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Research Article

Immunoregulatory Impact of miR-124 and miR-223 on Some Immunological Parameters in Rheumatoid Arthritis

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ABSTRACT

Rheumatoid arthritis (RA) is a long-lasting autoimmune disorder that causes inflammation of the synovium and the destruction of joints. This study evaluated the expression of miR-124 and miR-223 in RA patients and their association with key inflammatory markers: sCD28, IL-6, IL-17A, IL-40, MCP-1, and VEGF. One hundred eighty participants were enrolled, including 120 RA patients (newly diagnosed and biologically treated) and 60 healthy controls. Serum cytokine levels were measured using sandwich ELISA, and miRNA expression was quantified by qRT-PCR. Results revealed significant, distinct yet overlapping dysregulation of miR-124 and miR-223 in RA patients in comparison to controls, influenced by inflammatory status, therapeutic intervention, and patient-specific factors. These alterations correlated positively with raised serum levels of IL-6, IL-17A, and VEGF, indicating active inflammation, immune activation, and angiogenesis. Biological therapy significantly reduced miRNA dysregulation and inflammatory marker levels, reflecting effective disease control. The expression profiles of miR-124 and miR-223 closely reflected both inflammatory activity and therapeutic response. The correlation between expression of miR-223 and pro-inflammatory mediators reinforce its role in supporting T-cell activation and immunogenic signaling, while the contrary pattern of miR-124 indicates a regulatory role in modulating inflammatory cascades and angiogenesis. The immunological indicators sCD28, MCP-1, IL-40, and VEGF illuminate the reciprocal influence of immune activation, chemotaxis, and vascular remodeling in the pathogenesis of RA. The study's outcomes substantiate miR-124 and miR-223 as consistent diagnostic biomarkers of RA. Moreover, the essential roles of related immunological markers in disease activity and therapeutic response. This study contributes to enhancing the importance of using miRNA in modifying the immune reaction by influencing the concentration and function of inflammatory mediators to reduce the development and severity of RA and enhances the therapeutic response, with the feasibility of using them as diagnostic parameters and therapeutic targets.

Keywords: Immunoregulation; MicroRNAs; pro-inflammatory cytokines; sCD28

1. INTRODUCTION

Rheumatoid arthritis (RA) is systemic autoimmune illness marked by chronic synovial inflammation that ultimately results in progressive joint damage, discomfort, swelling, and

stiffness (Al-Rahim et al., 2023). RA originates from the interaction of hereditary immunological defects and surrounding factors. RA is characterized by impairment of immunological tolerance and generation of specific autoantibodies (Haarhaus and Klareskog,2024). The biological therapy has been greatly used to enhance dramatically treatment outcomes, particularly when utilized in the early phases of disease progression that specifically inhibit critical mediators involved in the pathogenesis of RA (Mohammed et al., 2023; Talib and Mohammed, 2024).

The pathophysiology of RA involves a complicated network of immunological mediators. Interleukin-6 (IL-6), a pivotal proinflammatory cytokine, facilitates B-cell differentiation and induces hepatic acute-phase responses. IL-6 mediates its effects in RA through classic and trans-signalling pathways involving membrane-bound and soluble IL-6 receptors, respectively, both converging on the gp130-JAK/STAT3 axis (Tylor et al., 2024). Trans-signalling, in particular, drives chronic synovial inflammation and hyperplasia by activating STAT3, which promotes proinflammatory gene expression, Th17 cell differentiation, and suppression of regulatory T cells (Kondo et al., 2021). Interleukin-17 (IL-17), secreted by Th17 cells, plays a pivotal role in RA which activates key signalling pathways (NF-κB, MAPKs) upon binding to its receptor on synovial cells, leading to the production of pro-inflammatory cytokines, that sustain synovial inflammation and tissue destruction (AlChalabi et al., 2024; Emerson et al., 2025). Interleukin-40 (IL-40), encoded by the *C17orf99* gene, has emerged as a significant elevated mediator in early seropositive RA for rheumatoid factor, anti-CCP and decreases following conventional or B-cell depletion therapy (rituximab) (Navrátilová et al., 2021). IL-40 exerts a multifaceted contribution in the pathogenesis of RA primarily produced by neutrophils, especially during NETosis, stimulates synovial fibroblasts to secrete pro-inflammatory chemokines like IL-8 and MCP-1, along with the matrix-degrading enzyme MMP-13, thereby promoting immune cell infiltration, inflammation, and tissue destruction (Dabbagh-Gorjani, 2024).

CD28, a co-stimulatory molecule on T lymphocytes, is crucial for full T-cell activation and survival, thus playing a critical role in the autoimmune cascade of RA. A soluble form of CD28 is a circulating form of the membrane-bound CD28 molecule that retains ligand binding ability but lacks signalling domains (Ding et al., 2023). It originates from alternative splicing or proteolytic shedding from activated T cells, which is increased in the serum and synovial fluid of RA patients. sCD28 may influence immunological responses because of interaction with CD80/CD86, interfering with co-stimulation and disturbing the balance of activation and control by increasing inflammatory immune mediators production and constraining suppressive signaling of CTLA-4, thus promoting autoimmunity (Su et al., 2025). The interaction of some drugs, like abatacept, consisting of CTLA-4 and the Fc region of IgG, operates by attaching to the CD80 and CD86 receptors on professional immune presenting cells and decreasing co-stimulatory signaling, suppressing T cell activation, and ultimately causing a diminution of RA activity (Edner et al., 2020; Khan et al., 2021). Monocyte chemoattractant protein-1 (MCP-1/CCL2) is a critical chemokine in RA by attracting $CCR2^{+}$ monocytes, memory T cells, and dendritic cells to arthritic joints, hence promoting chronic synovitis and tissue erosion (Tong et al., 2020). MCP-1 stimulates the formation of matrix metalloproteinases (MMPs) and VEGF, facilitating angiogenesis and cartilage degradation (Rania et al., 2021). Experimental models indicate that the suppression of the MCP-1/CCR2 axis diminishes inflammation and joint erosion, signifying therapeutic potential. (Li et al., 2021).

