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# Association of circulating Treg and plasma microRNA-21 with rheumatoid arthritis progression

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## Abstract

The etiology of rheumatoid arthritis (RA) is multifaceted. One of the hypothesized pathways that results in the progression of RA is regulatory T cell (Treg) dysfunction. The pro-osteoclastogenic and immunogenic characteristics of microribonucleic acid (microRNA)-21 (miR-21) suggest its role in RA progression. Hence, we investigated the significance of plasma miR-21 and Treg cell frequency as biomarkers for RA progression and assessed the link between miR-21 and Treg frequency in RA. This study enrolled 60 RA patients classified according to disease activity score 28-joint count with erythrocyte sediment rate (DAS28-ESR) to inactive cases (n = 30) and active cases (n = 30). Flow cytometer was used to assess Treg frequency. The Real-time quantitative PCR was used to measure the expression levels of miR-21 in plasma. When compared to the inactive group, the active group revealed significant up-regulation of miR-21 expression ($p$ = 0.004) and down-regulation of Treg frequency ($p$<0.001). While Treg frequency was negatively correlated, miR-21 fold change was positively correlated with DAS-28-ESR (r = -508, $p$ < 0.001 and r = 0.334, $p$ < 0.009, respectively). No correlation was detected between mirR-21 and Treg frequency. Treg distinguished the two groups at area under the curve (AUC) of 0.907 with 86.7% sensitivity and 73.3% specificity, whereas miR-21 up-regulation discriminated active from inactive RA patients at AUC of 0.717, with 83.3% sensitivity and 53.3% specificity. In conclusion, Treg frequency and miR-21 fold were differentially linked to DAS-28-ESR in RA. MiR-21 fold up-regulation changes and Treg frequency down-regulation can be suggested as biomarkers for RA activity.

**Keywords:** microRNA-21 (miR-21), regulatory T cell (Treg), Rheumatoid arthritis (RA) activity

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## Introduction

Rheumatoid arthritis (RA) is the result of an immune response by the body's immune system targeting healthy cells. The innate and adaptive immune systems' various cells and soluble constituents actively promote the development and maintenance of inflammatory processes and structural alterations affecting the bones and articular cartilages and synovial

membrane.[1] About 0.5–1% of the world's population is impacted by RA.[2] In Egypt, during 1999 and 2004, the prevalence of RA was approximately 0.3%, with a significant proportion of cases remained undiagnosed. Also, the mean age at onset of RA patients in the country was 38 years, which was significantly lower in females.[3] RA is accompanied by pain, edema, and stiffness in several joints, and with multiple organ disorders. Joint destruction advances quickly after RA begins, leading to physical dysfunction and distortion of the affected joints that cannot be reversed.[4]

Previous studies indicated a complex interaction between genetic, environmental, and immunological variables in the pathophysiology of RA.[5-7] An imbalance between proinflammatory T. helper cell (Th) and anti-inflammatory regulatory T cell (Treg) is thought to influence RA progression.[8] Forkhead box protein P3 (Foxp3)-expressing Treg cell is crucial for preserving immune system homeostasis by suppressing effector T cell activity.[9] After years of progressive immune system remodeling, joint inflammation develops as tissue tolerance wanes, tissue-invasive effector T cells proliferate. Synovitis changes from acute to a chronic damaging condition when synovial stromal cells transform into auto aggressive effector T cells.[8] Numerous autoimmune disorders are directly correlated with Treg cell numbers or functional deficiencies.[10]

Microribonucleic acid (MicroRNA, miR) is a non-coding, single-stranded RNA sequence that is between 18 and 25 nucleotides long. It has been demonstrated to roughly regulate 30% of the protein-coding genes, with its primary role being to change gene expression through post-transcriptional changes.[11] Depending on the degree of complementarity, the mature miRs binding to a complementary sequence on messenger RNA (mRNA) will either cause the mRNA to degrade or obstruct translation of gene expression.[5]

Many miRs have different expression patterns throughout the course of a disease, making it easy to identify disease activity.[12] The ability to better understand the pathways involved in the pathogenesis of autoimmune disorders may result from the recognition of changes in the status of miRs and their corresponding targets.[13] The exceptional stability of circulating miRs in body fluids makes them ideal non-invasive biomarkers.[14]

Numerous diseases, including RA, have been linked to miRs. The generation of inflammatory cytokines and T cell differentiation was the main focus of aberrant miR expression in RA.[11] Among the several classes of miRs, miR-21 is a prevalent miR, frequently elevated in numerous disorders, and shown to be crucial for cell division, apoptosis, and invasion. There are not many articles that show how miR-21 affects arthritis yet.[15]

Treg plays an important role in RA. A previous investigation raised the possibility of the presence of a link between miR-21 and Treg/Th17 imbalance, which impacts RA progression.[16] In the present investigation, we looked into the significance of peripheral blood circulating plasma miR-21 and the frequency of Treg cells in the progression of RA. Also, we aimed to assess if there is a link between miR-21 and Treg frequency in RA.

## Patients and Methods

This prospective cross-sectional comparative study included 60 RA patients divided into 2 groups. The first group included 30 RA patients with active disease and 30 RA patients in remission. All patients were recruited from the Outpatient Rheumatology clinic and the Inpatient Rheumatology Unit, Department of Internal Medicine, AL-Zahraa Hospital of Al-Azhar University in Cairo, Egypt.

The RA patients were diagnosed using the 2010 revised American College of Rheumatology/European League against rheumatism criteria for RA [17]. Disease activity was assessed using the 28-joint disease activity score (DAS28-ESR).[18]

Patients with infectious diseases, malignant diseases, cardiovascular complaints, or other inflammatory diseases were excluded from the study.

A venous blood sample (8 ml) was collected from each study subject and divided into four aliquots. The first blood aliquot (2 ml) was collected into an EDTA tube for a complete blood count and flow cytometry for detection of Treg frequency. The complete blood count was performed using an automated hematology analyzer (Sysmex, Kobe, Japan), according to the manufacturer's instructions. The second blood aliquot (1.6 ml) was transferred into a citrate tube for erythrocyte sedimentation rate (ESR) by the Wintrobe's method. The third blood aliquot (2 ml) was transferred to a yellow-capped vacationer for serum separation which was used for estimation of C reactive protein (CRP) and rheumatoid factor (RF) while anti-cyclic citrullinated peptide (Anti-CCP), data were collected from hospital records The fourth blood aliquot (2 ml) was placed into an EDTA tube and spun for 10 minutes at 1233 xg, after which the plasma was carefully withdrawn and centrifuged again for 10 minutes at 17000 xg. The supernatant was then moved to vials and kept at -80 $^{o}$C for RNA extraction and real-time quantitative PCR (qPCR) detection of miR-21 expression level.

