotrexate and a biologic DMARD, including TNF inhibitors, are frequently combined to increase their effectiveness in RA.[33] In line with a previous study, patients who received combination therapy were the best responders to treatment. Both of these theories that methotrexate's ability to prevent anti-drug antibodies and/or the synergy of methotrexate and biologics reducing inflammation via two pathways are plausible explanations for the increased response to combination treatments like MTX, anti-TNF and may even contribute to improved patient responses.[34]

This study estimated the impact of epigenetic processes on rheumatoid arthritis. miRNA-146a may be involved in autoimmunity since it can operate as a negative feedback mechanism during the initiation of immune responses.[9] Our findings demonstrated that serum of RA patients contains overexpressed miRNA-146a (p<0.0001). These outcomes concur with other studies, which showed that

RA cases had statistically greater levels of miRNA-146a expression in their synovial tissues and sera than controls.[35,36] In the same line, a study conducted in 2021, confirmed in comparison to the control, "miR-146a" was up-regulated in patients with RA and osteoarthritis.[37] It has been demonstrated that miR-146a is increased in IL-17-producing cells, which promotes Th17 differentiation and promoting RA pathogenesis through IL-17.[38] Serum miRNA-146a was found to be considerably overexpressed in RA patients "fold change 2.59±1.18" compared to controls "fold change 1.07±0.41" in a local research conducted in the province of Al-Najaf, Iraq ($p< 0.0001$). The expression efficiency of miR-146a was compared using the ROC curve analysis, which displayed high sensitivity 91.7% and high specificity (91.1%), at an AUC of 0.95 (95% CI 0.91-0.99), and a cut off value of 1.57.[39] Similarly, a local study conducted in Baghdad, found that miR-146a expression is linked to RA sickness and can affect the intensity and activity of the disease in the Iraqi population.[40] Furthermore, in Egypt, a previous study reported that synovial fluid and peripheral blood mononuclear cells from RA patients expressed more miRNA-146a than those from apparently healthy control subjects. They speculated that this increased expression of miR146a results in impaired function, which in turn increases prolonged TNF production.[41]

In conclusion, IL-17 may play a critical role in chronic inflammation. miRNA-146a is overexpressed in RA patients relative to healthy individuals. It may act as a negative regulator for IL-17. Both IL-17 and_miRNA-146a_can be used as diagnostic biomarkers for RA.

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Author Contributions

WAF, collected the data and wrote the draft of the manuscript. MFD, 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 study protocol was reviewed and approved by the Ethics Committee of the Faculty of Medicine, University of Kufa (reference no. HK/1052, dated 20/10/2022).

Informed consent

An informed written consent was obtained from each study subject before included in the study.

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