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CIRCULATING MIR-21 AND SERUM INTERLEUKIN-22 AS CANDIDATE BIOMARKERS IN RHEUMATOID ARTHRITIS: A CASE–CONTROL STUDY

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Abstract: The prevalence of rheumatoid arthritis (RA) is steadily increasing worldwide, and a subset of patients who test negative for rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP) antibodies cannot be reliably diagnosed using traditional serological markers. MicroRNA-21 (miR-21) and interleukin-22 (IL-22) have each been implicated in RA pathogenesis, but their combined behavior in an unselected clinical cohort is not well characterized. This study aims to quantify serum IL-22 and whole-blood miR-21 expression in patients with RA compared with healthy controls, and to examine their relationship with RF and anti-CCP serostatus. This case–control study enrolled 50 patients with RA diagnosed by treating rheumatologists across hospitals and clinics in Najaf, Iraq, and 40 age- and sex-comparable healthy controls. RF was assessed by latex agglutination; anti-CCP and IL-22 were measured by enzyme-linked immunosorbent assay (ELISA); miR-21 expression was quantified by stem-loop reverse-transcription quantitative PCR (RT-qPCR) with U6 small nuclear RNA as the reference gene, using the $2^{-}\Delta\Delta Ct$ method. The results showed that Serum IL-22 was significantly higher in RA patients than controls (53.2 ± 10.3 vs 27.5 ± 4.2 pg/mL; $p < 0.001$), whereas whole-blood miR-21 fold change was significantly lower in RA patients than controls (0.43 ± 0.16 vs 1.12 ± 0.54; $p < 0.001$). IL-22 and miR-21 were not significantly correlated within the RA group ($r = 0.12$, $p = 0.39$). ROC analysis showed AUC = 0.965 (95% CI 0.918–1.000) for IL-22 and AUC = 0.938 (95% CI 0.888–0.988) for miR-21 in discriminating RA from controls. Anti-CCP–positive RA patients had higher IL-22 and miR-21 levels than anti-CCP–negative patients. In conclusion, elevated serum IL-22 in RA is consistent with prior reports implicating the Th22/IL-22 axis in synovial inflammation. In contrast, the reduction in whole-blood miR-21 diverges from most previous studies reporting miR-21 upregulation in RA plasma, serum, or synovial tissue, and likely reflects differences in sample matrix (whole blood vs plasma/serum/synovium) and reference-gene normalization. This discordance should be explicitly investigated in future work rather than assumed away.

Keywords: Rheumatoid arthritis, Biomarker, Interleukin-22, MicroRNA-21, Anti-CCP antibody.

1. INTRODUCTION

Rheumatoid arthritis (RA) is a persistent, systemic inflammatory disorder marked by bilateral synovitis, advancing joint deterioration, and, in many individuals, extra-articular symptoms that diminish quality of life and elevate mortality risk [1]. Analyses of the Global Burden of Disease indicate that the prevalence of rheumatoid arthritis increased by around 14% from 1990 to 2020, with projections suggesting an additional 80% rise in prevalent cases by 2050, mostly due to population growth and ageing [2]. This escalating burden, together with geographical and age-related disparities in incidence and disability-adjusted life-years, highlights the ongoing need for indicators that facilitate earlier and more precise diagnosis [3,4,5].

Rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP) antibodies are fundamental serological markers for diagnosing rheumatoid arthritis (RA). Anti-CCP is often noted to possess more specificity than RF, with specificity ranging from 90% to 96% compared to RF's 70% to 85%, while both assays exhibit only modest sensitivity, failing to detect a significant number of genuine RA patients, especially in early or seronegative instances. This diagnostic deficiency has spurred an extensive quest for supplementary, non-invasive molecular biomarkers, with circulating microRNAs (miRNAs) and cytokines garnering significant recent focus [8,9].