*RNA extraction and real-time PCR*

Using 200 μl of plasma and miRNeasy commercial kits (Cat. No. 217004, Qiagen, Hilden, Germany), we extracted mature miRs from plasma, including miR-21, according to the manufacturer' instructions. The miScript II RT commercial Kits (Cat. No. 218161, Qiagen, Hilden, Germany) were used to reverse-transcribe the extracted RNA to complementary DNA, and miR expression was determined using the miScript SYBR Green PCR kit (Cat. No. 204145, Qiagen, Hilden, Germany) on a real-time PCR machine (quaint Studio 5 system, Applied Biosystems, USA), according to the manufacturer' instructions. An internal control of miR-SNORD68 was employed with a forward primer sequence (5'-CTCGCTTCGGCAGCACA-3'). The subsequent cycling conditions included heating at 95°C for 30 minutes, followed by 40 cycles each of 15 minutes at 94°C, 30 seconds at 55°C, and 30 seconds at 70°C. Δ cycle threshold (Ct) was calculated by subtracting the Ct values of SNORD68 from the Ct values of the target miR-21 in all patient samples together with samples from 10 previously collected, frozen and stored normal plasma samples kept to determine mean of normally expressed miRs to calculate the fold change. Fold change was calculated using 2-ΔΔCt for relative quantification.

*Flowcytometry assay*

A flow cytometer assay was conducted using a flowcytometry machine (BD FACSCalibur™, Biosciences, San Jose, USA). The software (Cell Quest Pro, BD Biosciences, San Jose, USA) was used for data analysis. A compensation setting was established before acquiring the samples using color calibrite beads (lot no. 5093879, BD, Biosciences, San Jose, USA).

A total of 100 μg of the EDTA blood was incubated with 5 μg of monoclonal antibody CD4 FITC-conjugated Ab (Catalog number: AO7750. Lot number 100, Immunotech, Beckman Coulter, Marsellia, France), CD25 APC-conjugated Ab (Cat. no. FAB1020A. lot. no. LXJ0215071, R&D systems, Minneopolis, Minnesota, USA) and PE-conjugated FoxP3 (Clone: 236A/E7. Cat. no 12-4777-42. lot. no. E11467-1633, eBioescience, Europe/ International, California, USA) using Foxp3 intercellular permeabilization concentrate reagent (Cat. no. 00-5123-43. lot. number 4273423, eBioescience, Europe/International, California, USA), according to the manufacturer's instructions.

In the gating strategy for Treg frequency detection, we used dot plot forward and side scatter (FS/SS), the mature lymphocyte gate was detected (R1). Another graph was taken for detection of CD4+ expressing T lymphocyte gate (R2). Then a quadrant plot was drawn from R1 and R2 representing CD25-APC on Y axes and FoxP3-PE on X axes. The gate of co-expression of CD25 surface marker and the intracellular PE-conjugated FoxP3 was considered as Treg frequency in the sample in the upper right quadrant referred to by blue arrows (Figure 1).

