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# Immunoregulatory Role of miRNA-26 in Modulating IL-32, IL-33, IL-34, and MMP-9 Expression in Rheumatoid Arthritis

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**Abstract**

Rheumatoid arthritis (RA) is a systemic autoimmune illness marked by abnormal cytokine expression and persistent inflammation. MicroRNAs (miRNAs), particularly miRNA-26, have been implicated in the regulation of inflammatory pathways and show promise as potential biomarkers for RA diagnosis and treatment monitoring. Aim: This study aimed to evaluate miR-26 expression about established clinical markers and pro-inflammatory cytokines, to identify potential predictive markers of treatment response and contribute to more personalized management strategies in RA. Method: 180 participants were enrolled and categorized into three groups: Newly diagnosed RA patients (NDRA), biologically treated RA patients (TRA), and Healthy controls (HC). Each group consisted of 60 Individuals. The miR-26 expression levels were quantified in blood specimens using quantitative real-time PCR (qRT-PCR). Additionally, serum concentrations of interleukins (IL-32, IL-33, IL-34) and matrix metalloproteinase-9 (MMP-9) were quantified using sandwich enzyme-linked immunosorbent assay (ELISA) techniques. Results: Both NDRA and TRA groups exhibited elevated miR-26 expression and significantly increased serum levels of IL-32, IL-33, IL-34, and MMP-9 relative to healthy controls ($p < 0.001$). The NDRA group showed the highest cytokine and MMP-9 levels, indicating active inflammation, while the TRA group had moderately reduced levels and a greater fold increase in miR-26 expression. IL-33 and MMP-9 demonstrated the most pronounced intergroup differences, suggesting their utility as early diagnostic biomarkers. The reciprocal relationship between miR-26 and inflammatory cytokines indicates a regulatory function of it. MiR-26 expression could be downregulated during peak of inflammation and partially restored with biotherapy treatment Conclusion: IL-33 and MMP-9 came out as prospective early diagnostic markers. The negative correlation between miR-26 and inflammatory mediators points to a regulatory, anti-inflammatory role for miR-26, which may be partially restored with treatment. These findings support the clinical utility of miR-26 and related cytokines in RA diagnosis, disease monitoring, and therapeutic targeting.

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## 1. Introduction.

Rheumatoid arthritis (RA) is a chronic autoimmune condition defined by an inflammation of the synovium, joint erosion, and systemic immune dysregulation. This disease is associated with overexpression of several immune mediators such as C-reactive protein (CRP), rheumatoid factor (RF), erythrocyte sedimentation rate (ESR), and various inflammatory cytokines [1-3]. Pro-inflammatory cytokines contribute to immune cell infiltration into the synovium, stimulate the activation of synovial fibroblasts and macrophages, drive synovial

membrane proliferation, and induce acute-phase protein synthesis, and support lymphocyte survival and differentiation. [4,5]. Interleukin-32 (IL-32) is identified as an inflammatory cytokine secreted by T cells, natural killer cells, monocytes, synovial fibroblasts, and epithelial cells. IL-32 contributes to the pathogenesis of several chronic immune-mediated disorders, including RA, by intensifying inflammation and promoting joint destruction [6,7]. IL-32 amplifies the secretion of key mediators that participate in the pathophysiology of RA. It acts synergistically with tumor necrosis factor (TNF) to establish a positive feedback loop that intensifies inflammatory responses. Additionally, IL-32 induces the secretion of matrix metalloproteinases MMP-1 and MMP-9, which contribute to cartilage degradation, and stimulates the activation of synovial fibroblasts, thereby promoting pannus formation and joint erosion [8]. Interleukin-33 (IL-33), a member of the IL-1 cytokine family, also has a significant impact on RA pathophysiology [9]. Elevated IL-33 levels have been linked to increased disease activity, as indicated by clinical markers such as the Disease Activity Score 28 (DAS-28), CRP, and ACCP antibodies. High concentrations of IL-33 have been observed in the serum and articular fluid of RA individuals. IL-33 mediates its effects through interaction with the ST2 receptor expressed on multiple immune cells, leading to the secretion of inflammatory-related mediators, upregulation of MMPs that degrade cartilage, and stimulation of osteoclast genesis contributing to joint destruction and bone erosion [10]. Interleukin-34 (IL-34) is another cytokine linked to the pathogenesis of autoimmune conditions, including RA. It modulates T-cell responses by promoting the proliferation of Th17 cells and enhancing the secretion of IL-17 [11]. IL-34 promotes proliferation of synovial tissue by induction of FLS expansion and inhibiting their apoptosis, stimulates production of VEGF and HIF-1α, [12]. Increased concentration of IL-34 has been related to increased disease activity and serves as a potential indicator of RA progression [13]. Matrix metalloproteinase-9 (MMP-9) is an endopeptidase produced by synovial fibroblasts and neutrophils, vital for RA immune-pathogenesis [14]. It promotes the degradation of collagen and gelatin, immune cell migration, and joint damage [15].

The miRNAs, a class of small non-coding RNAs, are emerging as markers for the identification and regulation of RA, because of their crucial effect in the regulation of inflammatory pathways [16]. The miRNAs modulate gene expression and influence both innate and specific immune responses, including antibody production and the secretion of inflammatory cytokines [17]. Dysregulated expression of many miRNAs has been associated with RA progression, via modulating inflammatory key signals like TLRs and NF-κB, and cytokine production [18]. miR-26 plays complicated and antagonistic roles in autoimmune and inflammatory conditions. In RA, upregulated expression miR-26 significantly effects the behavior of FLS, as well as inflammatory signaling, cytokine production, invasion, and resistance to apoptosis [19, 20]. The anti-inflammatory functions of miR-26b-5p suggest its candidate as medication target for RA [21]. This research aims to investigate the immunomodulatory effect of miRNA-26 in regulating levels of key inflammatory mediators IL-32, IL-33, IL-34, and MMP-9 in RA patients. Moreover, it seeks to assess the potential of miRNA-26 as a predictive indicator of disease activity and therapeutic response in RA.