Vascular endothelial growth factor (VEGF) is a crucial facilitator of pathological angiogenesis in RA, with increased concentrations in synovial tissue, fluid, and serum corresponding with disease severity (Mumtaz and Hussain, 2020). VEGF, synthesized by synovial fibroblasts, macrophages, and T cells in reaction to hypoxia and pro-inflammatory cytokines, stimulates endothelial cell proliferation, neovascularization, and heightened vascular

permeability, thereby enabling immune cell infiltration and perpetuating pannus formation (Weyand and Goronzy, 2021). VEGF additionally promotes the viability of synoviocytes and osteoclasts, facilitating cartilage and bone degradation. Its concentrations correlate with inflammatory activity markers such as DAS28, CRP, and ESR, reflecting its viability as a biomarker (Gao et al., 2024).

miRNAs are a type of non-coding RNA, short and post-transcriptionally regulate the expression of genes, directing mRNA transcripts, ending in mRNA degradation or translational inhibition. In RA, deviant expression of particular miRNAs has been associated with the modulation of immune responses and synovial inflammatory regulation (Daien et al., 2022). miRNA slows the cell cycle through the attenuation of cyclin-dependent kinases, and modifies macrophage activation with inhibition of immunostimulatory mediators. These features reinforce miR-124 as a potential epigenetic biomarker and therapeutic target (Zamzam et al., 2024). Likewise, miR-223 reveals a dual function by regulating both inflammatory responses and osteoclastogenesis. The abnormal expression in RA effects disease pathogenesis by modifying immune signaling and bone homeostasis (Jiao et al., 2021; Peng et al., 2023).

The objective of this investigation is to assess the expression levels of miR-124 and miR-223 in RA patients, examine their correlation with disease activity, and analyses their relationship with significant inflammatory and immunoregulatory markers (sCD28, IL-6, IL-17A, IL-40, MCP-1, and VEGF). The primary goal is to evaluate their potential as predictive indicators for the efficacy of treatment and the progression of the disease.

2. MATERIALS AND METHODS

2.1. Study Design

This case-control study aimed to evaluate the clinical significance of specific immunological parameters, including sCD28, IL-6, IL-17, IL-40, MCP1, and VEGF, in addition to the expression of miRNA 124 and miRNA 233, in affected individuals with RA as prospective diagnostic markers for disease detection and severity assessment.

2.2. Ethical Approval

The Iraqi Ministry of Health's Ethics Committee (Approval No. 54201, dated 25-10-2024) and the Council of the College of Biotechnology at Al-Nahrain University both authorized the study. All of them were given written informed consent. Demographical and clinical information was obtained via a structured questionnaire under medical supervision, and biological samples were obtained following ethical guidelines.

2.3. Sample Size and Subjects

A total of 180 individuals of both genders were selected based on specific inclusion criteria, comprising 120 individuals diagnosed with RA and 60 healthy controls. Recruitment was carried out at the National Center for Rheumatic Diseases in Baghdad between October 1, 2024, and January 31, 2025. Group I Newly Diagnosed RA Patients (ND), included 60 individuals who were recently diagnosed with RA and had not yet received any treatment. The mean age in this group was 48.03 years, comprising 18 males and 42 females. Group II Treated RA Patients (TP) consisted of 60 patients who were undergoing biological therapy, with a mean age of 47.90 years; this group included 20 males and 40 females. Group III Healthy Controls (HC) comprised 60 age and sex-matched healthy individuals with no prior history of autoimmune or inflammatory disorders. The mean age of the control group was 44.27 years,

including 14 males and 46 females. Participants were recruited by a consultant rheumatologist in accordance with the 2016 American College of Rheumatology/European League Against Rheumatism classification guideline for RA, with a minimum required score of ≥6 for study inclusion. Eligible individuals ranged in age from 35 to 85 years. To reduce confounding factors and enhance the validity of clinical and laboratory analyses, individuals with coexisting chronic or inflammatory conditions such as eczema, cardiovascular disorders, malignancies, other autoimmune diseases, or ongoing infections were excluded from the study.

2.4. Collection of Blood Specimens

Peripheral blood specimens were collected from all participants through venipuncture using sterile, disposable plastic syringes. The collected blood was divided into three portions. The first portion was transferred into EDTA tubes for measuring ESR, the second portion into serum separator (gel) tubes, and centrifuged to obtain serum, which was subsequently used for the quantitative determination of sCD28, IL-6, IL-17A, IL-40, MCP-1, and VEGF levels by ELISA. The third portion, comprising 200 µL of whole blood, was added to 600 µL of Triazole reagent in RNA-free microtubes, thoroughly mixed, and immediately processed for total RNA extraction.

2.5. Measurement of Erythrocyte Sedimentation Rate and Disease Severity

Peripheral blood specimens were collected from all participants, with a portion transferred into EDTA-containing tubes for erythrocyte sedimentation rate (ESR) analysis, which was performed using the ADVIA 2120i Hematology Analyzer. The DAS28-ESR is a standardized tool for assessing rheumatoid arthritis activity, calculated using tender and swollen joint counts (28 joints), ESR, and the patient's global health score. The formula is: DAS28-ESR = 0.56×TJC28 + 0.28×SJC28 + 0.70×ln (ESR) + 0.014×GH. Scores are interpreted as: <2.6 (remission), 2.6–3.2 (low), 3.2–5.1 (moderate), and >5.1 (high disease activity). This scoring system helps evaluate disease severity and treatment effectiveness.

2.6. Estimation of Serum Levels of Vitamin D and Immunological Parameters

The concentrations of Vitamin D, C-reactive protein (CRP), and rheumatoid factor (RF) using a Cobas analyzer (Cobas c311, Roche, Germany), while the concentrations of immunological parameter ACPA were calculated using an enzyme-linked immunosorbent assay (catalog numbers: MBS1601013, MyBiosource, British Columbia). Serum concentrations of sCD28, IL-6, IL-17A, IL-40, MCP-1, and VEGF were quantified using, in accordance with the manufacturer's protocols (Catalog Nos.: SL1596Hu, SL1001Hu1, SL2334Hu, SL3535Hu, SL1923Hu, and SL1811Hu; SunLong Biotech, China).