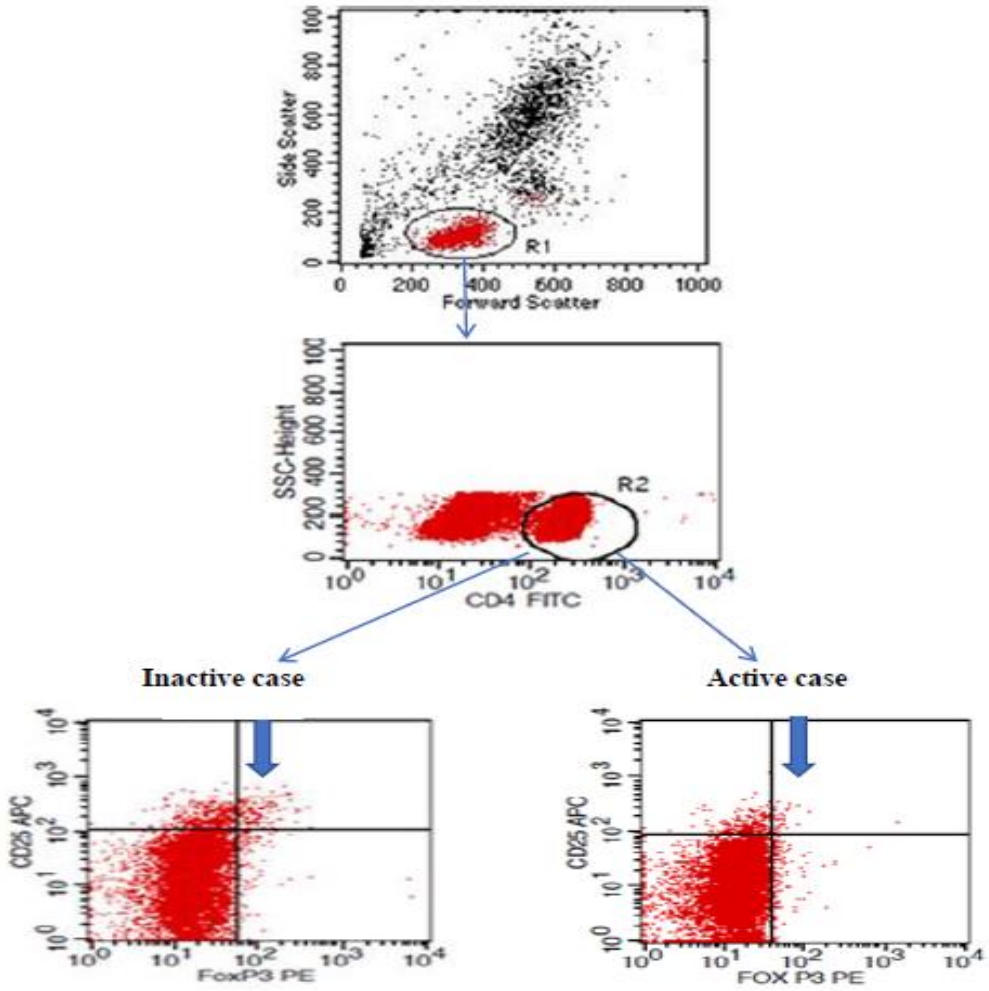


**Figure 1.** Gating strategy for Treg frequency.

*Statistical Analysis*

Data were coded and entered using the statistical package for the social sciences (SPSS) version 28 (IBM Corp., Armonk, NY, USA). Data were summarized using the mean and standard deviation (SD) for normally distributed quantitative variables, or median and interquartile range for non-normally distributed quantitative variables, and frequencies (number of cases) and relative frequencies (percentages) for categorical variables. For comparing categorical data, the Chi-square ($X^2$) test was performed. An exact test was used instead when the expected frequency was less than 5. Correlations between quantitative variables were done using the Spearman correlation coefficient. A receiver operating characteristic (ROC) curve was constructed with area under the curve analysis performed to detect the best cutoff value of significant markers for the detection of activity. A $p$ value ≤0.05 was considered statistically significant.

## Results

Demographic and clinical data of the study cases are shown in Table 1. When compared to the inactive group, the active group revealed a significant up-regulation of miR-21 ($p$=0.004) and down regulation of Treg percentage ($p$< 0.001), (Table 2).

**Table 1.** Comparison of demographic data, clinical data, and laboratory findings among the 60 rheumatoid arthritis (RA) patients.

| Studied parameters | Studied subjects | | *p*-value |
|---|---|---|---|
| | Inactive group | Active group | |
| Age /years (Mean ±SD) | 43.10 ±10.29 | 44.20±9.26 | NS |
| Sex | | | |
| Females no (%) | 28 (93.3%) | 27 (90.0 %) | NS |
| Males no (%) | 2 (6.7) | 3 (10 %) | |
| Disease duration /year | 6.50 | 5 .00 | NS |
| Median ($1^{st}$ -$3^{rd}$ quartile | (3.00-10.00) | (3.00-8.00) | |
| NLR Median | 1.23 | 8.58 | < 0.001 |
| ($1^{st}$ -$3^{rd}$ quartile) | (0.96-1.71) | (6.44 12.04) | |
| PLR Median | 100.00 | 250.50 | NS |
| ($1^{st}$ -$3^{rd}$ quartile) | (69.82 -196.36) | (182.00-316.00) | |
| ESR mm/hour | | | |
| Median | 29.00 | 35.00 | NS |
| ($1^{st}$ -$3^{rd}$ quartile) | (15.00-55.00) | (28.00-65.00) | |
| CRP (mg/l) | 5.00 | 13.00 | < 0.001 |
| Median ($1^{st}$ -$3^{rd}$ quartile) | (3.80-6.00) | (11.00-22.00) | |
| Anti CCP (u/ml) | 114.00 | 120.00 | NS |
| Median ($1^{st}$ -$3^{rd}$ quartile) | (111.00-132.00) | (112.00-153.00) | |
| Rheumatoid factor (Iu/ ml) | 44.00 | 63.50 | < 0.001 |
| Median ($1^{st}$ -$3^{rd}$ quartile | (35.00-55.00) | (54.00-69.00) | |

Data are expressed as median (interquartile range). Anti-CCP: Anti-cyclic citrullinated peptide; CRP: C reactive protein; DAS28-ESR: Disease Activity Score in 28 joints-erythrocyte sedimentation rates; ESR: Erythrocyte sedimentation rate; miR: micro-RNA; NLR: Neutrophil lymphocyte ratio; PLR: platelet lymphocyte ratio; TLC: total leukocyte count; Treg: regulatory T lymphocyte. $p > 0.05$ is not significant (NS).

**Table 2.** Comparison of fold change of miR-21 and Treg frequency between the 30 active and 30 inactive rheumatoid arthritis (RA) patients.

| | Inactive RA group | | | Active RA group | | | |
|---|---|---|---|---|---|---|---|
| | Median | $1^{st}$ quartile | $3^{rd}$ quartile | Median | $1^{st}$ quartile | $3^{rd}$ quartile | *p* value |
| Treg % | 3.40 | 2.60 | 5.10 | 1.30 | 0.78 | 2.10 | < 0.001 |
| miR-21 (fold change) | 4.03 | 0.14 | 34.49 | 26.73 | 10.25 | 301.92 | 0.004 |

MiR; micro-RNA; Treg: regulatory T lymphocyte. $p \leq 0.05$ is significant.

Treg percentage was negatively correlated with the DAS28-ESR score, rheumatoid factor (RF), and neutrophil lymphocyte ratio (NLR) (r = -0.508, $p < 0.001$; r = -0.434, $p = 0.001$ and r = -0.634, $p < 0.001$, respectively) (Table 3, Figure 2).

The miR-21 fold change was positively correlated with DAS28-ESR score, RF, and NLR (r = -0.334, $p = 0.009$; r = -0.4, $p = 0.002$; and r = -0.336, $p = 0.009$) (Table 3). No correlation was detected between mirR-21 and Treg frequency.

**Table 3.** Correlation between fold change of miR-21 and Treg frequency with the clinical, biochemical data in the 60 rheumatoid arthritis (RA) cases.

| | | Treg % | miR-21 (fold change) |
|---|---|---|---|
| miR-21 (fold change) | Correlation Coefficient | -0.194 | ------ |
| | *p* value | NS | ------ |
| Age/ year | Correlation Coefficient | -0.024 | 0.105 |
| | *p* value | NS | 0.423 |
| Disease Duration/ year | Correlation Coefficient | 0.005 | 0.040 |
| | *p* value | NS | 0.761 |
| ESR mm/hour | Correlation Coefficient | -0.181 | 0.139 |
| | *p* value | NS | 0.290 |
| TLC ($10^3/\mu$) | Correlation Coefficient | -0.122 | -0.026 |
| | *p* value | NS | 0.846 |
| NLR | Correlation Coefficient | -0.634 | 0.336 |
| | *p* value | < 0.001 | 0.009 |
| PLR | Correlation Coefficient | -0.028 | 0.243 |
| | *p* value | NS | 0.061 |
| CRP (mg/l) | Correlation Coefficient | -0.557 | 0.281 |
| | *p* value | < 0.001 | 0.030 |
| Rheumatoid factor (Iu/ ml) | Correlation Coefficient | -0.352 | 0.400 |
| | *p* value | 0.006 | 0.002 |
| DAS28-ESR Score | Correlation Coefficient | -0.508 | 0.334 |
| | *p* value | < 0.001 | 0.009 |

Anti-CCP: Anti-cyclic citrullinated peptide; CRP: C reactive protein; DAS28-ESR: Disease Activity Score in 28 joints-erythrocyte sedimentation rates; ESR: Erythrocytic sedimentation rate; miR: micro-RNA; NLR: Neutrophil lymphocyte ratio; PLR: platelet lymphocyte ratio; TLC: total leukocyte count; Treg: regulatory T lymphocyte. $p > 0.05$ is not significant (NS).

