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The Association between the Expression of MicroRNA-4270 and MicroRNA-4441 with some Metabolic Factors in Iranian Rheumatoid Arthritis Patients

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ABSTRACT

MicroRNAs (miRs) play a role in several diseases, such as rheumatoid arthritis (RA). The purpose of this study was to discover new microRNAs and investigate their involvement in RA, examining their connections with inflammation and metabolic markers.

New microRNAs related to RA were predicted using Mirbase and TargetScan databases based on RA target genes. The relationships between miRNAs and targets were visualized with Cytoscape software. Real-time polymerase chain reaction confirmed detectable miRNAs and metabolic factors were assessed using immunoassay and spectrometry methods in RA patients and healthy subjects. Four microRNAs (hsa-miR-153-5p, hsa-miR-4270, hsa-miR-4441, and hsa-miR-6754-5p) showed the highest correlation with RA target genes among millions of microRNAs.

The expression of miR-146b (fold change=1.8) and miR-4441 (fold change=1.7) was notably reduced, while miR-4270 showed upregulation (fold change=1.8) in plasma from RA patients compared to healthy individuals. MiR-6754 exhibited a decrease (fold change=1.3) but was statistically insignificant. MiR-153-5p expression was undetectable in plasma. Receiver operating characteristic (ROC) curve analysis indicated that miR-4441, with an area under the ROC curve (AUC) of 0.7728, and miR-4270 (AUC=0.7353) were promising biomarkers for RA. The expression of these studied miRNAs significantly correlated with essential clinical characteristics, including liver enzymes, cholesterol, phosphorus, and vitamin D3.

Our findings suggest that miR-4270 and miR-4441, present in the circulation, exhibit distinct expression patterns in RA. These microRNAs may serve as links between inflammation and metabolism and represent promising new biomarkers for this disease.

Keywords: Biomarkers, Inflammation, Metabolomics, MicroRNAs, Rheumatoid arthritis

INTRODUCTION

Rheumatoid arthritis (RA) is an inflammatory disease that destructs, deforms, and disables joints with heterogeneous manifestations. The pathogenesis of RA is still unclear; however, considerable data have

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demonstrated that the disease occurs commonly in middle-aged women with genetic predisposition exposed to various environmental factors such as microbial factors, smoking, living conditions, and other unknown factors.[1] Complex interactions between genes and the environment lead to a breakdown of immune tolerance, occurrence of inflammation, and induction of peptidyl arginine deaminases (PADs).[2] PADs modify peptides by converting arginine to citrulline. The modified proteins, in turn, are presented to T cells after processing by antigen-presenting cells (APCs).[3] These events lead to the local and systemic production of anti-citrullinated protein antibodies (APCAs), including rheumatoid factor (RF), anti-cyclic citrullinated peptide (anti-CCP), and anti-mutated citrullinated vimentin (anti-MCV) against the modified peptides.[4] On the other hand, studies have shown that metabolic alterations also occur in some people with RA.[5] Hence, it is suggested that their biochemical markers can be altered, such as blood sugar (BS), cholesterol (Chol.), triglyceride (TG), and liver enzymes like aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP).

MicroRNAs (miRNAs or miRs) are a conserved class of small noncoding RNAs that act as negative gene regulators. They have been found in human plasma and other body fluids, indicating their high stability in the extracellular environment and suggesting they may control cell-to-cell communication in healthy and disease circumstances.[6] Accumulated evidence has shown that microRNAs could be diagnostic and prognostic biomarkers for RA. They may be a critical epigenetic component in the breakdown of immune tolerance and progression toward RA disease onset.[7] In addition to autoimmune disease, miRNAs contribute to the pathogenesis of obesity[8] and impaired oxidative metabolism.[9] Epigenetic dysregulations partly drive these RA metabolic impairments.[10] However, it is unclear whether RA-related miRNAs contribute to the RA comorbidities of obesity and altered metabolism.[5]