MicroRNAs are short, non-coding RNAs that modulate gene expression post-transcriptionally and exhibit remarkable stability in blood and other biological fluids, making them appealing potential biomarkers for inflammatory and neoplastic conditions [12]. In rheumatoid arthritis (RA), several circulating miRNAs, such as miR-16, miR-146a, miR-155, miR-223, and miR-21, have been identified as dysregulated in peripheral blood, plasma, or synovial tissue, with most studies indicating that miR-21 upregulation correlates with disease activity and the severity of synovitis. miR-21 has been shown, at the tissue level, to affect the apoptosis, proliferation, and invasiveness of fibroblast-like synoviocytes (FLS), suggesting its role as an active mechanistic factor in synovial hyperplasia rather than just a passive indicator [13]. Nonetheless, the documented fold changes for miR-21 exhibit significant variability across studies, which has been associated in other disease contexts with discrepancies in sample origin (plasma vs serum vs whole blood vs tissue) and, importantly, with the selection of endogenous reference genes for normalisation [14,15].

Interleukin-22 (IL-22) serves as the primary effector cytokine for the T-helper 22 (Th22) fraction and transmits signals via the IL-22 receptor, found on synovial fibroblasts as well as several epithelial and stromal cell types [16,17]. Increased levels of IL-22 have been correlated with the activity of rheumatoid arthritis (RA) and have been mechanistically connected to the proliferation of fibroblast-like synoviocytes and RANKL-mediated osteoclastogenesis, establishing a biologically credible pathway from IL-22 elevation to the synovial hyperplasia and bone erosion typical of RA [16,17,18].

Despite substantial separate literatures on miR-21 and IL-22 in RA, comparatively few studies have examined both markers together in the same cohort, and fewer still have done so in Iraqi patients. The present study was therefore designed to (i) quantify serum IL-22 and whole-blood miR-21 expression in a cohort of RA patients recruited across multiple hospitals and clinics in Najaf, Iraq, compared with healthy

controls; (ii) examine the relationship of both markers with RF and anti-CCP serostatus; and (iii) assess whether IL-22 and miR-21 correlate with one another, which would be consistent with a shared regulatory pathway.

2. MATERIALS AND METHODS

2.1 STUDY DESIGN AND SETTING

This cross-sectional case control study conducted from July 2025 to December 2025 among patients attending, Najaf City, Teaching Hospitals, and a private rheumatology clinics.

2.2 STUDY PARTICIPANTS

Fifty patients with RA were enrolled after diagnosis by treating rheumatologists on the basis of clinical evaluation and standard diagnostic criteria. Forty apparently healthy, age- and sex-comparable individuals with no personal or family history of autoimmune disease and negative RF/anti-CCP serology served as controls.

2.3 SAMPLE COLLECTION

Venous blood was collected from each participant after informed consent. Whole blood (EDTA anticoagulant) was used for RNA extraction and miR-21 quantification. A separate serum sample, obtained after clotting and centrifugation, was used for RF, anti-CCP, and IL-22 measurement, aliquoted, and stored at −80 °C until assay, avoiding repeated freeze–thaw cycles.

2.4 RHEUMATOID FACTOR TESTING

RF was determined qualitatively by slide latex agglutination (RF-Latex, SPINREACT, Spain), in which latex particles coated with human gamma-globulin agglutinate in the presence of RF at concentrations ≥ 8 IU/mL. Positive and negative controls were run with each batch according to manufacturer's protocol.

2.5 ANTI-CCP ANTIBODY TESTING

Anti-CCP IgG was measured by a quantitative reverse-phase sandwich ELISA (Human Anti-Cyclic Citrullinated Peptide Antibody ELISA Kit, BT Lab), following the manufacturer's protocol: samples and controls were incubated on antigen-precoated plates, followed by HRP-conjugated detection antibody, TMB substrate development, and optical density (OD) measurement at 450 nm. Results were interpreted against the kit cutoff (mean OD of the negative control + 0.15); samples below the cutoff were classified negative and those at or above the cutoff, positive.

2.6 SERUM IL-22 MEASUREMENT

Serum IL-22 concentration was measured using a double-antibody sandwich ELISA (Human Interleukin-22 ELISA Kit, Melsin Medical Co., Ltd), with an assay range of 0–320 pg/mL and a minimum detectable dose below 1.0 pg/mL, per the manufacturer's specification. Standards and samples were assayed in duplicate; optical density was read at 450 nm and concentrations were interpolated from the standard curve, correcting for the 5-fold sample dilution specified in the kit protocol.