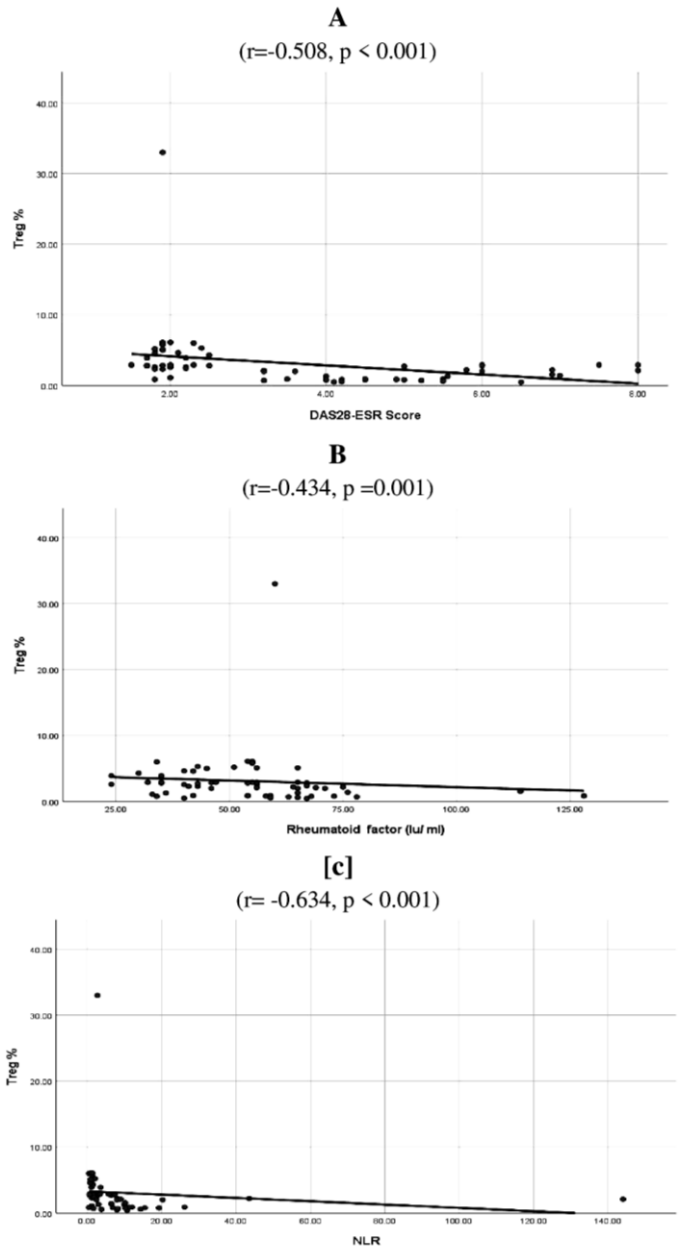


**Figure 2.** Treg frequency correlation with DAS-28ESR, Rheumatoid factor (RF) and neutrophil lymphocyte ratio (NLR) [A] Treg negatively correlated with DAS-28ESR [B] Treg negatively correlated with Rheumatoid factor (RF). [C] Treg negatively correlated with neutrophil lymphocyte ratio (NLR).

The ROC curve analysis regarding the discriminative power of circulating Treg percentage and miR-21 level of expression revealed that miR-21 can be used as a biomarker of severity. Such that miR-21 can distinguish between active and inactive RA patients at a cutoff of 5.44-fold change with an area under the curve (AUC) of 0.71, with 83.3% sensitivity, and 53.3% specificity. While the Treg percentage at a cutoff of 2.74 with an AUC of 0.91 yielded 86.7% sensitivity and 73.7% specificity (Table 4, Figure 3).

**Table 4.** Receiver operating characteristic (ROC) curve for Treg %, miR-21 as discriminators of active and inactive rheumatoid arthritis (RA) disease.

| | Area Under the Curve | *p* value | 95% Confidence Interval | | Cut off | Sensitivity % | Specificity % |
|---|---|---|---|---|---|---|---|
| | | | Lower Bound | Upper Bound | | | |
| Treg % | 0.907 | < 0.001 | 0.832 | 0.981 | 2.74 | 86.7 | 73.3 |
| miR-21 (fold change) | 0.717 | 0.001 | 0.588 | 0.847 | 5.4382 | 83.3 | 53.3 |

MiR; micro-RNA; Treg, regulatory T lymphocyte. $p \leq 0.05$ is significant.

