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ORIGINAL ARTICLE

Prognostic Value of miRNA-155 and Tumor Necrosis Factor-Alpha in Rheumatoid Arthritis Disease

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Key words:
Rheumatoid arthritis (RA), TNF-α, miRNA-155, Aggressive and mild stage

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ABSTRACT

Background *Rheumatoid arthritis (RA) is one of common autoimmune disorder globally affected 1% of population characterized by irregular cytokine production. microRNAs can influence the cytokine production that is associated with autoimmunity. Biomarkers found in local and systemic inflammation may give the solution for accurately diagnosing and treating arthritis.* ***Objective:*** *to evaluate TNF-α levels in synovial fluid and serum with serum miRNA-155 expression as prognostic markers in RA individuals.* ***Methodology:*** *80 RA patients were attended to Al-Saddar Medical City Rheumatology Unit in the Najaf Governorate and 40 healthy as control group were selected for case-control study. The mean patients' age was 50.6±16.5 and 42.5±9.24 years for healthy group. These patients were diagnosed by specialized physicians and given a diagnosis of rheumatoid arthritis then classified into mild (stage1 and 2) and aggressive stages (stage 3 and 4). Three ml of venous blood were taken from all participant from which 2 ml were placed in gel tube for serum separation to measuring RF and ACCP as confirmative diagnosis, the other 1ml was placed in Eppendorf tube with Triazole and immediately stored at -20 C◦ for miRNA -155 evaluation by RT-PCR, Knee synovial fluid was separated from Knee effusion of both RA patients and patients suffering from knee trauma only as control group. TNF-α was measured in serum and S.Fluid by ELISA assay.* ***Results****: that RA patients between the ages 45 - 67 years had a greater incidence and severity of the illness. TNF-α levels were substantially greater in patients with Aggressive-stage disease than in patients with mild-stage. According to ROC analysis appropriate cut-off value of miRNA-155 was 1.66, which had 0.944 % sensitivity, 0.889% specificity, AUC= 0.972, at P=0.000***. while the appropriate cut-off value of TNF-α appeared with high validity as prognostic factors in both serum and S. Fluid (87 % sensitivity, 97% specificity, AUC 0.992 in serum and 83.3% sensitivity, 86.7% specificity, AUC 0.915 in synovial Fluid.* ***Conclusion:*** *By using ROC analysis, it was possible to achieve good diagnosis accuracy for TNF-α levels in both serum and synovial fluid, as well as more sensitive and accurate discrimination of RA disease. TGF-β1 and miRNA-155 blood levels may help patients to avoid discomfort and stress instead of employing synovial fluid separation as a diagnosis for excellent prognosis in RA illness.*

INTRODUCTION

Rheumatoid arthritis (RA) is a prevalent autoimmune condition that affects around 1% of the world's population and is defined by local and systemic inflammation with progressive and irreversible joint destruction as a result of persistent synovitis that causes disability and increased mortality[1], it is caused by a wide variety of variables such as age, sex, infection, genetic and epigenetic susceptibility such as bacterial or viral infection[2,3]. The excessive production of cytokines plays a fundamental role in particular destruction and extra-articular manifestations associated with RA[4,5].

TNF-α is a cytokine that affects different kinds of cells in pleiotropic ways. It plays a key role in controlling inflammatory reactions and is connected to the etiology of several autoimmune and inflammatory disorders[1]. There are various ways in which TNF-α signaling contributes to the pathophysiology of RA. It causes proinflammatory cells including synovial fibroblasts and macrophages to be recruited, activate endothelial cells, and generates proinflammatory cytokines that cause broad multi-organ damage, encourage the development of pannus, and cause joint degradation damage[6].

One type of epigenetic factor that is important for controlling many biological processes is miRNAs. These processes include signal transmission, immunological response, organ development and apoptosis. miRNAs are noncoding small RNAs when dysregulate expression of miRNAs lead to pathological conditions such as malignancy, inflammatory and

autoimmune disorders[7]. Numerous body fluids, including blood, urine, saliva, cerebrospinal fluid, and synovial fluid have been shown to contain miRNAs. These biomarkers can accurately diagnose certain diseases, estimate disease development, assess therapy response, and predict medication responsiveness in individual patients are desperately needed in the care of people with RA.

The aim of this study was to measure the validity of TNF-α and miRNA-155 as prognostic factor in rheumatoid arthritis disease.