There is limited knowledge of the miRNAs in RA pathogenesis, particularly during the preclinical phase of the disease.[7] Previous studies have evaluated several miRNAs and target genes in diseases such as RA by alterations in their expression compared to healthy controls. However, none has been entirely approved as a biomarker for diagnostic and therapeutic purposes of RA until now. Based on this information, we examined some miRNAs not studied in RA disease. Thus, our primary goal was to investigate the plasma expression of miR-4270, miR-153-5p, miR-4441, miR-6754-5p, and miR-146b in Iranian patients with RA compared to healthy people. To help diagnose or treat the disease, we assessed the biomarker capability of those miRs in RA. In the next step, our minor objective was to compare RA plasma expression of each miRNA to disease activity and metabolic parameters. We hypothesized that some miRNAs would reflect RA disease activity and metabolic alterations.

MATERIALS AND METHODS

Participants

In this cross-sectional investigation, one hundred forty-four individuals were included: 72 patients diagnosed with RA and 72 healthy controls, among those referred to the pathobiology medical diagnosis Hakim laboratory, Tabriz-Iran, from October 2018 to March 2021. People with a history of autoimmune diseases, cardiovascular diseases, liver diseases, kidney diseases, malignancy, and other chronic diseases were excluded from the study to select the control group. Clinical and laboratory parameters for RA patients are shown in Table 1.

Blood Samples and Assessment of Biological Parameters

All the blood samples were processed to separate plasma or serum. Serological tests such as RF and CRP were monitored in serum using an immune turbidimetric assay (Pishtaz RF kit, Iran). The Westergren method measured the ESR (DragonMed 2010 ESR Analyzer, Malaysia, Shah Alam). Biochemical tests, including BS, Chol., TG, hepatic enzymes (AST, ALT, and ALP), calcium, and phosphorus, were measured by spectrometric assay (Pars Azmoon Assay kits, Iran; Alpha Classic Auto Analyzer, Iran). Anti-CCP and anti-MCV were measured by the Enzyme Immunoassay (EIA) technique (AESKULISA Elisa Test Kits, Germany). Thyroid-stimulating hormone (TSH) and vitamin D3 were measured using immunofluorescence (Biomerieux test kits, France; VIDAS immunoanalyzer, France).

Table 1. Participants' clinical and laboratory features are depicted.

Individual characteristics	RA samples (n=72)	Healthy control samples (n=72)
Sex (male/female)	1/6	1/3
Age, mean (range)	51.32 (30–78)	49 (28–75)
Disease duration (y), mean (range)	10.2 (1–25)	N/A
Smoking, number (%)	20%	3%
Serologic tests		
ESR (mm), mean	21.75	8.04
CRP (mg/dL), mean	16.07	5.10
Positive rheumatoid factor (u/mL), n (%)	4 (20%)	2 (10%)
Positive anti-CCP antibody (u/ml), n (%)	28 (87/5%)	N/A
Positive anti-MCV antibody (u/ml), n (%)	20 (71.43%)	N/A
Metabolic factors		
BS (mg/dL), mean (High %)	123 (7.3%)	90.5 (13%)
Cholesterol (mg/dL), mean (High %)	196.84 (17.1%)	182.14 (28%)
TG (mg/dL), mean (High %)	144.25 (12.2%)	167.12 (14%)
Hepatic Enzyme AST (U/L), mean (High %)	19.66 (4.9%)	17.63 (9%)
White blood cells (10^6/µL), mean	7.45	6.89
TSH (mU/L), mean (out of normal range %)	2.32 (12.55%)	3.17 (0.03%)
Vitamin D_3 (ng/mL), mean (less than normal range %)	37.97 (9.2%)	34.22 (10%)
Calcium (mg/dL), mean, n (%)	10.7 (55.05%)	9.78 (49.08%)
Phosphorus (mg/dL) , mean, n (%)	2.43 (22.6%)	3.55 (21.34%)

anti-CCP: anti-cyclic citrullinated peptide; anti-MCV: anti-mutated citrullinated vimentin; AST: aspartate aminotransferase; BS: blood suger; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; N/A: not applicable; TG: triglyceride; TSH: thyroid stimulating hormone.