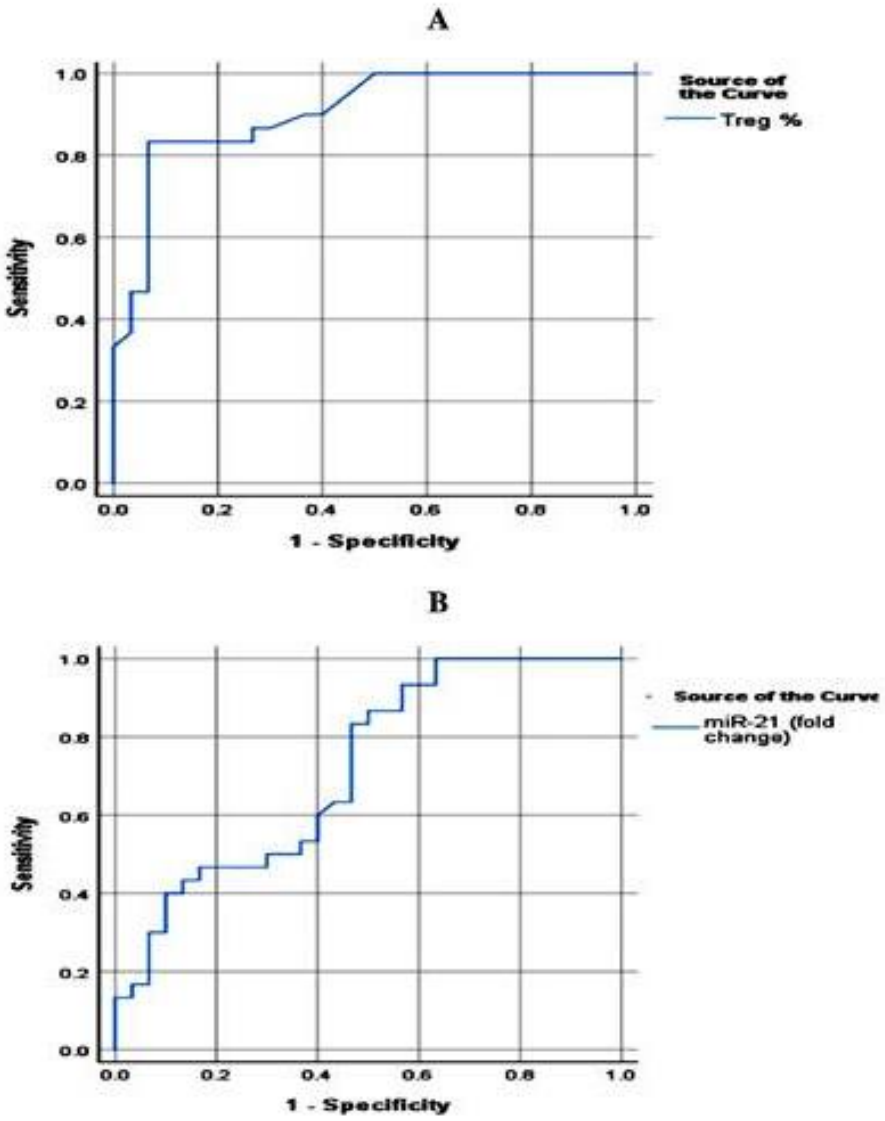


**Figure 3.** Receiver operating characteristic (ROC) curve of discriminative capacity of [A] Treg [B] miR-21 to differentiate between active and inactive rheumatoid arthritis groups.

## Discussion

Despite the complexity of RA pathophysiology, Tregs and miRs are receiving more attention for their crucial contributions to understanding how the illness develops.[19] Consequently, in the present study we investigated the significance of peripheral blood circulating plasma miR-21 and the frequency of Treg cells in the progression of RA. We also assessed whether there is a link between miR-21 and Treg frequency in RA.

In the current investigation, when we compared the active and inactive RA cases, Treg frequency was considerably lower in the active cases ($p$< 0.001). Our results concurred with those of the study by Kotschenreuther et al., 2022,[10] which emphasized contribution of decreased Treg cell frequencies and functions to the pathophysiology of RA. The study by Kotschenreuther et al., 2022, also observed a deviated differentiation of T helper cells in RA towards Th17 rather than suppressive Treg. The study by Wang et al., 2023,[20] documented that RA progression was due to proliferation of pro-inflammatory Th1 and Th17 and observed that therapeutic administration of suppressive Tregs shows tremendous promise in RA therapy.

Additionally, the study by Biniecka et al., 2010,[21] found that there was a higher proportion of Tregs in the synovial fluid of RA patients when compared to healthy controls. Such finding indicates an interesting contrast to a significant reduction in Treg frequency in the peripheral blood of patients with active RA when compared to RA patients in remission. Moreover, the study by Zafari et al., 2018,[22] found that RA patients have lower levels of overall FoxP3 gene expression as well as the regulatory T cell-specific demethylated region compared to healthy controls. The study by Weyand & Goronzy, 2021,[8] explained that the decline in T cell tolerance resulted from poor DNA repair, which also leads to aberrant cell cycle dynamics, telomere fragility, and mitochondrial DNA instability

According to our findings, active RA patients had considerably higher levels of miR-21 expression than the inactive group. Our data supported the conclusions made by the study of Xiong et al., 2016,[23] who added that miR-21 suppression, is accomplished through the transforming growth factor-1 (TGF-1)/Smad-4/7 signaling pathway, can suppress the invasiveness of fibroblast-like synoviocyte cells by down-regulating the production of the matrix metalloproteinase (MMP)-1, MMP-3, and MMP-13. Also, the study by Dong et al., 2014,[24] found that RA patients have considerably lower levels of miR-21 and play a role in the imbalance of Th17 and Treg cells.

On the contrary, the study by Liu et al., 2019,[25] reported that miR-21 decrees cause pro-inflammatory action in RA and added that miR-21 elevation can repress interlukin-6 (IL-6) and IL-8 expressions and improve the symptoms of RA by down-regulating the Wnt signal. In order to relive RA, the study by Li et al., 2021,[11] showed that miR-21 from bone marrow mesenchymal stem cells decreases Kruppel-like factor-4 by targeting the ten-eleven translocation methylcytosine dioxygenase-1, a member of the DNA demethylase family that controls the expression of many RA-related genes.

In the current study, a significant negative correlation was detected between Treg and the DAS28-ESR score (r =-0.508, $p$< 0.001). Also, Treg was negatively correlated with RF and NLR (r = -0.434, $p$ = 0.001 and r = -0.634, $p$ < 0.001, respectively). These findings support the observation by the study of Scheinecker et al., 2020,[26] which asserted that the collapse of immunological tolerance is a distinguishing feature of the inflammatory process in RA, and Treg cell decline in number represents one of the routes that were described.