2.7 RNA EXTRACTION

Total RNA was extracted from whole blood using the TransZol Up Plus RNA Kit (TransGen Biotech), which combines guanidinium-phenol lysis with silica-membrane spin-column purification. RNA concentration and purity (A260/A280) were assessed by spectrophotometry prior to reverse transcription.

2.8 REVERSE TRANSCRIPTION: STEM-LOOP cDNA SYNTHESIS

Total RNA from each sample was reverse-transcribed to cDNA in a single reaction using a pooled stem-loop reverse-transcription (RT) primer mix containing both the miR-21-specific and U6-specific stem-loop primers simultaneously, so that cDNA templates for both targets were generated together from the same RNA input. The stem-loop primers (Table 1) were used as the gene-specific primer (GSP) input for the EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech), following the manufacturer's GSP-primed synthesis protocol. The resulting cDNA was used as template for both the miR-21 and U6 real-time PCR reactions described below.

2.9 PRIMERS USED FOR REVERSE TRANSCRIPTION AND REAL-TIME PCR

Stem-loop RT primer sequences and real-time PCR primer sequences (Table 1) were adapted from a previously validated stem-loop RT-qPCR design for miR-21 and U6 [19].

TABLE 1. Stem-loop reverse-transcription primers and real-time PCR primers for miR-21 and U6.

Target	Primer role	Sequence (5'→3')
U6 snRNA	Stem-loop RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAAATATGG
miR-21	Stem-loop RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAACA
Universal	qPCR reverse primer (shared)	ATCCAGTGCAGGGTCCGAGG
U6 snRNA	qPCR forward primer	GCGGCGGCGCAAGGATGACACG
miR-21	qPCR forward primer	GCGGCGGTAGCTTATCAGACTG

2.10 REAL-TIME QUANTITATIVE PCR

Real-time PCR was performed separately for miR-21 and U6 from the same pooled cDNA, using AccuPower 2X GreenStar qPCR Master Mix (Bioneer Corporation), a ready-to-use SYBR Green-based (dsDNA-binding fluorescent dye) master mix, with the corresponding target-specific forward primer paired with the shared universal reverse primer (Table 1) in each reaction. Reactions were run per the manufacturer's recommended two-step protocol (pre-denaturation, 95 °C; denaturation, 95 °C; combined annealing/extension, 60 °C; 45 cycles), with melting-curve analysis performed on every run to confirm single-product specificity and exclude primer-dimer artifacts.

2.11 RELATIVE QUANTIFICATION

Relative miR-21 expression was calculated using the $2^{-}\Delta\Delta Ct$ method [22], with U6 small nuclear RNA as the endogenous reference gene and the mean control-group Ct values as the calibrator. Fold-change values below 1 indicate relative downregulation compared with the reference condition.

2.12 STATISTICAL ANALYSIS

Statistical analysis was performed using IBM SPSS Statistics version 29.0. Continuous variables were expressed as mean ± standard deviation and median. Levene's test, independent-samples t-test, and Mann–Whitney U test were used for group comparisons as appropriate. Categorical variables were analyzed using the chi-square test. Associations between IL-22 and miR-21 were assessed using Pearson's and Spearman's correlation tests. ROC curve analysis was performed to evaluate the diagnostic performance of serum IL-22 and whole-blood miR-21, with the area under the curve (AUC), 95% confidence interval, sensitivity, specificity, and cutoff values determined. A two-tailed p-value < 0.05 was considered statistically significant.

3. RESULTS

3.1 BASELINE CHARACTERISTICS

Fifty patients with RA (36 female, 14 male; mean age 46.6 ± 12.1 years) and 40 healthy controls (26 female, 14 male; mean age 45.8 ± 10.6 years) were enrolled. Age and sex distribution did not differ significantly between groups (age: $p = 0.73$; sex: χ^2 $p = 0.63$), indicating the two groups were reasonably comparable on these variables (Table 2). Within the RA group, 41/50 (82%) were RF-positive and 46/50 (92%) were anti-CCP-positive; all controls were RF- and anti-CCP-negative by design.