2.7 RNA Extraction and cDNA Synthesis

TRIzol ™ Reagent (Thermo Scientific, USA) was used in extracting total RNA in 500 1 of serum following the manufacturer's protocol. Chloroform was then used to conduct a phase separation, and isopropanol was used to sediment RNA. The 70% ethanol was used to purify the RNA pellet, which was air dried and homogenized in water that was free of any nuclease. Quantification of RNA and its purity was done with the help of the Quantus 180 Fluorometer (Promega, USA). Constructed cDNA was made out of the extracted RNA with the help of the GoScript ™ 180 Reverse Transcription System (Promega, USA). The reaction was done by a first round of denaturation of RNA and random primers at 70°C during a period of 5 minutes

and then a reverse transcription stage at 42°C, taking 60 minutes. The reaction was stopped by inactivation of the enzyme at 70°C for 15 minutes.

2.8. Primer Design

The sequences for miR-124 and miRNA233 were obtained from the 5iRbase database (https://www.mirbase.org/). To measure the abundance of miR-124 and miR-223 in RA patient samples, specific primers were designed based on their mature miRNA sequences. The forward primer for miR-124 was 5′-AACAGACGTGTTCACAGCGG-3′ (20 nucleotides), while the forward primer for miR-223 was 5′-AACACGTGCGTGTATTTGACAAG-3′ (23 nucleotides). A universal reverse primer (CR), 5′-CAGTGCAGGGTCCGAGGT-3′ (18 nucleotides), was employed for both miRNAs to facilitate consistent amplification. For normalization, the small nuclear RNA U6 was used as an endogenous control. U6 amplification was performed using a forward primer 5′-GTGCTCGCTTCGGCAGCA-3′ and a reverse primer 5′-CAAAATATGGAACGCTTC-3′, each 18.

2.9. Quantitative Reverse Transcription PCR (qRT-PCR)

qRT-PCR was conducted to quantify gene expression. The reaction mixture contained cDNA, gene-specific primers, and nuclease-free water, totaling 20 µL. Amplification cycles included initial denaturation at 95°C for 3 minutes, followed by 40 denaturation cycles (95°C, 15 s), annealing (55°C, 45 s), and extension (72°C, 60 s). Relative expression levels were calculated using the comparative ΔΔCt method, normalizing target gene expression to a housekeeping gene and expressing fold changes as

$$2^{\{-\Delta\Delta C_{\{t\}}\}}$$

2.10. Bioinformatics Analysis

The interaction between miR-124, miRNA223, and immunological parameters was predicted using miRTarBase and TargetScan databases. Protein-protein interaction networks involving sCD28, IL-6, IL-17, IL-40 MCP1, and VEGF were constructed via the STRING database. Functional enrichment analysis of the biological processes and signaling pathways was carried out with the help of the DAVID and Enrichr platforms.

2.11. Statistical Analysis

The study used SPSS 25.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA) to analyze data. The continuous variables will be provided in the form of mean Sa standard deviation (SD), or median with an interquartile range (IQR) depending on the normality evaluation with a Shapiro-Wilk test. One-way ANOVA or Kruskal-Klassen wallis tests with respective post hoc comparisons were used in the evaluation of group differences. The correlation coefficient looked at the relationship between variables using Pearson or Spearman correlation. The GraphPad Prism was used to analyse the ROC curve to determine the diagnostic performance of biomarkers. It was taken to be statistically significant $p<0.05$.

3. RESULTS AND DISCUSSION

3.1. Basic Characteristics

This study enrolled 180 participants, stratified equally into three groups: newly diagnosed RA patients (ND; n = 60), RA patients receiving biological treatment (TP; n = 60), and healthy control subjects (HC; n = 60). Table 1 presents the demographic and clinical profiles of the study population.

Table 1. Demographic and laboratory characteristics of investigated groups

Characteristic: median (IQR; 25-75%) or n (%)	**NP; n = 60**	**TP; n = 60**	**HC; n = 60**	***p*-value**
Age; years	51.5 (43.0-65.0)	47.5 (38.0-58.0)	45.0 (38.0-50.0)	
Sex				
Female	42 (70)	40 (66.7)	46 (76.7)	
Male	18 (30)	20 (33.3)	14 (23.3)	
ESR; mm/h	50 (45.0-60.0)	34.5 (29.0-38.0)	15.0 (13.0-17.0)	0.0001
CRP; mg/L	30.24 (25.44-34.56)	18.23 (15.0-23.0)	3.58 (3.24-4.60)	0.0001
RF; U/mL	55.0 (46.0-62.0)	28.0 (0.001-34.0)	-	-
ACPA; U/mL	228.9 (200-256)	144.6 (118.5-153.0)	-	-
DAS28	5.90 (5.50-6.80)	4.65 (4.0-5.1)	-	-
Vitamin D ng/mL	12.5 (9.07-19.0)	19.5 (15.0-22.0)	35.0 (33.0-37.0)	0.003

Newly Diagnosed RA Patients (ND), Treated RA Patients (TP), and Healthy Controls (HC)

The median age in the ND group was 51.5 years (interquartile range [IQR]: 43.0–65.0), which was slightly higher compared to the TP group at 47.5 years (IQR: 38.0–58.0) and the HC group at 45.0 years (IQR: 38.0–50.0). Females predominated across all cohorts, comprising 70.0% of ND, 66.7% of TP, and 76.7% of HC participants. Inflammatory indices, including ESR and CRP, were markedly elevated in RA patients compared to healthy controls, with the highest levels observed in the ND group, followed by the TP group, and the lowest in the HC group. These intergroup differences were statistically significant (p = 0.0001), underscoring heightened inflammatory activity in untreated RA cases. Serological autoantibodies RF, and ACPA antibodies were also elevated among RA patients. The ND group exhibited significantly higher RF levels [55.0 U/mL (IQR: 46.0–62.0)] relative to the TP group [28.0 U/mL (IQR: 0.001–34.0)]. Similarly, ACCP concentrations were elevated in the ND group [228.9 U/mL (IQR: 200–256)] compared to the TP group [144.6 U/mL (IQR: 118.5–153.0)]. The Disease activity was measured by the help of DAS28, which was significantly greater in the ND group [5.90 (IQR: 5.50–6.80)] versus the TP group [4.65 (IQR: 4.00–5.10)], indicating more effective disease control in patients under treatment. Additionally, serum vitamin D levels were significantly lower in RA patients than in healthy controls, with the ND group exhibiting the lowest concentrations [12.5 ng/mL (IQR: 9.07–19.0)], followed by the TP group [19.5 ng/mL (IQR: 15.0–22.0)] and the HC group [35.0 ng/mL (IQR: 33.0–37.0)] (p = 0.003).