Target Prediction and Selection of MicroRNAs Using Bioinformatics Tools

We used a unique method to select the appropriate microRNAs in RA disease. First, genes related to rheumatoid arthritis were identified [approximately eight genes, including protein tyrosine phosphatase non-receptor type 22 (PTPN22), cluster of differentiation 244 (CD244), class II, major histocompatibility complex, transactivator (CITTA), SPT20 homolog, SAGA complex component (SUPT20H), protein-arginine deiminase type-4 (PADIA4), solute carrier family 22, member 4 (SLC22A4), NF-kappa-B inhibitor-like protein 1 (NFKBIL1), interleukin 10 (IL10)] using NCBI and Genome Ensemble sites. Subsequently, by entering the gene data into the Target Scan software (version 8.0), microRNAs were predicted, and then using the Cytoscape software (3.9.0), the network connection plotted between them (Figure 1).

RNA Extraction

Total RNA extraction steps were performed according to the modified version of RiboEx's kit (Gene All, South Korea) method with the TRIzol reagent. The purity and concentration of the RNA obtained were determined through 260/280 nm absorbance measures using the NanoDrop spectrophotometer (DeNovix, USA).

cDNA Synthesis

cDNA was synthesized using the ExcelRT™ Reverse Transcription Kit (SMOBIO Technology, Germany), a stem-loop for each type of microRNAs (supplementary table), and Thermal Cycler (Bio-Rad, USA) were used for cDNA synthesis. According to the manufacturer's instructions, we first prepared ten µL of mixture A (RNA, primers, and dNTPs) and ten µL of mixture B (cDNA buffer). Finally, the mixture was incubated in a thermal cycler (25°C/10 minutes,

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followed by 85°C/5 min). One µL RNase H enzyme was added into each reaction at 37°C for 20 minutes to remove RNA.

Quantitative Real-time PCR Analysis

Quantitative real-time polymerase chain reaction (PCR) was performed using a StepOne thermal cycler (StepOne™ Real-Time PCR System, USA). Reactions were carried out in a total of 20 µL, including 10 µL SYBR Green PCR Master Mix (SMOBIO Technology, ExcelRT™ Reverse Transcription Kit, Germany), 0.5 µL forward primer, 0.5 µL universal reverse primer, 1 µL cDNA and, 8 µL nuclease-free H_2O. After adding the resulting mixture to the 96-well plate for the StepOne thermal cycler, the system program was set according to the manufacturer's recommendations. First, the initialization phase at 94°C for 2 minutes, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 seconds, and elongation at 72°C for 30 seconds. Normalized miRNA expressions were used to analyze the data measuring the expression of studied miRs. For this purpose, ΔCt was calculated. The internal control miRs Ct values were subtracted from the Ct of the studied miRNAs to calculate ΔCt.