It is important to note that, in our study, Foxp3 was the marker of identification to identify Treg, despite the fact that new technical developments have led to a new understanding of the heterogeneity of multiple Treg cell populations using the epigenetic signature of Treg cells.[26]

In the present study, MiR-21 was positively correlated with the DAS28-ESR score, RF, and NLR (r =-0.334, $p$< 0.009; r =-0.4, $p$ = 0.002; and r = -0.336, $p$ = 0.009, respectively). This agrees with findings of the study by Yang et al., 2021,[27] who documented that miR-21 corresponds with

RA disease activity. Also, the study by Churov et al., 2015, [28] proved that miR-21 is elevated in the plasma of RA patients, and one of the alluring non-invasive indicators that can be used for the diagnosis of RA. This could be explained by the findings of the study by Sekar, 2021,[12] who reported that miR-21 was strongly expressed in osteoclast precursors and that pro-osteoclastogenic properties of miR-21 make it a potential therapeutic target to treat RA. Also, the study by Yang et al., 2016,[29] demonstrated that the miR-21 pathway is essential for the survival of synovial fibroblasts in RA patients.

In the current investigation mirR-21 did not correlate with Treg. This disagreed with the findings of our previous study that miR-21 was correlated to Th17/Treg balance [6]. This may be explained by the smaller sample. The study by Yao et al., 2015,[30] found a connection between the amount of miR-21 expression and the Treg/Th17 ratio in hepatocellular cancer. The study by Sun et al., 2021, [31] reported that miR-21 did not control the ability of Tregs to suppress the immune system. They added that after Treg-specific deletion of miR-21, there was no difference in the progression of autoimmune disorders. However, the study by Dong et al., 2014,[24] study showed that miR-21 is linked to altered frequencies of Treg subset individually in RA patients. Furthermore, according to the study by Huang et al., 2021,[32] miR-21 causes Th17 cell differentiation by inhibiting Smad-7. A competitively binding inhibitory Smad protein called Smad-7 prevents the development of the Smad-2/3 complex and inhibits the interaction of Smad-4 with TGF-β1 receptor.

On the other hand, the study by Li et al., 2015,[33] reported that in peripheral blood mononuclear cells from patients with atherosclerosis, miR-21 can negatively influence the frequency of circulating Treg cells through a TGF-β1/ Smad-independent signaling pathway. The notion that each miR has multiple mRNA targets that can participate in various processes, including immune responses, cellular differentiation, cellular proliferation, metabolism, homeostasis, and apoptosis. These suggest that miR-21 may have a role in Treg other than differentiation and helps to explain why there is no correlation between Treg frequency and miR-21.[34]

Both Treg frequency and plasma miR-21 can be used as biomarkers of activity to distinguish between active and inactive RA patients. We observed that miR-21-fold change at cutoff = 5.44-fold change with AUC = 0.71, showed 83.3% sensitivity, and 53.3% specificity as determined by ROC curve analysis. However, the Treg percentage showed more discriminative ability between both groups at cutoff = 2.74% with AUC = 0.91, which yielded 86.7% sensitivity and 73.7% specificity, as shown in Table 4 and Figure 4. This observed high sensitivity with Treg percentage in detecting active cases which can be explained by the nature of Treg function reported by the study of Takeuchi et al., 2020,[35] emphasizing that granulocyte-macrophage, colony-stimulating factor-secreting effector of Th17 cells are activated by inadequate Treg suppression, which evokes arthritis activity. Myeloid cells, innate lymphoid cells, and synoviocytes in the joint are also activated, mediating chronic bone-destructive joint inflammation. As proposed by the study of Yao et al., 2022,[36] these open the doors for therapies depending on correction of the imbalance of Th17/Treg cells to alleviate joint inflammation and bone destruction in RA patients.

The current study limitations included that due to funding constraints, the study did not look into the function of Treg cell and its secreted cytokines. Moreover, the musculoskeletal ultrasonography joint score was not examined.

In conclusion, in RA patients, the miR-21-fold change and DAS-28-ESR had a positive correlation, while the frequency of Treg had a negative correlation. Up-regulation and down-regulation of the miR-21-fold in RA patients, and Treg frequency can be employed as sensitive indicators of RA activity. There was no correlation found between the number of Tregs in RA and the mirR-21 expression level. Despite the complexity of RA pathophysiology, Tregs and miRs are receiving more attention for their crucial contributions to understanding how the illness develops [19]. Consequently, in the present

study we investigated the significance of peripheral blood circulating plasma miR-21 and the frequency of Treg cells in the progression of RA. We also assessed whether there is a link between miR-21 and Treg frequency in RA.

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In the current study, a significant negative correlation was detected between Treg and the DAS28-ESR score ($r =-0.508$, $p< 0.001$). Also, Treg was negatively correlated with RF and NLR ($r = -0.434$, $p = 0.001$ and $r = -0.634$, $p < 0.001$, respectively). These findings support the observation by the study of Scheinecker et al., 2020,[26] which asserted that the collapse of immunological tolerance is a distinguishing feature of the inflammatory process in RA, and Treg cell decline in number represents one of the routes that were described.

It is important to note that, in our study, Foxp3 was the marker of identification to identify Treg, despite the fact that new technical developments have led to a new understanding of the heterogeneity of multiple Treg cell populations using the epigenetic signature of Treg cells.[26]

In the present study, MiR-21 was positively correlated with the DAS28-ESR score, RF, and NLR ($r =-0.334$, $p< 0.009$; $r =-0.4$, $p = 0.002$; and $r = -0.336$, $p = 0.009$, respectively). This agrees with findings of the study by Yang et al., 2021,[27] who documented that miR-21 corresponds with RA disease activity. Also, the study by Churov et al., 2015, [28] proved that miR-21 is elevated in the plasma of RA patients, and one of the alluring non-invasive indicators that can be used for the diagnosis of RA. This could be explained by the findings of the study by Sekar, 2021,[12] who reported that miR-21 was strongly

expressed in osteoclast precursors and that pro-osteoclastogenic properties of miR-21 make it a potential therapeutic target to treat RA. Also, the study by Yang et al., 2016,[29] demonstrated that the miR-21 pathway is essential for the survival of synovial fibroblasts in RA patients.