Findings of this study affirm the established inflammatory landscape of RA and reveal an additional perspective of the manner in which immune responses are affected by biological therapy. The epidemiological data that suggests that RA influences women two to three times more prevalently than men is consistent with the predominance of female patients across all categories. This is likely due to the influence of sexual hormones on immune function (Smolen et al., 2018). The slightly elevated median age in the newly diagnosed (ND) group may be indicative of delayed diagnosis, which is frequent in early RA due to subtle or nonspecific symptoms (Jassim et al., 2024). The acute phase response is characterized by elevated ESR and CRP levels in RA patients, particularly in the ND group, which emphasize the role of systemic inflammation, which is primarily driven by cytokine overproduction, particularly IL-6 and TNF-α. This aligns with Aletaha et al. (2010), who emphasized the association between untreated RA and elevated inflammatory burden. The observed reduction in these markers among biologically treated patients (TP group) highlights the capacity of anti-TNF or anti-IL-

6 agents to suppress systemic inflammation by interrupting key cytokine signalling pathways. Autoantibody analysis revealed higher RF and ACCP levels in ND patients, reinforcing their roles as early indicators of aggressive disease linked to B-cell hyperactivity and autoantigen presentation. As shown by Van der Helm-van Mil et al. (2005), high autoantibody titers correlate with erosive disease and poor prognosis. Their decline in treated patients reflects reduced autoreactive B-cell activity and less immune complex deposition under biologic therapy. DAS28 scores, significantly higher in ND patients, further confirm active disease. Their reduction in the TP group supports studies demonstrating that biologics reduce synovial inflammation, leukocyte infiltration, and cartilage destruction (Singh et al., 2016).

Moreover, the consistently low vitamin D levels, particularly in ND patients, highlight an immunomodulatory deficit. Vitamin D plays a regulatory role in suppressing Th17 differentiation and promoting T-regulatory cell activity. Cutolo et al. (2014) demonstrated that hypovitaminosis D exacerbates autoimmune processes in RA. The partial normalization of vitamin D levels in the TP group may result from improved disease control, enhanced absorption due to reduced inflammation, or physician-guided supplementation during treatment. Together, these results emphasize that both traditional biomarkers (ESR, CRP, RF, ACCP) and immunoregulatory factors like vitamin D are dynamically influenced by disease activity and treatment. They also suggest a mechanistic interplay between inflammatory cytokines, autoantibody production, and T-cell dysregulation in RA pathogenesis and remission.

3.2. Estimation Serum Levels of Immunological Parameters

Table 2 showed that the assessment of immunological biomarkers revealed statistically significant differences (p = 0.0001) across all evaluated parameters among the three study groups: newly diagnosed RA patients, treated RA patients, and healthy controls, as further illustrated in Figure 1.

Table 2. Comparative analysis of serum levels of immunological biomarkers among investigated groups

Immunological parameters median (IQR; 25-75%)	**NP; n = 60**	**TP; n = 60**	**HC; n = 60**
IL-6 (pg/mL)	183.99 (168.1-200.6)	85.28 (79.3-90.4)	34.87 (29.62-46.44)
IL-17 (pg/mL)	151.87 (140.1-213.5)	106.15 (89.76-112.7)	32.23 (29.76-38.75)
IL-40 (ng/mL)	30.68 (27.68-36.37)	17.45 (16.37-18.85)	10.23 (9.65-11.05)
sCD28 (ng/mL)	23.99 (18.16-26.19)	17.39 (16.21-18.60)	8.45 (7.90-9.36)
MCP-1 (pg/mL)	315.48 (298.6-345.6)	200.7 (188.7-218.0)	98.31 (94.48-103.16)
VEGF (pg/mL)	381.85 (301.0-443.3)	278.3 (253.3-300.1)	198.1 (189.5-206.9)

*p-value: 0.0001

IL-6 a key proinflammatory cytokine, exhibited the highest median concentration in newly diagnosed RA patients [183.99 pg/mL (IQR: 168.1–200.6)], followed by the treated group [85.28 pg/mL (IQR: 79.3–90.4)], with the lowest levels observed in healthy controls [34.87 pg/mL (IQR: 29.62–46.44)]. A similar pattern was noted for IL-17 with significantly elevated levels in the newly diagnosed group [151.87 pg/mL (IQR: 140.1–213.5)], moderate levels in treated patients [106.15 pg/mL (IQR: 89.76–112.7)], and minimal levels in the control group [32.23 pg/mL (IQR: 29.76–38.75)]. IL-40, was markedly elevated in patients with RA, with the highest median levels observed in newly diagnosed individuals [30.68 pg/mL (IQR: 27.68–36.37)]. This was significantly greater than levels in the treated group [17.45 pg/mL (IQR: 16.37–18.85)] and healthy controls [10.23 pg/mL (IQR: 9.65–11.05)]. Similarly, the co-stimulatory molecule sCD28 showed a stepwise decline across groups, with the highest concentrations in newly diagnosed RA patients [23.99 ng/mL (IQR: 18.16–26.19)], followed

by the treated group [17.39 ng/mL (IQR: 16.21–18.60)], and the lowest levels in the control group [8.45 ng/mL (IQR: 7.90–9.36)].MCP-1 levels were significantly elevated in the newly diagnosed RA group [315.48 pg/mL (IQR: 298.6–345.6)] compared to the treated patient (TP) group [200.7 pg/mL (IQR: 188.7–218.0)] and healthy controls [98.31 pg/mL (IQR: 94.48–103.16)], reflecting enhanced monocyte recruitment associated with active inflammation. Similarly, VEGF, a key regulator of angiogenesis and synovial vascular remodeling, was markedly increased in ND patients [381.85 pg/mL (IQR: 301.0–443.3)], followed by the TP group [278.3 pg/mL (IQR: 253.3–300.1)], with the lowest levels observed in the healthy control group [198.1 pg/mL (IQR: 189.5–206.9)].