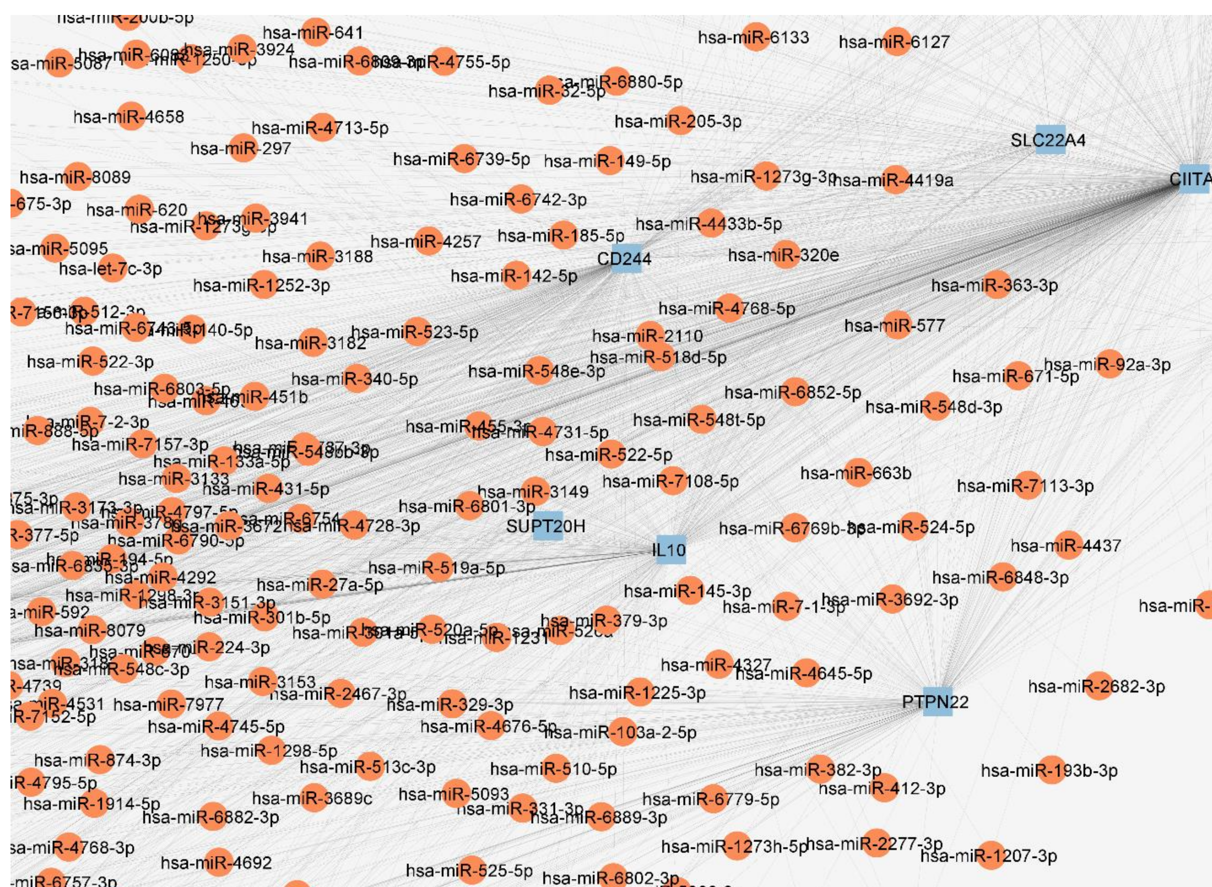

Figure 1. A network of gene-microRNA (miRNA) associations was generated using Cytoscape software. It reveals multiple miRNAs with connections to genes associated with rheumatoid arthritis. Blue rectangles represent genes, while miRNAs are depicted as orange circular shapes. Dark lines illustrate the relationships between them.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 8 software. Correlations between miRNA expressions and patients' clinical data were analyzed by Pearson correlation coefficient test using Minitab version 17 software. Receiver operating characteristic (ROC) curve analyses, plotting the true positive rate (sensitivity) vs. the false positive rate (1 − specificity) at various threshold settings were performed for plasma miRNAs, and the areas under the curve (AUCs) were calculated. Statistical significance was considered at a $p \leq 0.05$.

RESULTS

Bioinformatics Data Analysis

We found 34 miRNAs among millions of miRNAs that are more related to the target genes. Then, among these 34, only four microRNAs showed the highest correlation (hsa-miR-153-5p, hsa-miR-4270, hsa-miR-4441, and hsa-miR-6754-5p). Therefore, we investigated their expression profile in RA disease for the first time, and miR-146b was considered as a control in these experiments (Figure 2).