METHODOLOGY

Patients and control characterization:

A case-control study design was used on 120 participants, comprising ٤٠ healthy control group and 80 patients with rheumatoid arthritis, who were collected from the Rheumatology Unit in Al-Sadder Teaching Hospital in AL-Najaf Al-Ashraf attending province both groups match in age and sex with no significant difference between them. The patients were examined by a physician and diagnosed as having rheumatoid arthritis based on clinical, radiological, and serological parameters according to 2010 ACR/EULAR criteria from October 2023 till the end of March 2024.

Included and Excluded criteria:

All RA patients were of both sexes and ranged in age were included. Patients with co-occurring autoimmune diseases, chronic illnesses, and other forms of arthritis were excluded from in the study, although those with RA.

***Sample collection*:**

Three milliliters of venous blood were taken from the patients and controls. the sample were transferred to a gel tube for serum separation, which was used to measure RF and ACCP by ELISA testing. Synovial fluid was separated from Knee effusion of both RA patients and patients suffering from knee trauma only as control group and stored in blank tube until used . TNF-α was measured from serum and synovial fluid by ELISA assay according to (BT LAB/China). 1ml of blood was transferred into Eppendorf tube with 1 ml Triazole for miRNA-155 expression by RT-PCR. Total RNA extraction using the TransZol™ reagents following the manufacturer's protocol (Trans/China). Primers for miR-155 and U6 calibrators were designed by Macrogen /Korea. U6 expression were used for the normalization of miRNA expression , 1Step RT-qPCR reaction was prepared according to Promega company and the thermocycling conditions were: one cycle of Reverse transcription at 37˚C for 15 minutes, then one cycle of RT inactivation at 95˚C for 10 minutes and 40 cycle included Denaturation with 95 ˚C, 10 sec, Annealing (60 ˚C, 30 sec) then Extension for thirty seconds at 72˚C. Gene expression (gene fold) value was calculated[8].

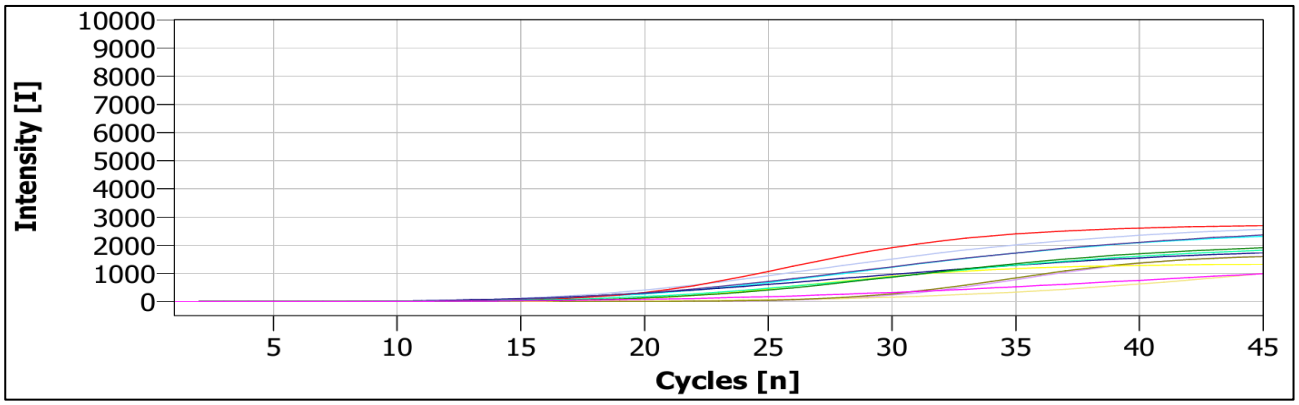


Fig. 1: Real time PCR image showing Ct value of microRNA-155 00

The average CT(cycle threshold) value for each triplicated sample was collected using an RT-PCR instrument to calculate the gene fold, then ΔCT
value determined as follows for every sample:
Δ CT = CT (tested miR326) - CT(reference geneU6)
ΔΔCT=ΔCT(tested sample)- ΔCT(reference gene)
Fold gene expression RQ=2-(ΔΔCT)

Statistical analysis

Data were expressed as means ±S.D. Statistical analyses were performed through SPSS 20 followed by an independent T-test, one-way ANOVA, ROC curve and P-value. The Receiver operating characteristic (ROC) curve was made to assign the best cutoff point that amplifies the sensitivity, specificity, and to identify its cutoff point that differentiates among patients and healthy control.