## 2. Materials and Methods.

### 2.1. Study population

This case-control study was carried out between June 1 and December 31, 2024, included 180 participants of both sexes recruited according to the inclusion criteria. The cohort categorized into three groups: newly diagnosed RA (NDRA) patients, biologically treated RA patients (TRA), and healthy controls (HC). Each group consisted of 60 individuals, with balanced distributions of age and sex across the categories. Patients were referred to the Rheumatology Unit at Al-Yarmouk Teaching Hospital. The severity of RA was assessed by a specialist rheumatologist using the DAS28 method. Individuals with RA who had concurrent malignancies, acute or chronic infections, or other autoimmune conditions were omitted from the study. Documented informed consent was gathered from all participants. Ethical approval for the research was permitted by the Ethics Committee of the Iraqi Ministry of Health (Approval No. 384, dated May 30, 2024).

### 2.2. Sample Collection

Blood samples were drawn via intravenous access from all participants. A volume of 250 µL was kept in Trizol at -80°C for RNA extraction, and 2 mL blood was transferred to an EDTA tube for complete blood count (CBC) and measuring ESR. The residual sample was transported to a gel tube for serum separation following centrifugation at 5000 rpm for 15 minutes, intended for the assessment of serum levels of total Vitamin D, RF, CRP, ACCP, MMP9 and Interleukins (IL-32, IL-33, IL-34).

### 2.3. miRNAs Isolation

Total RNA, including miRNAs, was extracted using a combined Trizol and silica membrane purification method. The quantity and quality of the RNA were estimated using a Nanodrop ND-2000, with all samples showing integrity ratios below 2.3. To prepare mature miRNAs, polyadenylation was performed using poly(A) polymerase (Tinzyme, China). cDNA synthesis was then carried out using a 5x RT kit (Tinzyme, China).

### 2.4. Quantitative Real-Time PCR (qRT-PCR) of miR-26

The expression levels of miR-26 were determined using the Luna SYBR Green Kit (Biolabs) using primers designed by http://www.srnaprimerdb.com/ and the sequence for mature miRNA obtained from https://www.mirbase.org/, the primer sequence for miR-26a was forward 5mir-26a-1 F AACACGTGTTCAAGTAATCCAGGAT-3, reverse 5-CR CAGTGCAGGGTCCGAGGT-3 -3 and the housekeeping was U6 F 5-GTGCTCGCTTCGGCAGCA-3, U6 R 5-CAAAATATGGAACGCTTC-3. A 20 µL reaction mixture, containing Luna master mix, specific primers, and cDNA, was subjected to qRT-PCR on an Applied Biosystems 7500 system. The cycling conditions were 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds. Melting curve analysis confirmed product specificity, and data were assessed using SDS Relative Quantification Software 2.3.

### 2.5. Routine Diagnosis

A small quantity of blood sample was analyzed using an automated hematology analyzer (Mindray BC-700 Series, China) to determine WBC count and measure ESR. Serum levels of total Vitamin D, CRP, RF were assessed using a Cobas analyzer (Cobas c311, Roche, Germany).

### 2.6. Immune Assay

The serum levels of immunological indicators were estimated using enzyme-linked immunosorbent assay (ELISA) kits. These included ACCP (catalog numbers: MBS1601013, MyBiosource, British Columbia), IL-32, IL-33, IL-34, and MMP9 (E-EL-H0216, E-EL-H2402, E-EL-H1650, and E-EL-H6075, respectively; ElabScience, USA).

### 2.7. Statistical Analysis

Statistical software was used for data analysis, with dplyr, ggpubr, and ggplot2 packages for visualization. Relative quantification (2^ΔΔCT method) was employed to determine miR-26 expression changes. Categorical variables were described by number and frequency, and significant differences were identified using Pearson's Chi-square test. The Shapiro-Wilk test was used to assess the normality of continuous data. The results indicated that these variables did not follow a normal distribution and were instead presented as median and interquartile range (IQR: 25–27%). Significant differences between variables were evaluated using the Kruskal-Walli's test (for comparisons involving more than two grou-ps) or the Mann-Whitney test (for comparisons involving two groups). The area under the curve (AUC) was estimated using receiver operating characteristic (ROC) curve analysis. For each pair of cytokines, the correlation coefficient (rs) was determined using Spearman's rank correlation analysis. Statistical analyses were conducted using GraphPad Prism version 9.2.0 (Boston, MA, USA) and IBM SPSS Statistics 25.0 (Armonk, NY: IBM Corp)