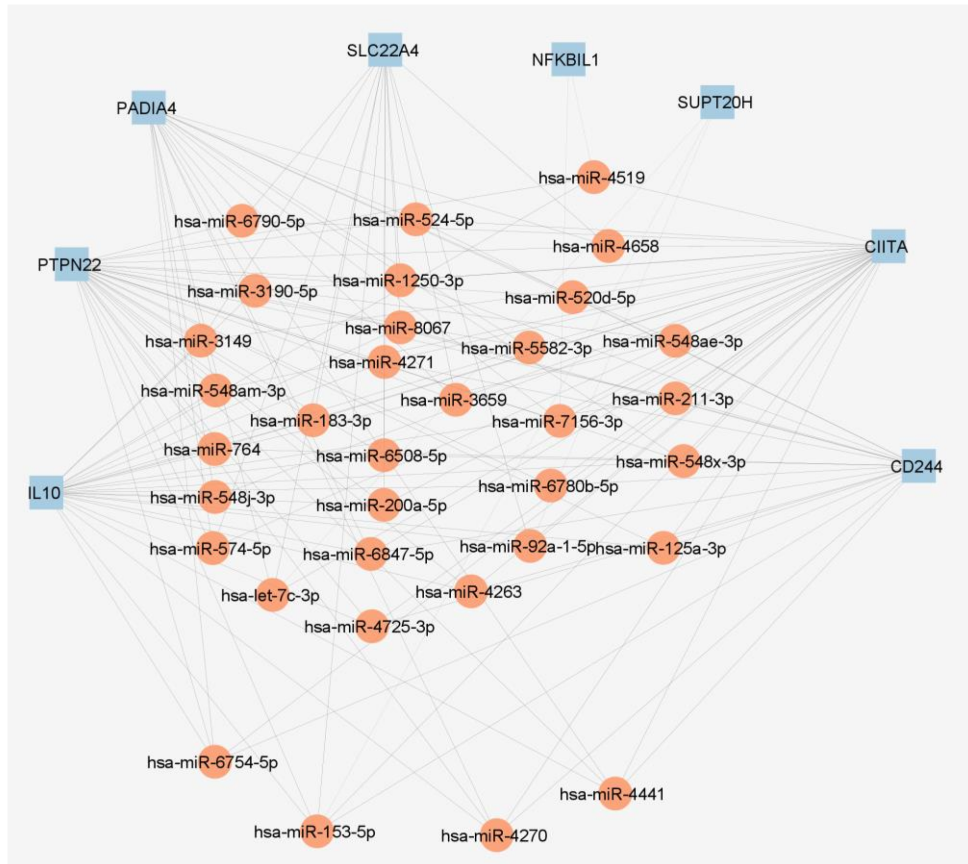

Figure 2. Identification of four microRNAs (miRNAs) that demonstrated the highest correlation with rheumatoid arthritis (RA) targets. After mapping the relationship of miRNAs with RA target genes using Cytoscape software, we found 34 miRNAs among millions of target genes with better connections than others. Four miRNAs showed the most association with the targets. Hence, we hypothesized that these four miRs are probably related to RA disease and can be investigated to select them as biomarkers seen in the lower part of the figure. The genes have been shown in blue rectangular, and the miRNAs in orange circular shapes. Associations have also been depicted with dark lines between them.

MicroRNA Profiles in RA Patients and Healthy Individuals

The results of PCR analysis of our data showed that the expression of miR-146b-5p was downregulated in RA plasma samples compared to healthy controls (Figure 3A). An AUC of 0.6744 was estimated for miR-146b (Figure 3B).

The results of PCR data about miR-153-5p showed that this miRNA could not be detected in plasma, so it is not present in plasma. Therefore, we could not measure the difference in its expression in RA compared to healthy people or its relationship with biochemical factors in this study. miR-4270 expression significantly increases in RA patients (p=0.0046) compared to normal controls (Figure 3C). Likewise, ROC analysis was conducted to investigate whether the expression of miR-4270 could be used as a diagnostic biomarker of RA. We found that AUC of 0.7353 was for miR-4270 (Figure 3D), suggesting that this microRNA may be referred to as a possible diagnostic biomarker.