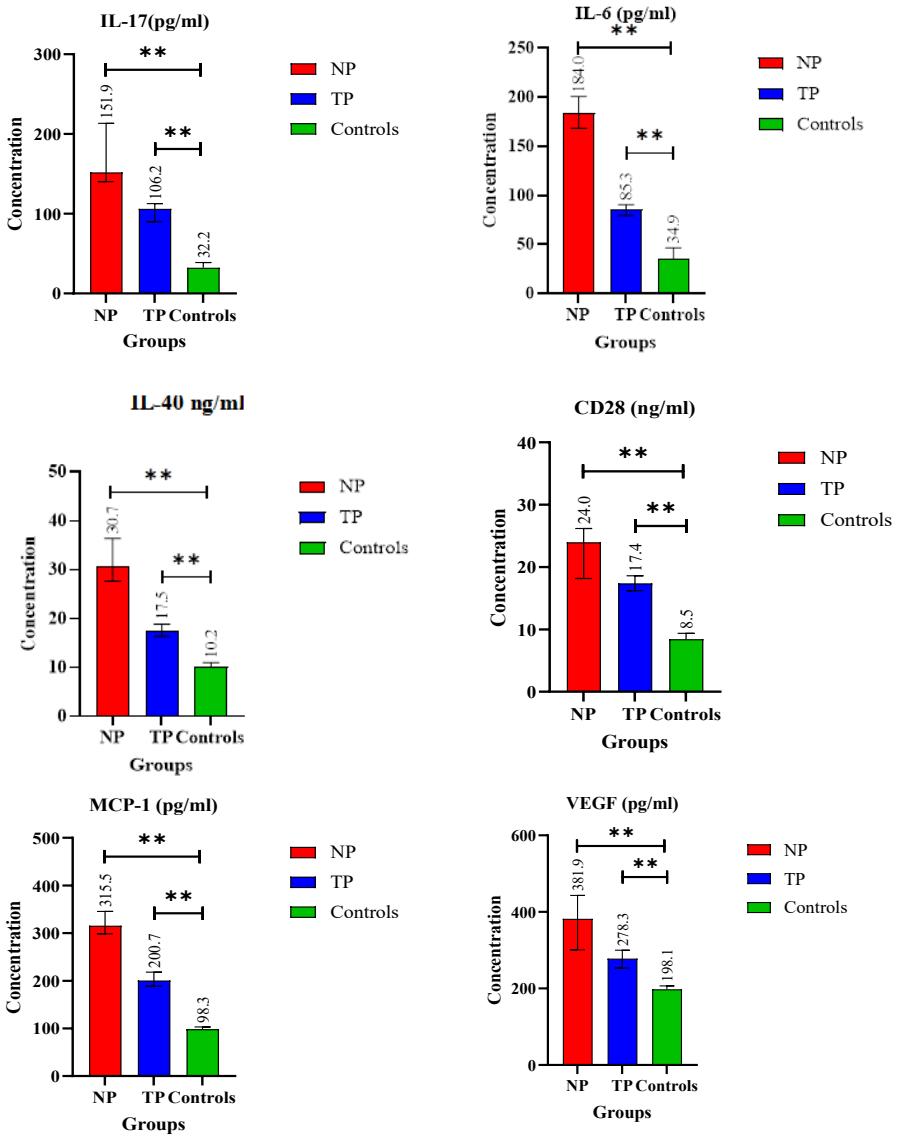

Figure 1. Serum levels of immunological parameters in newly diagnosed RA patients, treated patients, and healthy controls

The hyperactive immune response that characterizes the early stages of RA is driven by a complex interplay between cytokines, chemokines, and co-stimulatory molecules, which collectively promote synovial inflammation, joint damage, and systemic manifestations. Among these, IL-6 plays a pivotal role by stimulating osteoclastogenesis, promoting synovial hyperplasia, and contributing to anemia and fatigue. The significantly elevated IL-6 levels

observed in newly diagnosed patients align with findings by Alivernini et al. (2025), who demonstrated a strong association between elevated IL-6 and active synovitis in early RA. The lower IL-6 levels seen in treated patients reflect the effectiveness of biological therapies particularly IL-6 and TNF-α inhibitors in suppressing this cytokine's expression.IL-17, primarily secreted by Th17 cells, acts synergistically with IL-6 to upregulate matrix metalloproteinases, thus facilitating cartilage degradation. The increased IL-17 levels in ND patients reinforce the dominance of Th17-driven inflammation in early RA, consistent with previous findings (Robert and Miossec,2019; Dammacco et al., 2020). In parallel, IL-40 markedly elevated in the ND group. Abed et al. (2023) reported that IL-40 supports B-cell survival and promotes autoantibody production, particularly ACPA, suggesting its role in perpetuating humoral autoimmunity. The decreased levels of IL-40 in TP patients may indicate treatment-induced suppression of B-cells.

sCD28, a mediator of T-cell co-stimulation, was markedly increased in ND patients, demonstrating persistent T-cell activation. This study supports the results of Cui and Qian ,2023 who reported a correlation between raised sCD28 levels and both disease activity ratings and joint damage. The gradual decrease of sCD28 in the TP and HC groups illustrates the immunomodulatory effect of biologic therapy on T-cell signalling pathways. MCP-1, essential to monocyte recruitment to inflammatory synovia, was higher in ND patients, signifying active leukocyte infiltration and contributing to joint erosion. This observation aligns with Luo et al. (2024), who documented elevated MCP-1 levels in early rheumatoid arthritis and their subsequent downregulation following anti-TNF therapy. Furthermore, VEGF increased significantly in ND patients, confirming its role in synovial angiogenesis, a characteristic feature of RA. VEGF facilitates neovascularization, hence enhancing immune cell trafficking and pannus proliferation. The reduced VEGF levels in TP patients reflect the beneficial effects of biologics in suppressing angiogenic activity (Le and Kwon, 2021; Sakalyte et al., 2022). These findings demonstrate the complex contribution of immunomodulatory and costimulatory molecules in the progression of RA and emphasize their control as a key method of biological treatment. This work contributes to the expanding data supporting the use of these immunological markers as diagnostic instruments and indicators of therapy response in RA.