## 3. Results and Discussion

The study cohort was divided into three distinct groups: newly diagnosed rheumatoid arthritis patients (NDRA), treated RA patients (TRA), and healthy controls (HC), with 60 individuals in each group. Table 1 presents the baseline features of the investigated groups. The data for continuous variables are demonstrated as medians (interquartile range, IQR), and qualitative parameters are presented as counts (percentages). The median age for NDRA patients was 48.5 years (IQR: 31.0–63.0), and for TRA patients, it was 49.0 years (IQR: 34.0–54.0). Healthy controls had a median age of 45.0 years (IQR: 39.0–56.0). Depending on age, no detectable differences were observed between the three groups ($p = 0.526$). The sex of the NDRA patients, 48 (80%) females and 12 (20%) males. In the TRA group, 44 (73.3%) were females, and 16 (26.7%) were males. For the HC, 48 (80%) were females, and 12 (20%) were males. No marked differences in sex distribution between the groups. The DAS28 for NDRA patients was 5.75 (IQR: 5.0–8.1), and for TRA patients, it was 4.5 (IQR: 3.5–5.9). WBC × $10^9$/L: NDRA patients had a median WBC of 11.85 (IQR: 10.9–13.7), while TRA patients had a lower median WBC of 8.4 (IQR: 6.1–11.9). Healthy controls had a median WBC of 7.0 (IQR: 6.4–7.9). The differences in WBC between the groups were statistically significant ($p = 0.013$). ESR mm/h: the median ESR for NDRA patients was 66.0 (IQR: 45.0–75.0), while TRA patients had a median ESR of 31.0 (IQR: 26.5–35.0). Healthy controls had a much lower median ESR of 15.0 (IQR: 15.0–17.0). The differences in ESR between the groups were highly significant ($p$

< 0.001). The median CRP for NDRA patients was 58.0 mg/L (IQR: 47.0–68.3), and for TRA patients, it was 25.3 mg/L (IQR: 21.0–32.0). The HC group had a median CRP of 4.0 (IQR: 3.0–5.0). The differences in CRP levels were highly significant ($p < 0.001$). ACCP U/mL: The median ACCP for NDRA patients was 87.4 (IQR: 80.0–115.0), and for TRA patients, it was 45.3 (IQR: 33.4–52.0). RF IU/mL: NDRA patients had a median RF of 56.0 (IQR: 48.0–62.0), and TRA patients had a median RF of 28.0 (IQR: 23.0–34.0). Healthy controls had a median RF of 10.0 (IQR: 10.0–15.0). The differences in RF levels were highly significant ($p < 0.001$). Vitamin D (ng/mL): The median Vitamin D level for NDRA patients was 15.2 (IQR: 10.7–21.4), and for TRA patients, it was 19.5 (IQR: 14.0–22.4). Healthy controls had a much higher median Vitamin D level of 35.0 (IQR: 33.0–37.0). The differences in Vitamin D levels were highly significant ($p < 0.001$).

Results in Table 2 demonstrate the concentrations of IL-32, IL-33, IL-34, and MMP9 in the serum of the studied groups. The concentrations of these immunological markers were analyzed using the Mann-Whitney U test to assess the significance of differences between medians. The results are expressed as median (interquartile range, IQR), and p-values are reported for each comparison.IL-32 (pg/mL): Newly diagnosed RA patients showed significantly higher levels of IL-32 (137.4 pg/mL; IQR: 124.7–143.7) compared to both treated RA patients (85.9 pg/mL; IQR: 78.4–97.1) and healthy controls (58.37 pg/mL; IQR: 53.3–67.97), with a $p$-value of < 0.001. This indicates a remarkable elevation of IL-32 in NDRA patients, suggesting it could play a role in early-stage RA. Similarly, serum level of IL-33(pg/mL) was significantly elevated in NDRA patients (115.0 pg/mL; IQR: 85.9–127.8) compared to both the TRA patients (60.7 pg/mL; IQR: 47.9–69.5) and HC (43.2 pg/mL; IQR: 38.85–48.07), with a $p$-value of < 0.001. The elevated IL-33 levels suggest an inflammatory response that is particularly heightened in ND RA.IL-34 (pg/mL) levels were significantly high in NDRA patients (135.4 pg/mL; IQR: 123.7–140.0) compared to TRA patients (90.0 pg/mL; IQR: 85.9–97.5) and HC (57.17 pg/mL; IQR: 54.62–59.43), with a $p$-value of < 0.001. This further emphasizes the heightened immune response in early RA, which might correlate with disease activity. The concentration of MMP9(ng/mL) was also significantly elevated in NDRA patients (11.4 ng/mL; IQR: 10.0–12.2) compared to TRA patients (7.32 ng/mL; IQR: 6.14–7.65) and HC (5.45 ng/mL; IQR: 4.56–5.57), with a $p$-value of < 0.001. MMP9, an enzyme involved in tissue remodeling, could be indicative of ongoing joint damage in RA, particularly in newly diagnosed cases. The data from Table 2 highlight significant differences in the serum levels of IL-32, IL-33, IL-34, and MMP9 between newly diagnosed RA patients, treated RA patients, and healthy controls. These outcomes indicate that IL-32, IL-33, IL-34, and MMP9 may be crucial markers for assessment of disease activity and could be potential targets for therapeutic intervention in RA, specifically in the incipient phase of the disease.