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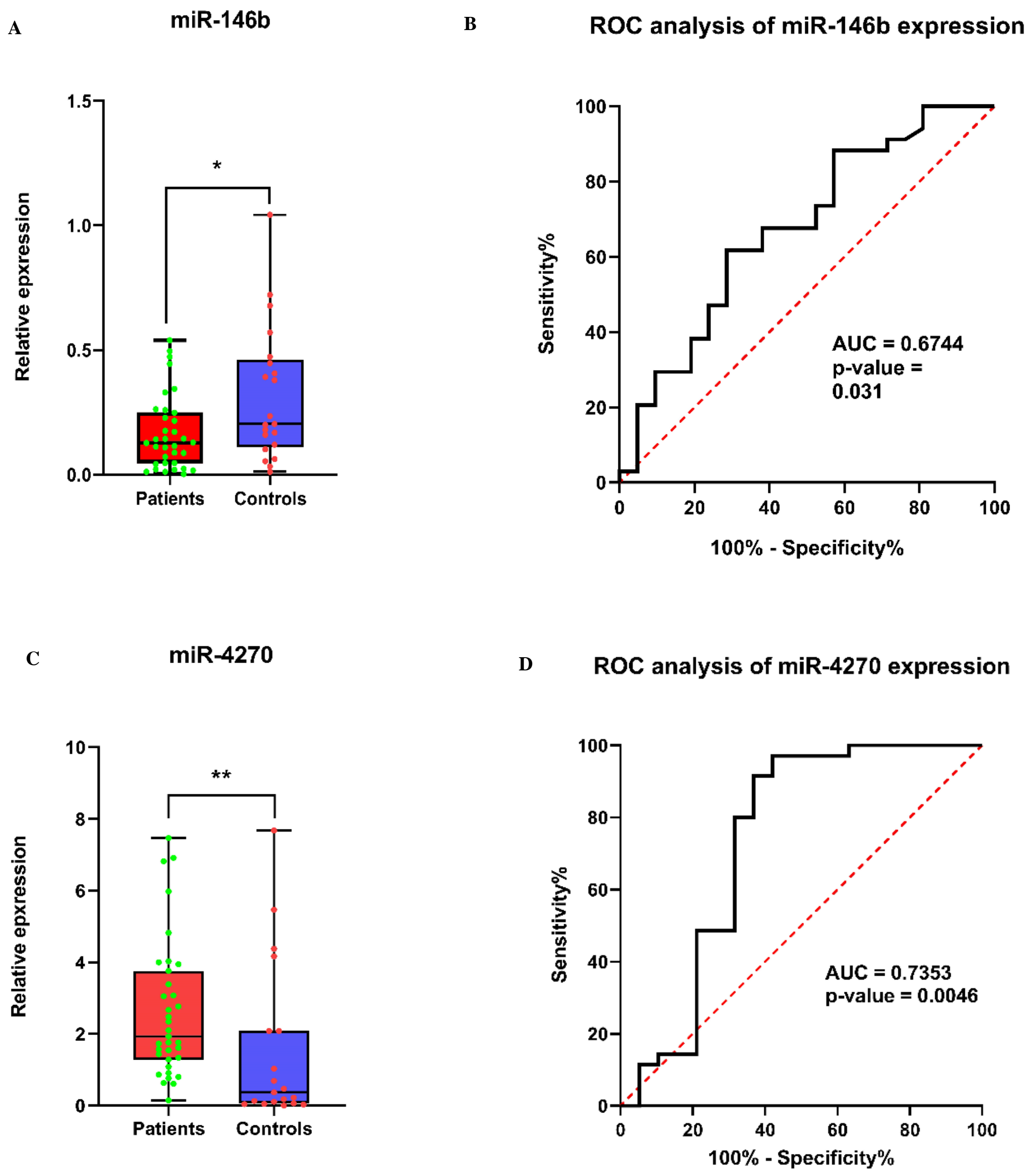
In our results, the expression miR-6754 in RA plasma samples showed a slight decrease compared to the healthy control group, but this change was insignificant (Figure 3E). In addition, the ROC curve analysis of miR-6754 showed that it did not confirm the biomarker potential of miR-6754 with an AUC of 0.5778 and *p*=0.3711 (Figure 3F). Finally, the expression of miR-4441 is significantly decreased (*p*=0.0022) in RA patients compared to the control group (Figure 3G). We found that the AUC was 0.7728 for miR-4441, the best AUC among studied miRNAs (Figure 3H), like miR-4270, which suggests it may be a biomarker for diagnosing this disease.