TABLE 2: Baseline demographic and serological characteristics of RA patients and controls.

Characteristic	RA (n = 50)	Control (n = 40)	p-value
Age, years (mean ± SD)	46.6 ± 12.1	45.8 ± 10.6	0.73
Female, n (%)	36 (72%)	26 (65%)	0.63
Male, n (%)	14 (28%)	14 (35%)	—
RF-positive, n (%)	41 (82%)	0 (0%)	—
Anti-CCP-positive, n (%)	46 (92%)	0 (0%)	—

3.2 SERUM IL-22 CONCENTRATION

Serum IL-22 was significantly higher in RA patients than in controls (53.2 ± 10.3 pg/mL vs 27.5 ± 4.2 pg/mL; median 51.5 vs 27.5 pg/mL). Levene's test indicated unequal variances ($F = 21.97$, $p < 0.001$), so the Welch (equal variances not assumed) result is reported: $t(67.4) = 16.09$, $p < 0.001$, mean difference 25.76 pg/mL (95% CI 22.56–28.95). The Mann-Whitney U test confirmed the difference ($U = 2000.0$, $p < 0.001$), representing an approximately 1.9-fold elevation in RA (Table 2).

3.3 WHOLE-BLOOD MIR-21 EXPRESSION

Whole-blood miR-21 fold change was significantly lower in RA patients than in controls (0.43 ± 0.16 vs 1.12 ± 0.54; median 0.37 vs 0.95) (Figure 1). Levene's test again indicated unequal variances ($F = 38.04$, $p < 0.001$); Welch's $t(44.6) = -7.79$, $p < 0.001$, mean difference −0.68 (95% CI −0.86 to −0.51). The

Mann-Whitney U test confirmed the difference (U = 124.0, p < 0.001), corresponding to an approximately 2.6-fold relative reduction in RA (Table 3).

Table 3: Serum IL-22, whole-blood miR-21 fold change, and anti-CCP OD in RA patients versus controls. Welch's t-test reported given significant Levene's test for equality of variances in all three comparisons; degrees of freedom (df) adjusted accordingly.

Marker	RA (n = 50) Mean ± SD (Median)	Control (n = 40) Mean ± SD (Median)	Levene's p	t (df)	p-value
IL-22 (pg/mL)	53.2 ± 10.3 (51.5)	27.5 ± 4.2 (27.5)	< 0.001	16.09 (67.4)	< 0.001
miR-21 ($2^{-}\Delta\Delta Ct$ fold change)	0.43 ± 0.16 (0.37)	1.12 ± 0.54 (0.95)	< 0.001	−7.79 (44.6)	< 0.001
Anti-CCP (OD450)	0.69 ± 0.34 (0.64)	0.10 ± 0.04 (0.10)	< 0.001	12.22 (50.4)	< 0.001