ROC curve results, as shown in Figure (1) were used to appraise the diagnostic efficiency of immunological markers IL-32, IL-33, IL-34, or MMP9 for differentiating between RA patients and healthy controls based on specificity, sensitivity, and AUC. For IL-32, the AUC was 0.978, demonstrating outstanding diagnostic validity. The optimal cut-off value for IL-32 was estimated to be 74.7 pg/ml, which yielded a sensitivity of 93.3% (95% CI: 0.956–1.0) and a specificity of 93.3%. These outcomes indicate that IL-32 is a highly effective marker for distinguishing between the groups. For IL-33, AUC was 0.973, indicating excellent diagnostic value. At the optimal cut-off value of 51.8 pg/ml, IL-33 demonstrated a sensitivity of 83.3% (95% CI: 0.891–0.982) and a specificity of 92.2%. These findings confirm IL-33 as a dependable marker in the diagnosis of RA. AUC of IL-34 was 0.989, reflecting remarkable diagnostic accuracy. The optimal cut-off value of 74.7 pg/ml yielded a sensitivity of 93.3% (95% CI: 0.973–1.0) and a specificity of 93.3%. These results emphasize IL-34 as an excellent marker for RA diagnosis. AUC of MMP9 was 0.707 (95% CI: 0.600–0.814), indicating a moderate level of diagnostic accuracy. At the optimal cut-off value of 5.6 ng/ml, MMP-9 showed a sensitivity of 70.0% and a specificity of 68.3%. These findings suggest that while MMP-9 has some diagnostic value, its performance is less robust compared to other evaluated biomarkers.

Heatmap correlation assessment was conducted to analyze the relationship between IL-32, IL-33, IL-34, and MMP9 with various clinical and laboratory variables in patients with RA, as shown in Fig.. The following key findings were observed: IL-32 showed strong positive correlations with IL-33 (rs = 0.639, $p < 0.001$), IL-34 (rs = 0.504, $p < 0.001$), and MMP9 (rs = 0.791, $p < 0.001$). It also correlated significantly with clinical markers, including the DAS28(rs = 0.506, $p < 0.001$), WBC (rs = 0.737, $p < 0.001$), ESR (rs = 0.629, $p < 0.001$), CRP (rs = 0.669, $p < 0.001$), ACCP (rs = 0.698, $p < 0.001$), and RF (rs = 0.730, $p < 0.001$). A negative correlation was observed with

Vitamin-D levels (rs = -0.254, $p = 0.051$), though this was not statistically significant.
IL-33 demonstrated significant positive correlations with IL-32 (rs = 0.639, $p < 0.001$), IL-34 (rs = 0.363, $p = 0.004$), and MMP9 (rs = 0.671, $p < 0.001$). Clinical variables, including DAS28 (rs = 0.457, $p < 0.001$), WBC (rs = 0.545, $p < 0.001$), ESR (rs = 0.641, $p < 0.001$), CRP (rs = 0.538, $p < 0.001$), ACCP (rs = 0.670, $p < 0.001$), and RF (rs = 0.601, $p < 0.001$), all exhibited strong positive correlations with IL-33. Vitamin-D levels also showed a negative correlation (rs = -0.237, $p = 0.068$), which was not significant.
IL-34 correlated positively with IL-32 (rs = 0.504, $p < 0.001$), IL-33 (rs = 0.363, $p = 0.004$), and MMP9 (rs = 0.552, $p < 0.001$). It also demonstrated significant positive associations with clinical variables including DAS28 (rs = 0.305, $p = 0.018$), WBC (rs = 0.629, $p < 0.001$), ESR (rs = 0.632, $p < 0.001$), CRP (rs = 0.612, $p < 0.001$), ACCP (rs = 0.657, $p < 0.001$), and RF (rs = 0.604, $p < 0.001$). A negative but statistically significant correlation was found with Vitamin D levels (rs = -0.281, $p = 0.03$).
MMP9 showed strong positive correlations with IL-32 (rs = 0.791, $p < 0.001$), IL-33 (rs = 0.671, $p < 0.001$), IL-34 (rs = 0.552, $p < 0.001$), and clinical markers, including DAS28 (rs = 0.483, $p = 0.018$), WBC (rs = 0.786, $p < 0.001$), ESR (rs = 0.627, $p < 0.001$), CRP (rs = 0.681, $p < 0.001$), ACCP antibody (rs = 0.741, $p < 0.001$), and RF (rs = 0.741, $p < 0.001$). A negative correlation was observed with Vitamin-D levels (rs = -0.089, $p = 0.499$), though this was not significant.

In summary, IL-32, IL-33, IL-34, and MMP9 were all significantly correlated with various laboratory and diagnostic indicators of disease activity in RA cases. IL-32, IL-33, IL-34, and MMP9 demonstrated strong positive correlations with inflammatory markers, such as CRP and ESR, as well as with clinical disease activity measures, including DAS28 and WBC count. The correlations between these biomarkers and Vitamin D levels were generally weak and not statistically significant.

Table 1: Baseline characteristics of rheumatoid arthritis patients and healthy controls.

| Characteristics: median (IQR) or n (%) | | Newly Diagnosed RA patients n=60 | Treated RA patients n = 60 | Healthy control n = 60 | *p*-value |
|---|---|---|---|---|---|
| Age; years | | 48.5(31.0-63.0) | 49.0 (34.0-54.0) | 45.0 (39.0-56.0) | 0.526 |
| Sex | Females | 48(80%) | 44 (73.3) | 48 (20.0) | NA |
| | Males | 12(20%) | 16 (26.7) | 12 (20.0) | NA |
| DAS28 | | 5.75(5-8.1) | 4.5 (3.5-5.9) | NA | NA |
| WBC; × $10^9$/L | | 11.85(10.9-13.7) | 8.4 (6.1-11.9) | 7.0 (6.4-7.9) | 0.013 |
| ESR: mm/h | | 66.0(45.0-75.0) | 31.0 (26.5-35.0) | 15.0 (15.0-17.0) | < 0.001 |
| CRP; mg/L | | 58.0(47.0-68.3) | 25.3(21.0-32.0) | 4.0 (3.0-5.0) | < 0.001 |
| ACCP antibody; U/mL | | 87.4(80-115) | 45.3(33.4-52) | NA | NA |
| RF; IU/mL | | 56.0(48.0-62.0) | 28.0 (23.0-34.0) | 10.0 (10.0-15.0) | < 0.001 |
| Vitamin-D; ng/mL | | 15.2(10.7-21.4) | 19.5(14-22.4) | 35.0 (33.0-37.0) | < 0.001 |

IQR: Interquartile range (25-75%); RA: Rheumatoid arthritis; HC: Healthy controls; DAS28: Disease Activity Score 28; WBC: White blood cell count; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; ACCP: Anti-cyclic citrullinated peptide; RF: Rheumatoid factor; NA: Not applicable; p: Probability (significant p-value is bold-marked). The Mann-Whitney U test was used to assess significant differences between medians, while Pearson's chi-square test was used to evaluate significant differences between frequencies.