ROC curve analysis was performed to estimate the diagnostic performance of selected immunological parameters in differentiating RA patients from healthy controls (Table 3). The area under the curve (AUC) values showed excellent discriminatory power for all tested parameters. IL-6, IL-40, sCD28, and VEGF each demonstrated perfect diagnostic accuracy with an AUC of 1.0, achieving 100% sensitivity and specificity at optimal cut-off values of 109.47 pg/mL, 17.91 pg/mL, 13.292 pg/mL, and 253.23 pg/mL, respectively ($p<0.0001$). IL-17 exhibited an AUC of 0.998, exhibiting 100% sensitivity and 96.7% specificity at a threshold of 78.81 pg/mL ($p<0.0001$). MCP-1 exhibited high diagnostic accuracy, with an AUC of 0.967, 100% sensitivity, and 69.7% specificity at a threshold of 161.445 pg/mL ($p<0.0001$). These data demonstrate that these biomarkers represent substantial potential as diagnostic tools for RA. This work highlights the considerable diagnostic capability of specific immunological biomarkers IL-6, IL-40, sCD28, VEGF, IL-17, and MCP-1 in differentiating rheumatoid arthritis patients from healthy subjects. ROC curve analysis demonstrated that four markers IL-6, IL-40, sCD28, and VEGF, achieved perfect diagnostic accuracy (AUC = 1.0) with 100% sensitivity and specificity, underscoring their robustness as diagnostic tools at defined cut-off values. IL-17 also exhibited near-perfect performance (AUC = 0.998), while MCP-1 maintained high sensitivity (100%) but showed slightly reduced specificity (69.7%) with an AUC of 0.967.

Table 3. ROC analysis of selected immunological parameters in RA diagnosis

Immunological Parameters:	AUC	Specify	Sensitivity	Cut-off-Value
IL-6 (pg/mL)	1.0	100%	100%	109.47
IL-17 (pg/mL)	0.998	100%	100%	78.81
IL-40 (ng/mL)	1.0	96.7%	100%	17.91
sCD28 (ng/mL)	1.0	100%	100%	13.292
MCP-1 (pg/mL)	0.967	69.7%	100%	161.445
VEGF (pg/mL)	1.0	100%	1%	253.23

*p-value: 0.0001

These findings align with and extend prior research conducted in Iraq. For example, Jaleel et al. (2022) found high serum levels of IL-6 in RA patients, though without defining diagnostic thresholds. Likewise, Hussein and Al-Fatlawi (2022) identified associations between IL-6 gene polymorphisms (−174 G/C) and increased cytokine levels in RA, emphasizing its pathogenic relevance but without ROC-based evaluation. The current study uniquely addresses this gap by establishing quantitative diagnostic thresholds. With regard to IL-17. Selimov et al. (2023) observed increased IL-17A levels and associations with anti-CCP and vitamin D deficiency, particularly in obese RA patients. However, diagnostic accuracy based on ROC analysis was not previously reported. To the best of our knowledge, this is the first research in Iraq assessing the diagnostic performance of IL-40, sCD28, VEGF, and MCP-1 in RA using ROC metrics. These biomarkers have not yet been validated in local cohorts, marking a novel contribution to RA biomarker research in Iraq. Collectively, the findings support the clinical utility of IL-6, IL-40, sCD28, and VEGF as reliable, sensitive, and specific biomarkers for RA diagnosis, with potential implications for early detection and personalized therapeutic strategies.

3.3. Expression of miRNA124 and miRNA223

Table 4 shows that quantitative expression profiling of miRNA-124 using the ΔΔCt method revealed distinct regulatory patterns in both patient cohorts. In the newly diagnosed RA (NP) group (n = 60), miRNA-124 was upregulated (ΔΔCt < 0) in 12 patients (20%), while a substantial majority of 48 patients (80%) exhibited downregulation (ΔΔCt > 0), indicating a marked suppression of miRNA-124 expression in early-stage RA. Similarly, in the treated patient (TP) group (n = 60), 20 patients (33.3%) showed upregulated expression levels, whereas 40 patients (66.7%) remained downregulated. These data suggest that miRNA-124 is predominantly downregulated in RA patients; however, treatment appears to partially reverse this trend, potentially reflecting therapeutic modulation of miRNA-124 expression and its involvement in the underlying pathophysiology of RA. Biologically, miRNA-124 functions as a negative modulator of inflammation, particularly through inhibition of the NF-κB pathway. Its downregulation is associated with elevated production of related mediators such as TNF-α, IL-6, and IL-1β**,** which promote to joint damage and synovial proliferation in RA. Treatment with DMARDs or biologics appears to alleviate inflammatory pressure, promoting the re-expression of miRNA-124 (El-Din et al, 2022; Qamar et al., 2025).

Table 4. Percentage of upregulated and downregulated miRNA124 expression among RA patients based on ΔΔCt analysis

Group	Total Patients	Upregulated (ΔΔCt < 0)	% Upregulated	Downregulated (ΔΔCt > 0)	% Downregulated
NP	60	12	20%	48	80%
TP	60	20	33.3%	40	66.7%

Table 5 presents the expression analysis of miRNA-223 using the ΔΔCt method, which revealed differential regulation patterns among the patient groups. In the ND cohort, miRNA-223 was upregulated (ΔΔCt < 0) in 22 patients (36.7%), whereas 38 patients (63.3%) demonstrated downregulation (ΔΔCt > 0), indicating a predominant reduction in miRNA-223 expression at disease onset. In contrast, among the TP, 26 individuals (43.3%) exhibited upregulated miRNA-223 expression, while 34 patients (56.7%) showed continued downregulation. These findings suggest that although miRNA-223 remains largely downregulated in RA patients, therapeutic intervention may contribute to partial restoration of its expression, potentially reflecting treatment-associated modulation of inflammatory regulatory mechanisms. Similarly, miRNA-223 showed a dominant downregulation trend in RA patients, demonstrating therapeutic responsiveness. It plays an efficient role in modulating innate immunity, particularly by modulating myeloid cell differentiation and cytokine production (e.g., IL-1β, STAT3). Its downregulation may enhance inflammatory cell activation in RA, whereas treatment helps restore its regulatory role (Li et al., 2010; Chen et al., 2014).