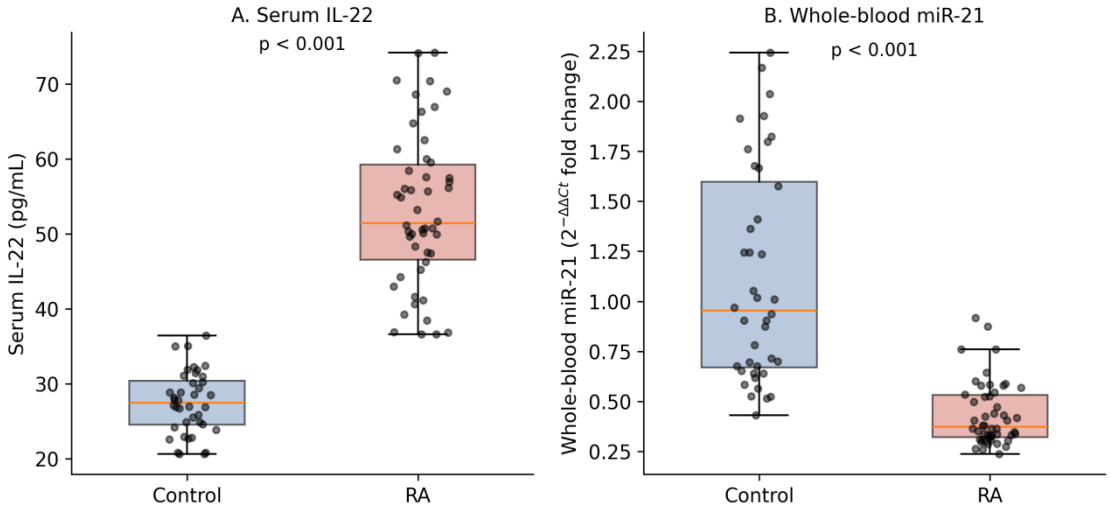


Figure 1: Distribution of (A) serum IL-22 and (B) whole-blood miR-21 fold change in RA patients versus healthy controls. Boxes show median and interquartile range; individual data points are overlaid.

3.4 ASSOCIATION WITH RF AND ANTI-CCP SEROSTATUS WITHIN RA

Within the RA group, RF-positive patients (n = 41) showed numerically higher IL-22 (54.5 ± 10.0 vs 47.5 ± 10.2 pg/mL) and miR-21 (0.45 ± 0.16 vs 0.37 ± 0.15) than RF-negative patients (n = 9), but neither difference reached statistical significance (IL-22 p = 0.086; miR-21 p = 0.19), likely reflecting the small RF-negative subgroup. Anti-CCP-positive patients (n = 46) had significantly higher IL-22 (54.6 ± 9.5 vs 37.1 ± 0.9 pg/mL; p < 0.001) and significantly higher miR-21 (0.44 ± 0.16 vs 0.33 ± 0.02; p < 0.001) than anti-CCP-negative patients (n = 4), although the anti-CCP-negative subgroup was very small and this comparison should be interpreted cautiously.

3.5 CORRELATION BETWEEN IL-22 AND MIR-21

In the RA cohort, IL-22 and miR-21 exhibited no significant correlation (Pearson r = 0.125, p = 0.388; Spearman ρ = 0.199, p = 0.165), suggesting that their measured circulating levels were not strongly coupled under the conditions examined (Figure 2). The regression slope was small and not significantly different from zero (β = 0.0019, p = 0.388), and the 95% confidence band around the fitted line was wide relative to its slope, consistent with the weak, non-significant association.

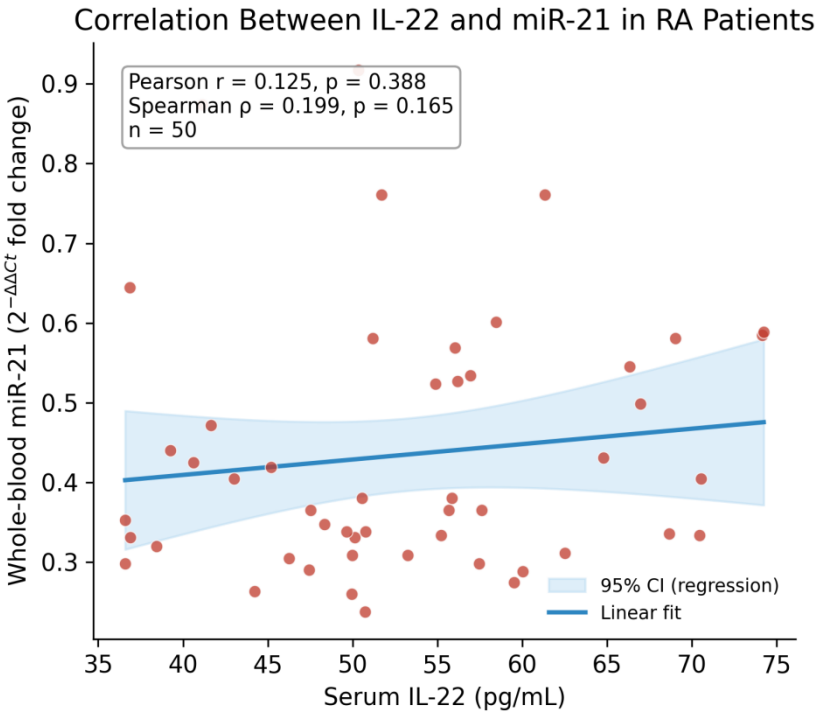


Figure 2: Scatter plot of serum IL-22 versus whole-blood miR-21 fold change in RA patients (n = 50), with linear regression line and 95% confidence band. No significant correlation was observed.