Table 2: Serum levels of IL-32, IL-33, IL-34, and MMP9 among investigated groups.

| Immunological parameters | NDRA patients Median (IQR) | TRA patients Median (IQR) | Healthy control Median (IQR) | *p*-value |
|---|---|---|---|---|
| IL-32;( pg/mL) | 137.4 (124.7-143.7) | 85.9 (78.4-97.1) | 58.37(53.3- 67.97) | < 0.001 |
| IL-33; (pg/mL) | 115.0 (85.9-127.8) | 60.7 (47.9-69.5) | 43.2(38.85-48.07) | < 0.001 |
| IL-34;( pg/mL) | 135.4 (123.7-140.0) | 90.0 (85.9-97.5) | 57.17(54.62-59.43) | < 0.001 |
| MMP9; (ng/mL) | 11.4 (10.0-12.2) | 7.32 (6.14-7.65) | 5.45(4.56-5.57) | < 0.001 |

IQR: Interquartile range; IL: Interleukin; MMP9: matrix Metalloproteinase 9; ND: Newly-diagnosed; p: Probability (significant *p*-value is bold-marked). The Mann-Whitney U test was used to assess significant differences between medians.

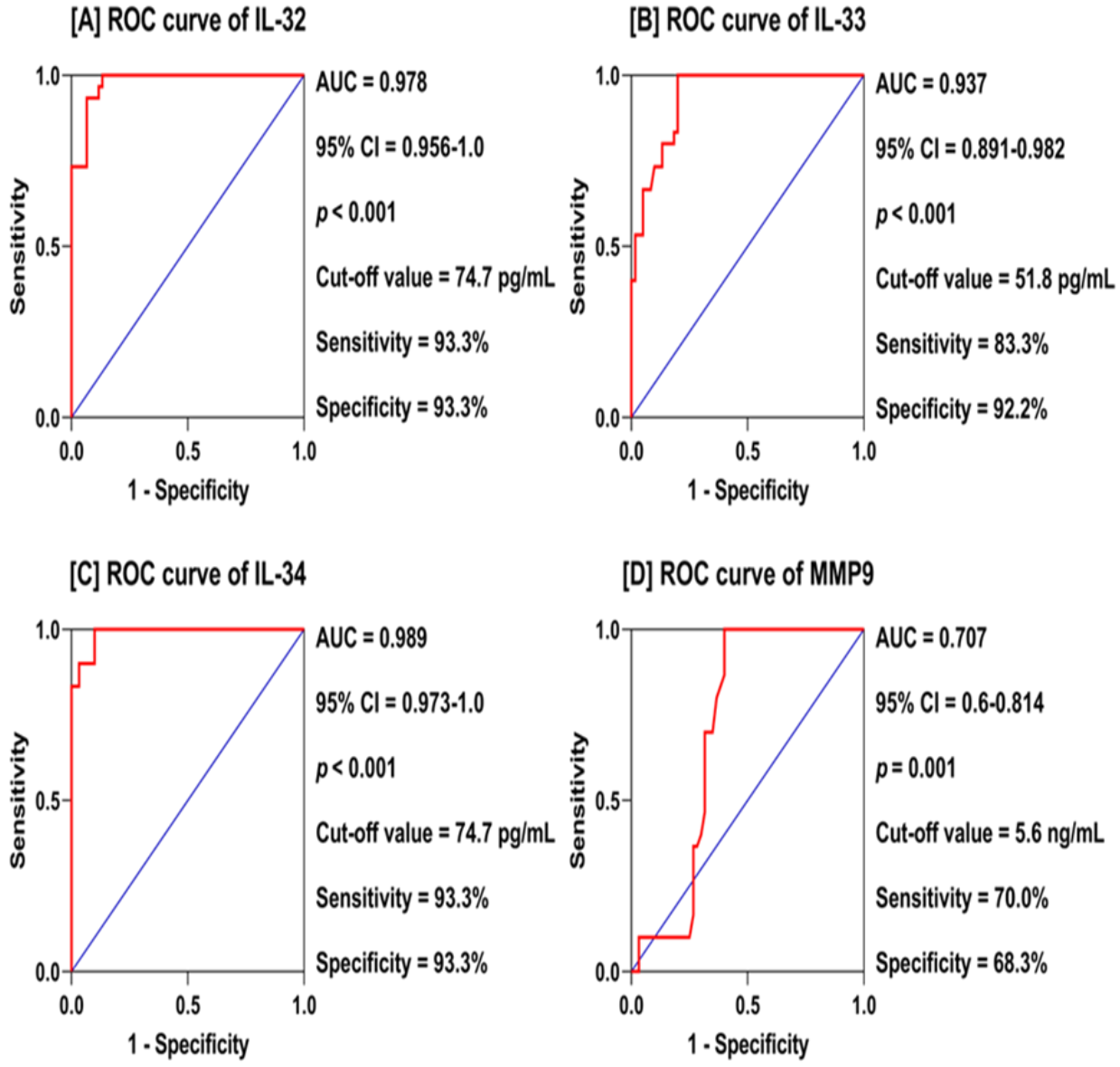


Figure 1: Receiver operating characteristic (ROC) curve analysis of interleukin (IL)-32, IL-33, IL-34, and matrix metalloproteinase 9 (MMP9) in patients with rheumatoid arthritis versus healthy controls. AUC: Area under the curve; CI: Confidence interval; p: Probability.