In the current investigation mirR-21 did not correlate with Treg. This disagreed with the findings of our previous study that miR-21 was correlated to Th17/Treg balance [6]. This may be explained by the smaller sample. The study by Yao et al., 2015, [30] found a connection between the amount of miR-21 expression and the Treg/Th17 ratio in hepatocellular cancer. The study by Sun et al., 2021,[31] reported that miR-21 did not control the ability of Tregs to suppress the immune system. They added that after Treg-specific deletion of miR-21, there was no difference in the progression of autoimmune disorders. However, the study by Dong et al., 2014,[24] study showed that miR-21 is linked to altered frequencies of Treg subset individually in RA patients. Furthermore, according to the study by Huang et al., 2021,[32] miR-21 causes Th17 cell differentiation by inhibiting Smad-7. A competitively binding inhibitory Smad protein called Smad-7 prevents the development of the Smad-2/3 complex and inhibits the interaction of Smad-4 with TGF-β receptor I.

On the other hand, the study by Li et al., 2015,[33] reported that in peripheral blood mononuclear cells from patients with atherosclerosis, miR-21 can negatively influence the frequency of circulating Treg cells through a TGF-1/ Smad-independent signaling pathway. The notion that each miR has multiple mRNA targets that can participate in various processes, including immune responses, cellular differentiation, cellular proliferation, metabolism, homeostasis, and apoptosis. These suggest that miR-21 may have a role in Treg other than differentiation and helps to explain why there is no correlation between Treg frequency and miR-21.[34]

Both Treg frequency and plasma miR-21 can be used as biomarkers of activity to distinguish between active and inactive RA patients. We observed that miR-21-fold change at cutoff = 5.44-fold change with AUC = 0.71, showed 83.3% sensitivity, and 53.3% specificity as determined by ROC curve analysis. However, the Treg percentage showed more discriminative ability between both groups at cutoff = 2.74% with AUC = 0.91, which yielded 86.7% sensitivity and 73.7% specificity, as shown in Table 4 and Figure 4. This observed high sensitivity with Treg percentage in detecting active cases which can be explained by the nature of Treg function reported by the study of Takeuchi et al., 2020,[35] emphasizing that granulocyte-macrophage, colony-stimulating factor-secreting effector of Th17 cells are activated by inadequate Treg suppression, which evokes arthritis activity. Myeloid cells, innate lymphoid cells, and synoviocytes in the joint are also activated, mediating chronic bone-destructive joint inflammation. As proposed by the study of Yao et al., 2022,[36] these open the doors for therapies depending on correction of the imbalance of Th17/Treg cells to alleviate joint inflammation and bone destruction in RA patients.

The current study limitations included that due to funding constraints, the study did not look into the function of Treg cell and its secreted cytokines. Moreover, the musculoskeletal ultrasonography joint score was not examined.

In conclusion, in RA patients, the miR-21-fold change and DAS-28-ESR had a positive correlation, while the frequency of Treg had a negative correlation. Up-regulation and down-regulation of the miR-21-fold in RA patients, and Treg frequency can be employed as sensitive indicators of RA activity. There was no correlation found between the number of Tregs in RA and the mirR-21 expression level.

## Author Contributions

MG, performed the publication process and helped in paper writing; OA, provided the whole supervision of the work; SSA, performed the sample collection and the clinical data curation; AE, revised the paper and performed the English editing; RH, wrote the protocol, performed sample processing, practical methdodlogy by Flow cytometry and the paper writing.

## Declaration of Conflicting Interests

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## Ethical approval

The protocol of the study was reviewed and approved by the Research Ethics Committee of the Faculty of Medicine, Al-Azhar University (Study No. 958, approval dated August 2021).

## Informed consent

A written informed consent was obtained from each study participant before being included in the study.

## References

1. D'Orazio A, Cirillo AL, Greco G., et al. (2024). Pathogenesis of rheumatoid arthritis: one year in review 2024. Clin Exp Rheumatol. 42(9):1707-1713. doi: 10.55563/clinexprheumatol/0307ed.

2. Jiang Y, Zhong S, He S., et al. (2023). Biomarkers (mRNAs and non-coding RNAs) for the diagnosis and prognosis of rheumatoid arthritis. *Front Immunol.* 14:1087925. doi: 10.3389/fimmu.1087925.

3. Usenbo A, Kramer V, Young T, Musekiwa A. (2015). Prevalence of Arthritis in Africa: A Systematic Review and Meta-Analysis. PLoS One. 10 (8): e0133858. doi: 10.1371/journal.pone.0133858.

4. Yao X, Wang Q, Chen C., et al. (2022). External Treatment Improves Th17/Treg Balance in Rheumatoid Arthritis. *Evid Based Complement Alternat Med*. 2022:5105545 doi: 10.1155/2022/5105545

5. Renman E, Brink M, Ärlestig L,. et al. (2021). Dysregulated microRNA expression in rheumatoid arthritis families-a comparison between rheumatoid arthritis patients, their first-degree relatives, and healthy controls. *Clin Rheumatol.* 40(6), 2387-2394.

6. Hammad R, Eldosoky MA, Lambert C., et al. (2024). Hsa-miR-21-5p reflects synovitis and tenosynovitis components of musculoskeletal ultrasonography Seven-joint scores in rheumatoid arthritis disease and predicts the disease flare. Pathol Res Pract. 253:154960. doi: 10.1016/j.prp.2023.154960.

7. Eldosoky M.A.E.R., Hammad R.H.M., Kotb H.G., Eldesoky N.A.R., Elabd H.A. (2018). Cell Free DNA and CD38 Expression on T helper Cells as Biomarkers of Disease Activity in Rheumatoid Arthritis. Am. J. Biochem. 8:60–68. doi:10.5923/j.ajb.20180803.03

8. Weyand CM, Goronzy JJ. (2021). The immunology of rheumatoid arthritis. *Nat Immunol*. 22(1), 10-18

9. Pereira, L. M. S., Gomes, S. T. M., Ishak, R., et al., (2017). Regulatory T Cell and Forkhead Box Protein 3 as Modulators of Immune Homeostasis. *Frontiers in immunology* 8:605. doi.10.3389/fimmu.2017.00605

10. Kotschenreuther K, Yan S, Kofler DM. (2022). Migration and homeostasis of regulatory T cells in rheumatoid arthritis. *Front Immunol*. 13:947636. doi: 10.3389/fimmu.2022.947636

11. Li, G.Q., Fang, Y.X, Liu, Y.,.et al. .(2021). MicroRNA-21 from bone marrow mesenchymal stem cell-derived extracellular vesicles target TET1 to suppress KLF4 and alleviate rheumatoid arthritis. *Ther Adv Chronic Dis*.; 12:20406223211007369. doi: 10.1177/20406223211007369