RESULTS

Demographic Distribution of Rheumatoid Arthritis patients and control subjects

Based on figure (2), the current work shows that among the 80 RA patients, 64 (80%) were female and 16 (20%) were males. and healthy, 31 (77.5%) were females. The mean age of RA patients was 50.6±16.5 years, whereas 42.5±9.24 years in healthy individuals.

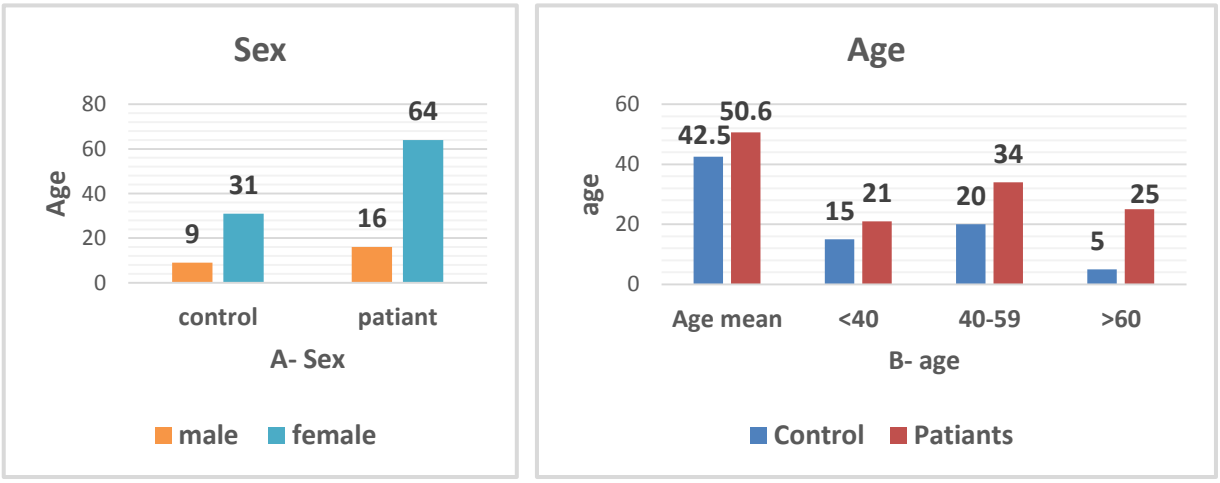


Fig. 2: Show the distribution of study subjects according to (A) Sex (B) Age

Evaluation the level of ACCP and RF factor in RA patients and control group

The results revealed higher significance ACCP levels (170.12±155.94 U/ml) for RA patients in comparison to control group (10.6 ± 3.59U/ml) at P value= 0.0001, RF of all patients were positive compareing with the negative results in all healthy groups as shown in table (1).

Table 1: Level of ACCP and RF auto antibody in patients and control

variable	control group	RA patients
	N(%)	N(%)
ACCP mean	10.6 ± 3.59U/ml	170.123 ± 155.94 U/ml
RF auto-antibody	negative (-)	positive (+)

Estimation of TNF-α level in RA patients and healthy controls:

The current results indicate that RA patients had significantly higher blood and S. fluid TNF-α levels (138.9 ± 6.9; 355.7 ± 28.7) ng/ml than the control group (29.2 ± 1.8 ng/ml; 78.5 ± 23.6) at p value (<0.0001***) as shown in Table (2). The appropriate cut-off value of TNF-α was (43.8 ng/ml), which had 87.5 % sensitivity, 97.5% specificity, AUC 0.992 (95% CI 0.975 - 1.000), and was highly significant at (0.000***). While in S. fluid the cutoff value was 187.7 ng/ml which had 83.3% sensitivity, 86.7% specificity, AUC 0.915 (95% CI 0.810 - 0.999), and was highly significant at (0.000***) fig 3 and Table 3).