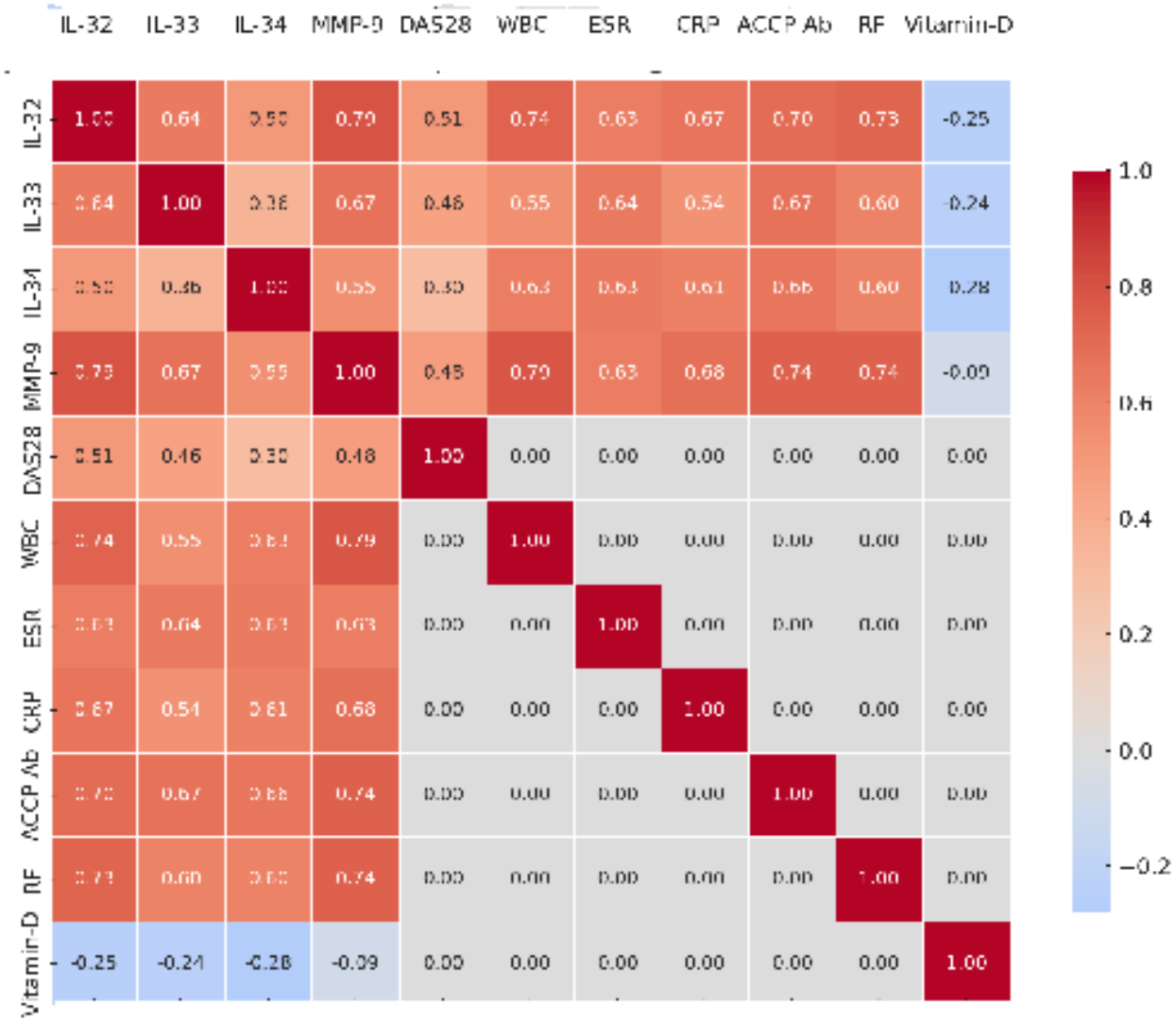


Figure 2: Correlation Heat Map between Inflammatory mediators and Immunological Parameters.

Table 3: Expression of miRNA26 (Fold Change using 2^-ΔΔCt method).

| Group | Fold Change (Mean ± SD) | State of Expression | P-value |
|---|---|---|---|
| Healthy Control | 1.00±0.0 | | - |
| Newly Diagnosed RA patients | 2.578 ± 4.91 | ↑ Compared to HC | 0.0055 * |
| Treated RA patients | 3.030 ± 6.42 | ↑ Compared to HC | 0.0004** |

* Statistically significant (P < 0.01), ** Highly significant (P < 0.001)