12. Bonek K, Kuca Warnawin E, Kornatka A, et al. (2022). Circulating miRNA Correlates with Lipid Profile and Disease Activity in Psoriatic Arthritis, Rheumatoid Arthritis, and Ankylosing Spondylitis Patients. *Biomedicines*. 10(4):893. doi: 10.3390/biomedicines10040893

13. Tavasolian F, Abdollahi E, Rezaei R., et al. (2018). Altered Expression of MicroRNAs in Rheumatoid Arthritis. *J Cell Biochem*. 119(1), 478-487

14. Hammad R, Eldosoky MA, Elmadbouly AA, et al. (2023). Evaluation of circulating miR-16-5p and miR-223-5p in association with musculoskeletal ultrasonography seven-joint score in the assessment of rheumatoid arthritis activity. *Egypt J Immunol*. 30(1),20-30

15. Sekar D. (2021). Implications of microRNA 21 and its involvement in the treatment of different types of arthritis. *Mol Cell Biochem.* 476(2),941-947.

16. Jin S, Chen H, Li Y., et al., (2018). Maresin 1 improves the Treg/Th17 imbalance in rheumatoid arthritis through miR-21. *Ann Rheum Dis*. 77(11),1644-1652

17. Aletaha D, Neogi T, Silman AJ, et al. (2010). Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheum.* 62(9),2569-81

18. Hirabayashi Y, Ishii T; (2013). Michinoku Tocilizumab Study Group. The DAS28-ESR cutoff value necessary to achieve remission under the new Boolean-based remission criteria in patients receiving tocilizumab. *Clin. Rheumatol*. 32(1), 123-7

19. Myasoedova E, Davis JM, Achenbach SJ, et al. (2018). OP0009 rising prevalence of functional disability in patients with rheumatoid arthritis over 20 years. *Ann Rheumatic Dis.* 77(Suppl 2):54–4. doi: 10.1136/annrheumdis- 2018-eular.3788

20. Wang L, Wang Y, Liu C, et al. (2023). Treg-targeted efficient-inducible platform for collagen-induced arthritis treatment. *Mater Today Bio.* 19:100557. doi: 10.1016/j.mtbio.2023.100557

21. Biniecka M, Kennedy A, Fearon U, et al. (2010). Oxidative damage in synovial tissue is associated with in vivo hypoxic status in the arthritic joint. *Ann Rheumatic Dis.* 69(6), 1172–8

22. Zafari P, Yari K, Mostafaei S, et al., (2018). Analysis of Helios gene expression and Foxp3 TSDR methylation in the newly diagnosed rheumatoid arthritis patients. *Immunol. Invest.* 47(6), 632–42

23. Xiong G, Huang Z, Jiang H,et al. (2016). Inhibition of microRNA-21 decreases the invasiveness of fibroblast-like synoviocytes in rheumatoid arthritis via TGFβ/Smads signaling pathway. *Iran J. Basic Med. Sci.* 19(7), 787-93

24. Dong L, Wang X, Tan J, et al., ( 2014). Decreased expression of microRNA-21 correlates with the imbalance of Th17 and Treg cells in patients with rheumatoid arthritis. *J. Cell Mol. Med*. 18(11), 2213-24

25. Liu XG, Zhang Y, Ju WF, et al. (2019). MiR-21 relieves rheumatoid arthritis in rats via targeting Wnt signaling pathway. *Eur. Rev*. *Med. Pharmacol. Sci*. 23(3 Suppl), 96-103

26. Scheinecker C, Göschl L, Bonelli M. (2020). Treg cells in health and autoimmune diseases: New insights from single cell analysis. *J. Autoimmun*. 110:102376. doi: 10.1016/j.jaut.2019.102376

27. Yang J, Wang S, Liu L, et al. (2021). Long non-coding RNA NEAT1 and its targets (microRNA-21 and microRNA-125a) in rheumatoid arthritis: Altered expression and potential to monitor disease activity and treatment outcome*. J. Clin. Lab. Anal.* 35(12), e24076. doi: 10.1002/jcla.24076 .

28. Churov AV, Oleinik EK, Knip M. (2015). MicroRNAs in rheumatoid arthritis: altered expression and diagnostic potential. *Autoimmun Rev*. 14(11), 1029-37

29. Yang S, Jiang S, Wang Y, et al. (2016). Interleukin 34 Upregulation Contributes to the Increment of MicroRNA 21 Expression through STAT3 Activation Associated with Disease Activity in Rheumatoid Arthritis. *J. Rheumato.* 43(7), 1312-9

30. Yao SX, Zhang GS, Cao HX, et al. (2015). Correlation between microRNA-21 and expression of Th17 and Treg cells in microenvironment of rats with hepatocellular carcinoma. *Asian Pac. J. Trop. Med*. 28(9), 762-5

31. Sun J, Liu R, He X, et al. (2021). MicroRNA-21 Regulates Diametrically Opposed Biological Functions of Regulatory T Cells. *Front. Immunol*. 12:766757. doi: 10.3389/fimmu.2021.766757.

32. Huang J, Xu X, Yang J., (2021). miRs Alter T Helper 17 Cell Fate in the Pathogenesis of Autoimmune Diseases. *Front. Immunol.* 12:593473. doi: 10.3389/fimmu.2021.593473

33. Li S, Fan Q, He S, et al., (2015). MicroRNA-21 negatively regulates Treg cells through a TGF-β1/Smad-independent pathway in patients with coronary heart disease*. Cell Physiol. Biochem*. 37(3), 866-78

34. Chang L, Yao H, Yao Z, et al. (2021). Comprehensive Analysis of Key Genes, Signaling Pathways and miRNAs in Human Knee Osteoarthritis: Based on Bioinformatics. *Front. Pharmacol.* 12:730587. doi: 10.3389/fphar.2021.730587.

35. Takeuchi Y, Hirota K, Sakaguchi S. (2020). Impaired T cell receptor signaling and development of T cell-mediated autoimmune arthritis. *Immunol Rev*. 294(1), 164-176

36. Yao X, Wang Q, Chen C, et al., (2022). External Treatment Improves Th17/Treg Balance in Rheumatoid Arthritis. *Evid. Based Complement Alternat Med*. 2022:5105545. doi: 10.1155/2022/5105545.