Table 2: The level of TNF-α in serum and S.Fluid of RA patients and healthy controls

TNF-α	Control	Patients	P-value
Serum	29.2 ± 1.8	138.9 ± 6.9	<0.0001***
S. fluid	78.5 ± 32.6	355.7 ± 28.7	<0.0001***

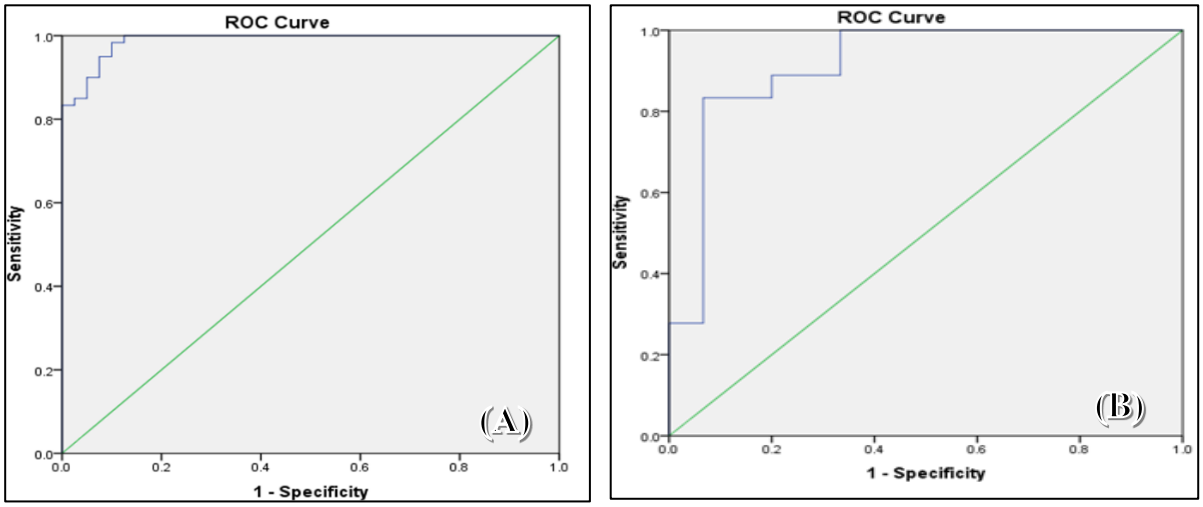


Fig. 3: ROC curve analysis of TNF-α in (A)serum and (B)S. Fluid of RA patient's vs control.

Table 3 Sensitivity and specificity of TNF-α in serum and S.fluid in rheumatoid arthritis (RA) patients.

TNF-α	Cutoff	AUC	Sen%	Spec%	Std. Error	CI 95%	P-value
serum	43.8	0.992	87.5 %	97.5%	0.007	0.975 - 1.000	0.000***
S. Fluid	187.7	0.915	83.3 %	86.7%	0.054	0.810 - 0.999	0.000***

Evaluation the level of TNF-α in rheumatoid arthritis patients according to the stages of the disease

The present study showed that TNF-α serum level was highly increased among patients in aggressive-stage (197.8 ± 38.3ng/ml) while less increased in patients within mild-stage (80.1±22.8 ng/ml) (P value <0.0001***) as shown in figure (4).

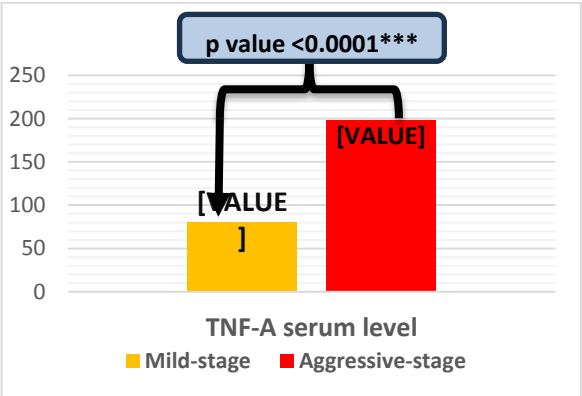


Fig. 4: The level of TNF-α in patients according to stages of disease

MiRNA-155 serum level in Rheumatoid Arthritis patients and control group

The mean expression level miRNA-155 was significantly higher in the RA patients' group (3.51 ± 0.33), while in control (Healthy) group was (1.16 ± 0.091) at P-value (P<0.0001 ***) as shown in figure (5). The appropriate cut-off value of miRNA-155 was 1.665, which had 94.4 % sensitivity, 88.9% specificity, AUC= 0.972 at (P=0.000***) showed in figure (6)