3.6 DISCRIMINATORY PERFORMANCE: ROC CURVE ANALYSIS

ROC curve analysis was performed to evaluate the ability of serum IL-22 and whole-blood miR-21 to discriminate RA patients from healthy controls (Figure 3). Serum IL-22 showed an AUC of 0.965 (95% CI 0.918–1.000); at the Youden-optimal cutoff of 36.6 pg/mL, sensitivity and specificity were (93.3%, 96.7%) respectively. Whole-blood miR-21 showed an AUC of 0.938 (95% CI 0.888–0.988); at the Youden-optimal cutoff of 0.601-fold, sensitivity was 90.0% and specificity was 85.0% (Table 4).

TABLE 4: ROC curve diagnostic performance of IL-22 and miR-21 for discriminating RA from healthy controls.

Marker	AUC (95% CI)	cutoff	Sensitivity	Specificity
Serum IL-22 (pg/mL)	0.965 (0.918–1.000)	> 36.6	93.3%	96.7%
Whole-blood miR-21 (fold change)	0.938 (0.888–0.988)	< 0.601	90.0%	85.0%

*AUC = area under the curve.

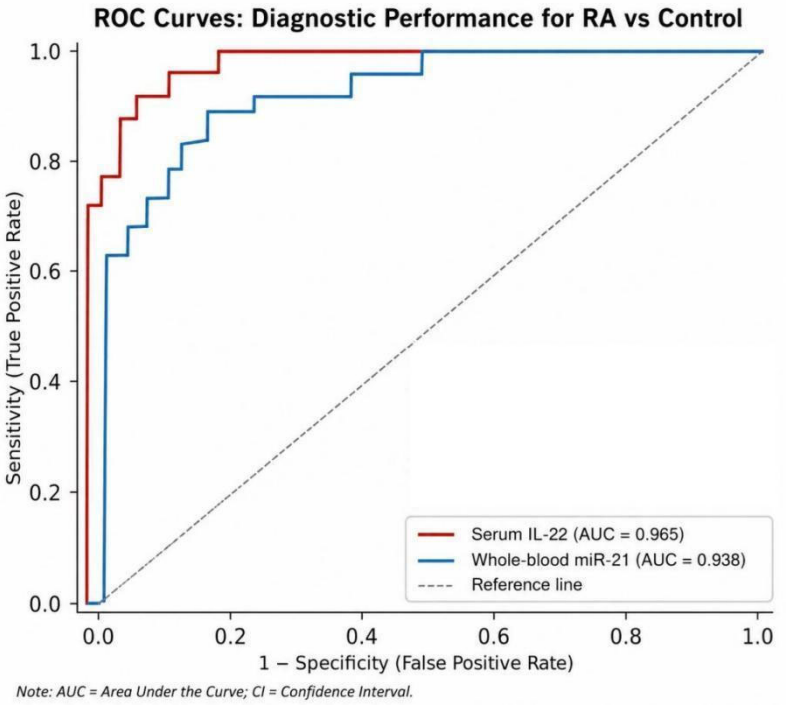


Figure 3: Receiver operating characteristic curves for serum IL-22 (red) and whole-blood miR-21 (blue) in discriminating RA patients and healthy controls.

4. DISCUSSION

This study found significantly elevated serum IL-22 and significantly reduced whole-blood miR-21 expression in RA patients compared with healthy controls, with no significant correlation between the two markers and a positive association between anti-CCP seropositivity and both marker levels.