Table 5. Percentage of upregulated and downregulated miRNA223 expression among RA patients based on ΔΔCt analysis

Group	Total Patients	Upregulated (ΔΔCt < 0)	% Upregulated	Downregulated (ΔΔCt > 0)	% Downregulated
NP	60	22	36.7%	38	63.3%
TP	60	26	43.3%	34	56.7%

Table 6 showed that the mean ΔΔCt values for miRNA-233 in the NP and TP groups were 1.16 and 0.93, respectively, compared to 0 in healthy controls, indicating a relative downregulation in patients, which was more pronounced in the newly diagnosed group. In contrast, miRNA-124 showed a higher mean ΔΔCt of 3.61 in the NP group, suggesting substantial downregulation at disease onset, whereas treated patients exhibited a reduced mean ΔΔCt of 1.20, indicating partial restoration of miRNA-124 expression following treatment. Fold change analysis corroborated these findings, where miRNA-233 demonstrated a mild upregulation in the NP group (mean fold change ~1.11) and a significant increase in the TP group (mean fold change ~3.77), implying a possible treatment-associated upregulation or compensatory mechanism.

Table 6. ΔΔCt and fold change values of miRNA-233 and miRNA-124 in newly diagnosed, treated and healthy subjects

Group	Mean ΔΔCt miRNA233	Mean ΔΔCt miRNA124	Mean Fold Change miRNA233	Mean Fold Change miRNA124
NP	1.1583	3.6090	1.1077	0.4256
TP	0.9253	1.2037	3.7687	2.2720
HC	0.0000	0.0000	1.0000	1.0000

Conversely, miRNA-124 exhibited marked downregulation in the NP group (mean fold change ~0.43) and notable upregulation post-treatment (mean fold change ~2.27), further supporting the therapeutic impact on miRNA expression profiles. Healthy controls-maintained baseline expression (fold change = 1) for both miRNAs. These results suggest that miRNA-233 and miRNA-124 are differentially expressed in RA patients and modulated by treatment, highlighting their potential roles as biomarkers for disease activity and therapeutic response. Though not the central focus, miRNA-233 displayed a different trend. Despite mild downregulation based on ΔΔCt values, its fold change increased significantly post-treatment, suggesting an adaptive or compensatory upregulation in response to inflammation resolution or tissue recovery. The observed inter-individual variability where some patients exhibit

upregulation while others show downregulation of these miRNAs is likely due to heterogeneity in disease severity, treatment response, immune environment, genetic background, and epigenetic regulation. In early RA, elevated inflammatory cytokines can suppress anti-inflammatory miRNAs, while effective therapy may reverse this suppression. Additionally, genetic polymorphisms**,** epigenetic alterations (e.g., DNA methylation), and differences in cell type specific miRNA expression (e.g., in T cells, macrophages, synoviocytes) contribute to expression variability. The clinical diversity of RA ranging from Th17-driven to macrophage-dominant phenotypes also influences miRNA regulation patterns (Das and Rao, 2022).

3.4. Correlation Analysis of miRNAs and Immunological Biomarkers

A Pearson correlation heatmap was generated to evaluate the relationship between miRNA expression levels (miRNA124 and miRNA223) and key immunological biomarkers (IL-6, IL-17, IL-40, sCD28, MCP-1, and VEGF) in patient samples. The analysis revealed a moderate to strong positive correlation between IL-6 and IL-17**,** suggesting coordinated regulation of these pro-inflammatory cytokines in disease pathogenesis. Additionally, sCD28 exhibited a positive correlation with MCP-1 and VEGF**,** implying a potential link between T-cell costimulatory signaling and angiogenic or chemotactic pathways. miRNA223 revealed a reverse association with IL-40, supporting a potential regulatory function for modifying anti-inflammatory or homeostatic signals. In contrast, miRNA124 showed weak to moderate adverse interactions with VEGF and IL-6, revealing its role in the regulation of inflammatory and vascular responses.

These obtained results underline the complex interaction between miRNAs and immune mediators, indicating their importance as diagnostic or prognostic parameters in inflammatory conditions. miRNA-124 had an inverse connection with VEGF, MCP-1, and sCD28, indicating a possible inhibitory function on angiogenesis, monocyte recruitment, and T-cell costimulation, respectively (Figure 2). The unfavorable relationships underscore miRNA-124 as a potential inhibitor of chronic inflammatory pathways in rheumatoid arthritis (RA). miRNA-223 exhibited a robust positive connection with IL-6 and IL-17, two characteristic proinflammatory cytokines in the etiology of RA. This indicates that miRNA-223 may be increased in response to or actively involved in Th17-mediated inflammatory signaling, hence reinforcing its function as a contributor to synovial inflammation. Conversely, IL-6 and IL-17 exhibited a positive correlation with VEGF and MCP-1, substantiating the idea that inflammatory cytokine signaling is intricately associated with angiogenic activity and monocyte chemotaxis in the RA synovium. These findings underscore the therapeutic potential of targeting miRNAs to modulate inflammation in RA. Specifically: Inhibition of miRNA-223 could attenuate IL-6/IL-17-driven inflammation, thereby reducing tissue destruction and joint degradation.