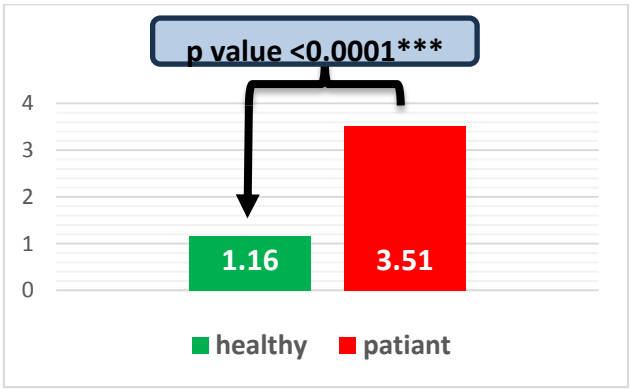
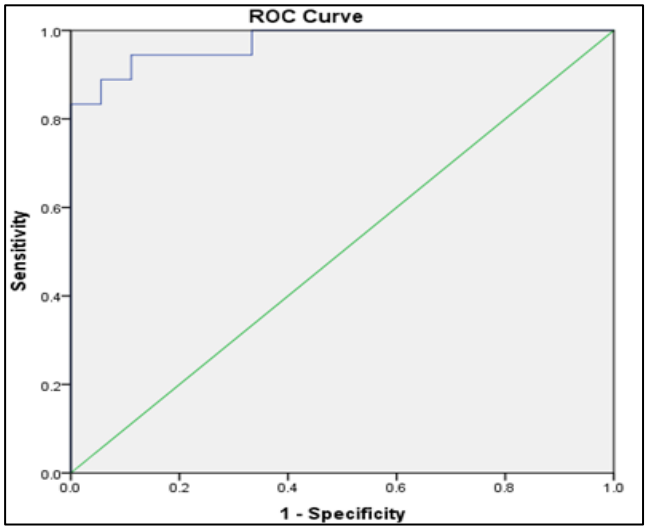


Fig. 5: MiRNA-155 expression level on blood of patient and control (healthy)



AUC	**0.972**
P-value	**0.000*** **
cut-off	**1.66 ng/ml**
sensitivity	**94.4 %**
specificity	**88.9%**

Fig. 6: ROC curve for MiRNA-155 level of control (healthy) and patient blood

DISCUSSION

Like other autoimmune illnesses, RA primarily affects women; the ratio of women to men is (2:1 to 3:1)[9]. Although RA can strike at any age, most cases start between the ages of 30 and 50 years. Similar present at[10], observed that RA can develop at any age but it most commonly begins between the ages of 30 - 60.

Women are more likely to develop RA than men, with a ratio of approximately 3:1 [11]. The impact of age on the severity of rheumatoid arthritis is a complex topic. Some studies have suggested that older patients with RA may experience more severe symptoms and a more rapid disease progression may be due to factors such as decreased immune function, comorbidities, and the accumulation of joint damage over time usually at th age 45-75 years [12] .

Citrullination is a normal process that occurs during tissue turnover and repair, but in patients with RA, citrullination occurs excessively in the joints leading to the production of autoantibodies, including ACPA[13]. Other result found the ACPA titer was significantly associated with higher RF, CRP and ESR levels which regard as important marker for the increase the activition of the RA disease and observed that ACCP antibody with high level in the serum of RA patients versus healthy control with high significant difference[14]. Also local study by Abd[15] they indicated that both autoantibodies RF and ACPA are elevated in the majority of RA patients which used as diagnostic and management markers for the RA disease and provide information about disease activity .In a study conducted in Medical City hospital in Baghdad province by Li x[16] the RF and ACCP were significantly and higher in patients with high disease activity compared to those with moderate disease activity ACCP associated with more severe disease outcomes, including increased radiographic damage and joint erosions. [16-17] they mention that ACCP increased in RA patients and illustrated that ACPA have pathogenic role in RA by promotion of pro-inflammatory cytokine production and osteoclast genesis, leading to bone and cartilage damage.

In our work, we attempted to measure the amount of TNF-α in the blood and synovial fluid of patients with knee effusion, both RA and non-RA. Although TNF-α levels in blood were greater in RA patients than healthy persons, TNF-α levels in synovial fluid were higher in RA patients than in non-RA patients. As an issue of fact, TNF-α plays a crucial role in a typical immune response. However, abnormal or high TNF-α production can be harmful and cause illness, it is thought to be a key component in the pathogenic progression of RA [18]. RA patients had statistically significant higher serum TNF-α levels than healthy Wang Q[8] reported that TNF-α-highly increased in RA patients and related to the severity of the disease . The main function of synovial fluid act as a biological lubricant in the joint, decreasing friction between the surfaces of cartilage in the joint [19]. the amount of TNF-α is present in the synovial fluid of people with RA were twice fold as in people without this disease [20]. Elevated serum TNF-α levels, especially in RA patients with higher titer of anti-CCP and/or RF test associated with progressive of disease and may be more helpful in patients with knee effusion [21]. In study done by Hassany A et al[22] they observed an increased level of TNF-α in both blood and joint fluid of patients with rheumatoid arthritis compared to healthy controls. Similarly, a study of shin et al [23] measured the levels of TNF-α in the synovial fluid of 80 RA patients and 40 osteoarthritis patients (as controls) who reported that the TNF-α levels were significantly elevated in the RA group compared to the control group ($p<0.001$). Jiang L