The result of gene expression analysis seems to be increased in both newly diagnosed and treated RA individuals relative to healthy controls. Where fold change is computed using the 2^(-ΔΔCt) method. The Healthy Control group is used as the reference; hence, its fold change is 1.00 by definition. Newly diagnosed RA patients show a ~2.6-fold increase in gene expression compared to healthy controls. Treated RA patients show an even higher (~3.0-fold) increase. No significant difference between newly

diagnosed and treated RA patients, although treated patients have a slightly higher mean. The large SD values suggest high variability in gene expression among individuals in the RA groups. This could be This study was designed to evaluate the differential expression of miR-26 and its association with inflammatory profiles in R0A patients treated with infliximab, categorizing them as responders and non-responders, and comparing them to healthy controls. Our findings reveal several critical insights into the complex interplay between miR-26 expression, inflammatory biomarkers, and treatment response in RA. According to the results of baseline features, the median age for Newly Diagnosed RA patients was 48.5 years (IQR: 31.0–63.0), while Treated RA patients had a median age of 49.0 years (IQR: 34.0–54.0). Healthy Controls presented with a slightly lower median age of 45.0 years (IQR: 39.0–56.0). Non-meaningful differences in age distribution among the three groups (p = 0.526). These findings indicate that the study groups were age-matched, minimizing the likelihood of age acting as a confounding variable in subsequent analyses. The comparable age profiles strengthen the internal validity of the study, allowing for more accurate attribution of observed differences in clinical, immunological-related parameters to disease status or treatment effects rather than age-related factors. RA can occur at any age, but onset before 30 or after 65 is less common. Most individuals are diagnosed between the ages of 40 and 60. Women are often diagnosed earlier than men, sometimes as early as their 30s or 40s. The peak age of onset is typically in the fifth decade [22, 23]. RA is commonly significant in women many times than in men because several factors such as changing levels of female sex hormones estrogen and progesterone, especially during menopause, which play a pivotal role in the regulation of immune homeostasis that increases the risk of autoimmune diseases like RA. In addition, the structure of females' immune system has stronger responses than males, which increases the risk of autoimmune diseases. Environmental triggers such stress, infection and fatigue, play in harmony with some genes linked to RA are more frequently expressed or triggered in women may increase susceptibility in females [6]. In this study, the DAS28 was notably higher in Newly Diagnosed RA patients (median: 5.75, IQR: 5.0–8.1) compared to Treated RA patients (median: 4.5, IQR: 3.5–5.9). The elevated DAS28 in the newly diagnosed group likely represents the initial, uncontrolled inflammatory activity that characterizes early RA. At this stage, patients typically present with active joint inflammation, elevated acute-phase reactants ESR or CRP, and due to biological variability, treatment differences, or sample size.

## 4. Discussion

prominent symptoms such as pain, swelling, and morning stiffness. In contrast, treated patients had already received disease modifying antirheumatic drug or biotherapy treatment, which aim to suppress inflammation and prevent joint damage. The lower DAS28 in this group indicates a partial to good therapeutic response, reflecting better disease control, although some patients may still have moderate disease activity depending on treatment efficacy, duration, and adherence. Different study found that the mean DAS28 score at baseline for newly diagnosed patients was approximately 5.1, which decreased to around 3.5 after treatment initiation [1, 24]. This study reported that both newly diagnosed and treated patients tested positive for ESR, CRP, RF, and ACCP signifying systemic inflammation and the presence of autoantibodies typical of RA. The levels of these markers were markedly increased in the NDRA group relative to the TRA group and considerably higher than HC, who exhibited normal or baseline levels. The high ESR and CRP values in NDRA patients represent active systemic inflammation, consistent with early, untreated RA. In contrast, TRA patients demonstrated lower levels, likely due to the suppressive effects of DMARDs and/or biologics, which reduce inflammatory activity over time [25]. Similarly, RF and ACCP antibodies were positive in all RA patients, confirming the autoimmune nature of the disease. Although these markers are generally more stable over time than acute-phase reactants, higher titers in newly diagnosed patients may indicate a more active or aggressive disease phenotype at presentation [26]. Results recorded a difference in WBC count among investigated groups was statistically significant ($p$=0.013), indicating a clear association between RA disease activity and elevated leukocyte counts. The elevated WBC count in NDRA patients reflects a hallmark of early and untreated inflammation stimulates the production and mobilization of WBC as part of the immune response, leading to leukocytosis, particularly in the presence of high cytokine activity (e.g., IL-6, TNF-α) [27]. In contrast, TRA patients exhibited lower WBC counts, suggesting a therapeutic effect of immunomodulatory treatments or biologics. These therapies are designed to suppress immune overactivity and reduce systemic inflammation, often resulting in normalization or reduction of elevated WBC levels. These findings reinforce the utility of WBC count as a supportive marker for determination

of disease progression and treatment response in RA patients influenced by infection, medications, or comorbid conditions [28, 29]. The observed significantly lower Vitamin D levels in both ND RA patients (median: 15.2 ng/mL) and TRA patients (median: 19.5 ng/mL) compared to HC (median: 35.0 ng/mL) ($p < 0.001$) reflect a well-documented phenomenon in RA and other autoimmune diseases. The reasons for these lower levels can be attributed to several key factors: Chronic inflammation, which is central to RA, is known to disrupt Vitamin D metabolism, inflammatory cytokines like TNF-α, IL-6, and IL-1 are elevated in RA and can increase the activity of enzymes such as cytochrome P450 that inactivate Vitamin D. This results in lower levels of active Vitamin D, contributing to Vitamin D deficiency [30]. TRA patients who are on medications such as steroids (e.g., prednisone) or biologic therapies (e.g., TNF inhibitors, methotrexate) are often at risk for Vitamin D deficiency. Corticosteroids in particular, can impair calcium absorption and interfere with Vitamin D metabolism, leading to lower levels. The effects of biologic drugs on Vitamin D levels are variable but may also contribute to a reduction in circulating Vitamin D [31].