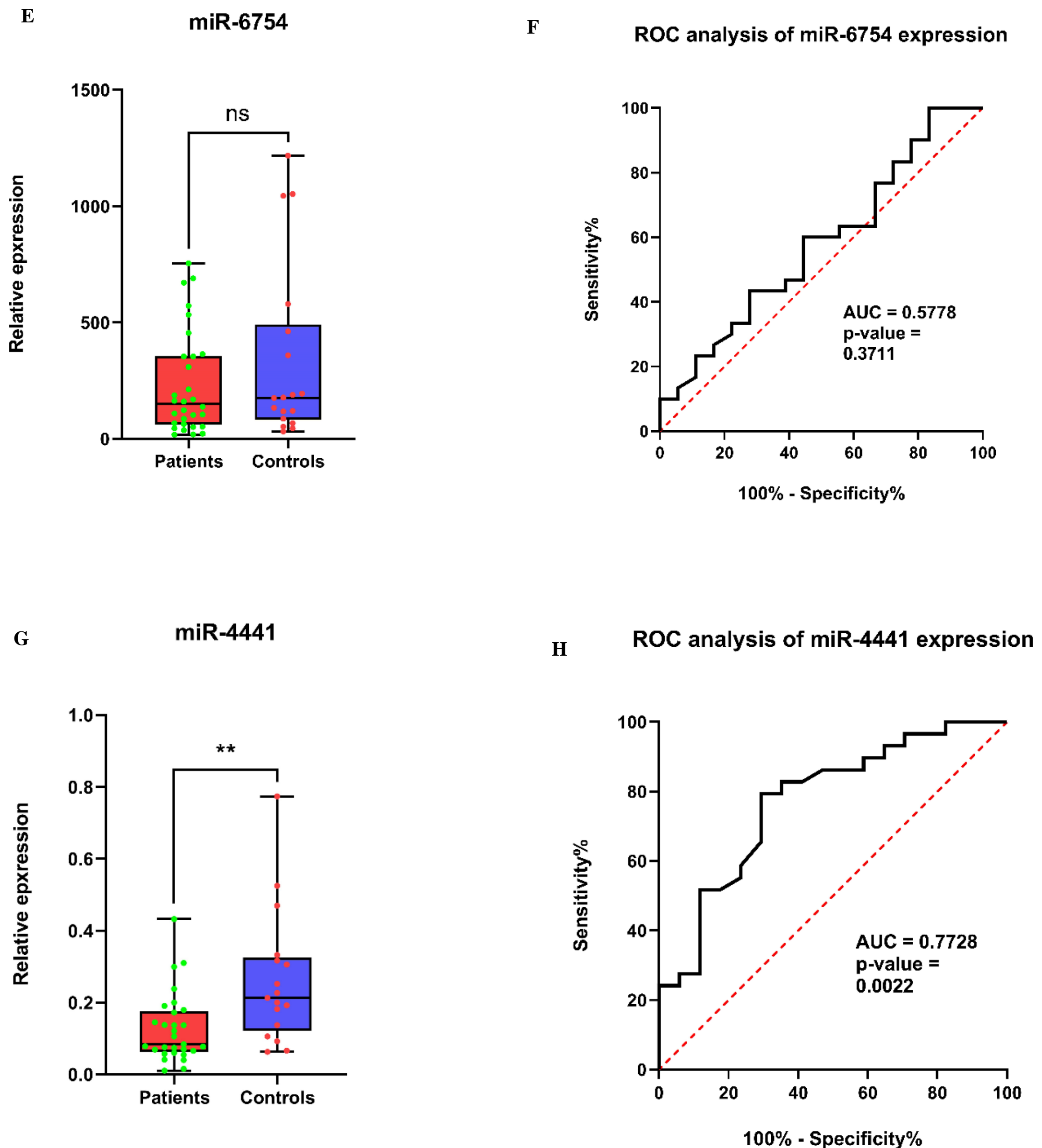


Figure 3. Relative mean expressions of miR-146b, miR-4270, miR-6754-5, and miR-4441 in plasma of rheumatoid arthritis patients and healthy subjects (A, C, E, and G). As can be seen, except for miR-6754-5, all the miRs selected in the study indicated a significant difference among the patients compared to the healthy control subjects. ROC curve analysis was used to estimate the biomarker potential value of miRs (B, D, F, and H). Among the four microRNAs, miR-4270 and miR-4441 have good areas under the curve (AUCs), and therefore, these two can be introduced as possible biomarkers for RA disease. AUC: the areas under the curve; ROC: receiver operating characteristic, RA: rheumatoid arthritis.

Associations between RA Disease and Clinical Markers

MiR-146b expression was associated with RF and erythrocyte sedimentation rate (ESR) (Figures 4A and 4B), and miR-4270 expression was associated with ESR (Figure 4C). In addition, the expression level of miR-4441 showed a positive correlation with c-reactive protein (CRP) and anti-CCP (Figures 4D and 4E). Plasma miR-146b was positively associated with AST and negatively related to ALP (Figures 4F and 4G). MiR-4270 was positively associated with cholesterol and AST (Figures 4H and 4I). miR-6754-5p showed significant relationships to AST and serum phosphorus (Figures 4J and 4K). Finally, miR-4441 significantly correlated with vitamin D3 (Table 2 and Figure 4L).