The IL-22 finding is consistent with a substantial body of prior work implicating the Th22/IL-22 axis in RA pathogenesis [20]. IL-22 receptor is expressed on synovial fibroblasts, and IL-22 has been shown to promote fibroblast-like synoviocyte proliferation and RANKL-driven osteoclastogenesis, providing a plausible mechanistic link between elevated IL-22 and the synovial hyperplasia and bone erosion characteristic of RA [16,17,18]. Our 1.9-fold rise in RA serum IL-22 is within, if not below, the range of elevations reported elsewhere and supports IL-22 as a consistent characteristic of active RA across groups.

Interpreting miR-21 takes additional attention. Most investigations in plasma, serum, or synovial fluid/tissue reveal miR-21 overexpression in RA, implicating it in fibroblast-like synoviocyte apoptosis resistance, proliferation, and invasiveness [10,11,13,14]. Our whole-blood result, which decreased significantly, deviates from this trend. First, sample matrix matters: whole blood comprises all circulating leukocyte populations, whereas most RA miR-21 research employed plasma or serum. Cell-type-specific miRNA expression may create opposing net signals depending on sample compartment. Second, the choice of endogenous reference gene for normalisation can shift both the magnitude and direction of a reported fold change. Since the current research normalised to U6 alone, a different, more stable reference gene may provide a different result. This should be addressed directly in follow-up work utilising methods like NormFinder or geNorm across numerous possible reference genes. Third, disease duration, treatment status (DMARD or biologic exposure), and disease activity were not systematically captured in this cohort, but these variables have been shown to modulate circulating miRNA levels. Without these

variables, we cannot determine whether the discordant direction reflects a treated, more chronic population or an untreated, early-disease population like some prior cohorts.

Both biomarkers have good ROC curve discrimination. Isolating RA patients from healthy controls was very accurate with serum IL-22 and whole-blood miR-21 AUCs of 0.965 and 0.938, respectively.serum IL-22 showed a numerically higher AUC than whole-blood miR-21 in this dataset. IL-22 levels are higher in RA patients and correlated with disease activity and radiographic progression, suggesting its potential value as a biomarker of RA-associated inflammatory activity [20]. Recent ROC analysis has shown that circulating/exosomal miRNAs may diagnose RA, supporting the use of miRNA-based biomarkers for RA discrimination [21]. Biomarker performance varies by sample type, population, illness stage, and treatment status, hence the high AUC values in this research should be confirmed in larger independent cohorts.

The lack of a significant correlation between IL-22 and miR-21 suggests that IL-22 acts predominantly through the Th22/STAT3/RANKL axis in synovial tissue, while miR-21's role in whole blood may be more reflective of circulating immune cell composition than local synovial activity. The stronger association of both markers with anti-CCP positivity than RF positivity is consistent with anti-CCP's superiority as a serological correlate of RA-associated immune activation [6,7,8], but the small anti-CCP-negative subgroup (n = 4) limits this comparison.

This study has several limitations that should guide interpretation and future replication. It is cross-sectional and cannot establish temporal or causal relationships between IL-22, miR-21, and disease onset or progression. Disease activity (e.g., DAS28), disease duration, and current treatment were not incorporated into the analysis, precluding adjustment for these established confounders. The RF-negative and anti-CCP-negative RA subgroups were small, limiting the power of seronegative-specific comparisons. miR-21 was measured in whole blood rather than in isolated cell populations, plasma, or synovial tissue, which as discussed above likely contributes to the divergence from prior literature and should be explicitly tested through parallel matrix comparisons in future studies. Finally, normalization relied on a single reference gene (U6); multi-reference-gene normalization strategies would strengthen confidence in the reported fold changes.

5. CONCLUSION

In this cohort of RA patients from Najaf, Iraq, serum IL-22 was significantly elevated and whole-blood miR-21 was significantly reduced relative to healthy controls, with anti-CCP positivity associated with higher levels of both markers. The IL-22 finding is consistent with previous reports associating increased IL-22 with RA related inflammation. The miR-21 finding diverges from most prior reports of miR-21 upregulation in RA and should not be treated as confirmatory of a downregulation model; rather, it highlights the importance of sample matrix and reference-gene selection in circulating miRNA studies and warrants replication using multiple sample types (whole blood, plasma, and, where feasible, synovial tissue) and multiple candidate reference genes before either direction of change is treated as established.

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