The present study demonstrates markedly elevated levels of IL-32, IL-33, IL-34, and MMP-9 in both NDRA and TRA patients compared to HC. These findings underscore the effect of inflammatory cytokines and degrading enzymes in the mechanism of RA and highlight differences in immune activity based on treatment status. IL-32 and IL-33 levels were markedly higher in NDRA patients, reflecting the strong inflammatory state associated with active RA. IL-32 is known to induce key cytokines such as TNF-α, IL-1ß, and IL-6, all of which patriciate in synovial inflammation and joint erosion[32] In TRA patients level of IL-32and IL-33 in serum reduced but still elevated levels suggest that chemical and biological treatment can suppress their expression, it may not fully normalize cytokine profiles in all individuals, possibly due to partial treatment response or residual disease activity.IL-33 is released upon cellular stress and injury, amplifying inflammation through activation of mast cells and Th2-type immune responses. Its higher expression in NDRA patients suggests more severe synovial inflammation and tissue damage compared to treated individuals [33]. Similarly, IL-34 was significantly raised in RA patients, especially those not receiving treatment. IL-34 plays a crucial role in monocyte and macrophage persistence and function, because of chronic inflammatory environment of RA joints is characterized by elevated levels of inflammatory mediators like TNF-α, IL-1ß, and IL-6, which stimulate IL-34 expression, especially in FLS, which are highly active in RA synovium in addition to increased recruitment and survival of macrophages in the synovium. The production of more MMPs worsens joint damage. This creates a positive feedback loop: inflammation to more IL-34 production and severe inflammation [34].

MMP-9 was also significantly elevated in NDRA patients, indicating active tissue remodeling and joint degradation. Because of elevated levels of inflammatory cytokines in combination with high neutrophil activity, especially in synovial fluid, the Neutrophils release MMP-9 upon activation, adding to the elevated levels seen in serum [35]. Even after treatment like methotrexate or biotherapy, low-grade inflammation may persist, especially in early phases, which can maintain elevated MMP-9 levels, though usually reduced from the pretreatment peak suggest that current therapeutic approaches in modulating immune responses may not completely normalize inflammatory markers. This underscores the need for more targeted or aggressive interventions in certain patients to achieve better immunological remission [14, 36].

The observed significant upregulation of miR-26 in both NDRA and TRA cohorts relative to the control group suggests a vital role for miR-26 in the mechanism of RA. Specifically, the NDAR group exhibited high levels of miR-26, accompanied by significantly elevated ESR, WBC, ACCP, RF, CRP, IL-32, IL-33, IL-34, and MMP-9, alongside a high DAS28 score. In contrast, the TRA group, also showing elevated miR-26 compared to controls, displayed significantly lower levels of inflammatory markers and disease activity scores. This implies miR-26 in TRA patients may exert a compensatory or anti-inflammatory effect, potentially mitigating the inflammatory burden induced by RA. The miR-26 functions as a regulatory microRNA, playing a negative role in inflammation and tissue degradation by targeting pathways like PI3K/AKT, and possibly directly or indirectly suppressing pro-inflammatory cytokines and degrading enzymes [37,38] In NDRA, immune dysregulation is at its peak, and inflammatory signaling suppresses miR-26 expression, removing its inhibitory effect on cytokines and MMP9. The loss of miR-26-mediated control allows for unchecked cytokine production and joint degradation. In treated patients, there is a slight upregulation or restoration of miR-26, because of a moderate reduction in IL-32, IL-33, IL-34, and MMP9 compared to NDRA, though levels may still be elevated compared to healthy controls. Treatment

(often involving DMARDs or biologics) reduces inflammatory activity, which may allow miR-26 levels to partially recover. This suggests a therapeutic benefit and a potential anti-inflammatory role for miR-26, possibly helping to suppress cytokine and MMP9 expression [39, 40]. In RA, miR-26 is often upregulated as a feedback mechanism in response to inflammatory cytokines, possibly to limit or regulate inflammation but it may also contribute to pathology depending on the cellular context. Cytokines like IL-1ß, TNF-α, IL-6, IL-32, IL-33, and IL-34 are abundantly produced in RA and can directly or indirectly affect the expression of various miRNAs, including miR-26. The signaling pathway NF-κ may promote miR-26 upregulation as part of a broader cellular response to chronic inflammation. miR-26 has been shown in some contexts to modulate MMPs and VEGF expression. In RA, its upregulation may be linked to increased MMP-9 expression or serve as a compensatory mechanism to limit matrix degradation. miR-26 can influence FLS proliferation, apoptosis, and cytokine production, all key aspects of RA pathology [41,42].

One limitation of this study is the relatively small sample size, which may limit the generalizability of our findings. Future studies with larger cohorts are needed to validate these results and explore the underlying mechanisms by which miR-26 modulates inflammatory pathways in RA. Additionally, longitudinal studies are warranted to assess the predictive value of miR-26 and other biomarkers for long-term treatment response and disease progression.

## 5. Conclusion

This study underlines the modulatory function of miR-26 in RA through its association with raised levels of IL-32, IL-33, IL-34, and MMP-9. miR-26 expression was significantly upregulated in RA patients and associated inversely with inflammatory activity, proposing a potential anti-inflammatory role that may be restored with medication. IL-33 and MMP-9 demonstrated strong diagnostic performance, supporting their value as early indicators. The outcomes emphasize the benefit of miR-26 and related cytokines in RA diagnosis, monitoring, and as possible therapeutic targets.

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