et al[24] investigated the relationship between TNF-α levels and disease activity in 150 RA patients and found that patients with higher disease activity (DAS28 scores) Compared to those with lower disease activity, were those with considerably higher blood TNF-α levels ($p<0.001$).Also , Another study noted [25] that the serum and plasma levels of TNF-α were significantly higher in RA patients compared to healthy controls.

In a study reported that early stage of disease is still asymptomatic but when the levels of TNF-α elevated in correlation with elevated ACCP and RF is suggesting its involvement in the initiation of the autoimmune response [26]. Another reported finds that in patients with severe or refractory RA persistently elevated levels of TNF-α may contribute to the development of extra-articular manifestations, such as cardiovascular disease, pulmonary involvement, and osteoporosis[27]. A systematic review and meta-analysis in 2020,showed that TNF-α levels are connected with both disease severity and functional impairment and are consistently increased in individuals with established RA [28] also the acute stage of RA appears with higher level of TNF-α which stimulate an inflammatory response lead to clear pathogens and damage synovium . From our study and previous research the elevated level of TNF-α in both serum and synovial fluid in RA disease and highly elevated in serum with increased severity of disease and confirmed that measure the TNF-α level in serum considered accurate biomarker with high sensitivity and specificity and excellent AUC .

Su l et al[30] mention that MiR-155 has a detrimental and inflammatory role in RA via stimulating the production of TNF-α, IL-1B and decreasing IL-10 in peripheral blood $CD14^+$ cells. It was [31] illustrated that elevated levels of miR-155 in RA peripheral blood mononuclear cells associated with disease activity by enhanced expression of TNF-α and IL-1β. Recent research has suggested that the expression of the miRNA-155 may be altered in RA patients, and this could have implications for disease management and found that upregulation of miRNA-155 appears to be correlated with disease activity and severity in RA patients [32]. Another study reported that serum levels of miRNA-155 were significantly higher in RA patients compared to healthy controls suggesting its potential as a non-invasive biomarker for RA [33]. In study by Paoletti Aet al [34] found that miR-155 was significantly upregulated in the synovial tissue and peripheral blood mononuclear cells of RA patients and the author demonstrated that miR-155 promotes the differentiation of Th17 cells and inhibits the generation of regulatory T cells contributing to the inflammatory phenotype and concluded that miR-155 play critical roles in the pathogenesis of disease. On the same line Liao et al[35] who explain that in comparison to healthy individuals, miRNA-155 significantly increased in the synovial tissue and peripheral blood of RA patients. Circulating miRNA-155 levels have been elevated in RA and Psoriatic arthritis patients and it may reflect the underlying inflammatory and autoimmune processes.[36]

CONCLUSION

TNF-α level increased in serum and S. fluid of RA patients than healthy control group and among patients' group highly increased in aggressive group than mild group. Both TNF- α and miRNA-155 show excellent AUC with high sensitivity and specificity so may be used as a potential biomarker for RA diagnosis and monitoring disease progression. This study conclude the use of TNF-α serum level rather than S. fluid to reduce patient discomfort and stress during diagnosis and patient monitoring.

Recommendations

Examining the connection between miRNA155 gene expression and cytokines involved in the progression of Rheumatoid arthritis and recomanded more researches about new MiRNA that could act as indicators. Researching new miRNAs that could qualify as indicators for RA susceptibility

Ethical approval

The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data, the authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent, the authors have obtained the written informed consent from the patients

The corresponding author is in possession of this document.

Use of artificial intelligence for generating text, the authors declare that they have not used any type of generative artificial intelligence for the writing of this manuscript, nor for the creation of images, graphics, tables, or their corresponding captions.

Conflict of Interest

The authors declare that they have no financial support. The authors declare no conflicts of interest for research, authorship and publication of this article.

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