Conversely, enhancing miRNA-124 expression may suppress VEGF- and MCP-1-mediated pathways, offering a dual anti-inflammatory and anti-angiogenic strategy. Given their central roles in regulating both cytokine production and immune cell recruitment, miRNA-124 and miRNA-223 represent promising therapeutic targets in RA. Modulating their expression may not only help in disease control but also minimize systemic side effects associated with broad-spectrum immunosuppression. The correlation analysis reveals that miRNA-124 and miRNA-223 are closely associated with key inflammatory and angiogenic mediators in RA. miRNA-223 shows a strong positive correlation with pro-inflammatory cytokines IL-6 and IL-17, indicating its role in promoting Th17-mediated inflammation and synovial immune activation In contrast, miRNA-124 exhibits negative correlations with VEGF, MCP-1, and sCD28, suggesting it functions as a suppressor of angiogenesis, monocyte recruitment, and T-cell co-stimulation (Wu et al., 2023). These findings highlight miRNA-124's potential as an anti-inflammatory and anti-angiogenic regulator, whereas miRNA-223 may contribute to

inflammatory amplification. Therapeutically, inhibiting miRNA-223 could attenuate IL-6/IL-17-driven inflammation, while enhancing miRNA-124 expression might suppress pathological vascular and immune responses, offering promising strategies for RA treatment (Aziz, 2016; Zhang et al., 2023).

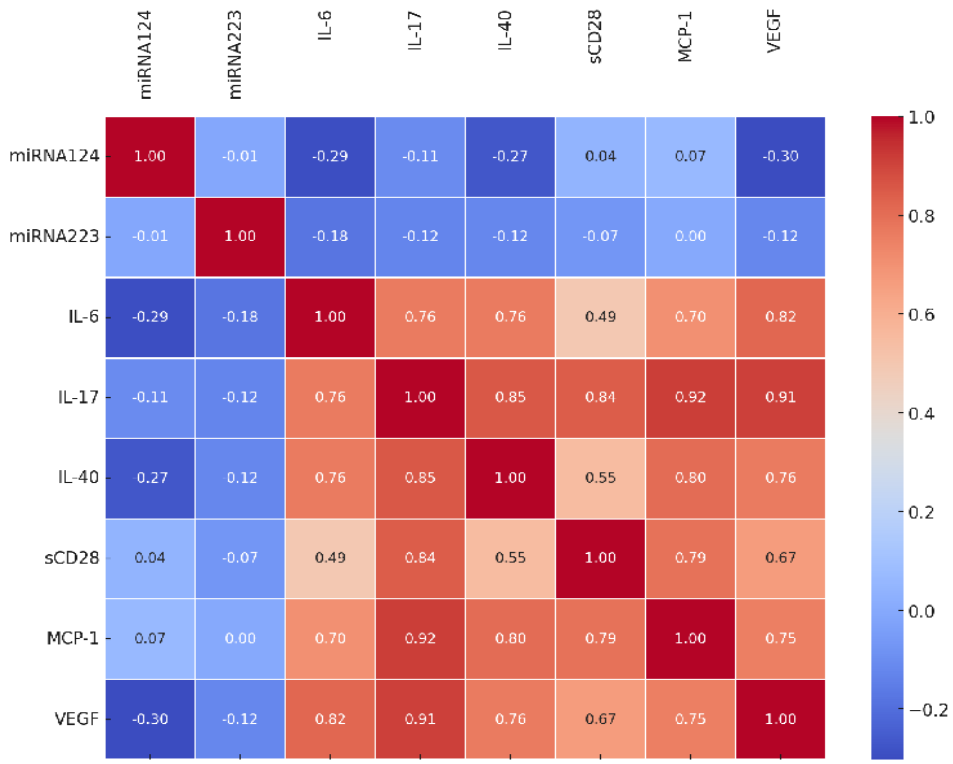

Figure 2. Correlation heat map between miRNA124, miRNA223 and immunological parameters

Several limitations deserve consideration in the future. The relatively small sample size, especially within the stratified subgroups, may limit the statistical power and generalizability of the results. Being a single centre study conducted in Baghdad, the findings may not be fully representative of broader or more diverse populations. Furthermore, the lack of detailed stratification by disease severity, duration, or comorbidities may have influenced the observed biomarker and miRNA expression patterns. These factors highlight the need for larger, multicentre, and longitudinal studies to validate and extend these findings. Subsequent investigations should encompass larger, more heterogeneous cohorts and utilize longitudinal study designs to monitor temporal alterations in biomarker profiles. Comprehensive functional analyses are warranted to elucidate the mechanistic interplay between microRNAs and immunological mediators. The integration of multi-omics data will facilitate a more holistic understanding of the underlying pathophysiological mechanisms. Rigorous validation of candidate biomarkers in independent clinical populations is crucial prior to their translational application. Furthermore, the therapeutic modulation of specific microRNAs and cytokines represents a promising avenue for targeted intervention. The adoption of cutting-edge analytical methodologies and the exploration of gene-environment interactions will further refine biomarker discovery and enhance diagnostic precision. Validation of identified biomarkers in independent clinical populations is essential to confirm their diagnostic and prognostic utility. Moreover, therapeutic targeting of dysregulated microRNAs and cytokines holds potential for precision medicine. Future studies should also incorporate advanced analytical technologies and investigate gene-environment interactions to improve biomarker discovery and diagnostic accuracy.

4. CONCLUSION

This study indicated that serum levels of IL-6, IL-17A, IL-40, sCD28, MCP-1, and VEGF were markedly raised elevated in newly diagnosed RA patients compared to treated patients and healthy controls, reflecting active inflammation, immune activation, and angiogenesis. Notably, miR-124 exhibited negative correlations with sCD28, MCP-1, and VEGF, suggesting a suppressive role in T cell co-stimulation, monocyte recruitment, and vascular remodelling. Conversely, miR-223 showed positive correlations with IL-6 and IL-17A, indicating its involvement in amplifying Th17-driven inflammatory responses. These associations underscore the interplay between miRNA regulation and immunological pathways in RA, supporting the utility of miR-124 and miR-223 as biomarkers for disease activity and potential therapeutic targets.

Conflict of Interest
Researchers confirm that they have nothing to declare in relation to the publication of the paper.

Author Contribution Statement
Aya Sabah Hameed: Specimens collection, conceptualization and methodology. Rawaa AlChalabi: Data curation, writing original draft, review and editing.

Data Availability Statement
The authors ensure that data supporting its findings are included within the article.

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