Table 2. Plasma microRNAs relationships in rheumatoid arthritis

Variables	miR-146b		miR-4270		miR-4441		miR-6754	
	Correlation	*p*	Correlation	*p*	Correlation	*p*	Correlation	*p*
Age	0.09	0.60	0.08	0.63	-0.06	0.69	-0.09	0.53
CRP	0.06	0.68	-0.002	0.99	0.42	0.008**	0.06	0.73
RF	0.36	0.03*	-0.20	0.22	0.30	0.07	-0.15	0.36
ESR	0.32	0.04*	0.32	0.04*	-0.04	0.80	-0.18	0.27
Anti-CCP	-0.08	0.65	-0.11	0.55	0.38	0.03*	-0.22	0.22
Anti-MCV	-0.23	0.71	-0.84	0.07	-0.47	0.43	-0.82	0.09
WBC	0.002	0.99	-0.15	0.49	0.23	0.29	-0.09	0.66
BS	-0.10	0.59	-0.09	0.64	0.08	0.69	-0.29	0.13
Chol.	-0.34	0.26	0.53	0.04*	-0.05	0.88	0.05	0.88
TG	-0.07	0.81	0.35	0.19	-0.51	0.08	-0.08	0.79
AST	0.48	0.008**	0.35	0.05*	-0.03	0.86	0.38	0.03*
ALT	-0.16	0.38	0.19	0.29	0.15	0.44	0.13	0.46
ALP	-0.75	0.01*	0.24	0.50	-0.17	0.64	-0.52	0.12
Calcium	0.17	0.60	-0.02	0.95	-0.53	0.09	-0.39	0.24
Phosphorus	-0.49	0.17	0.47	0.19	0.47	0.21	0.72	0.03*
Vitamin D_3	0.65	0.15	0.14	0.78	0.85	0.03*	0.19	0.72
TSH	0.13	0.53	-0.09	0.66	-0.13	0.53	-0.07	0.73

CRP: C - reactive protein; RF: rheumatoid factor; ESR: Erythrocyte Sedimentation Rate; anti-CCP: anti-cyclic citrullinated peptide; anti-MCV: anti-mutated citrullinated vimentin; WBC: White blood cells; BS: blood sugar; Chol.: Cholesterol; TG: Triglyceride; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; TSH: thyroid stimulating hormone.

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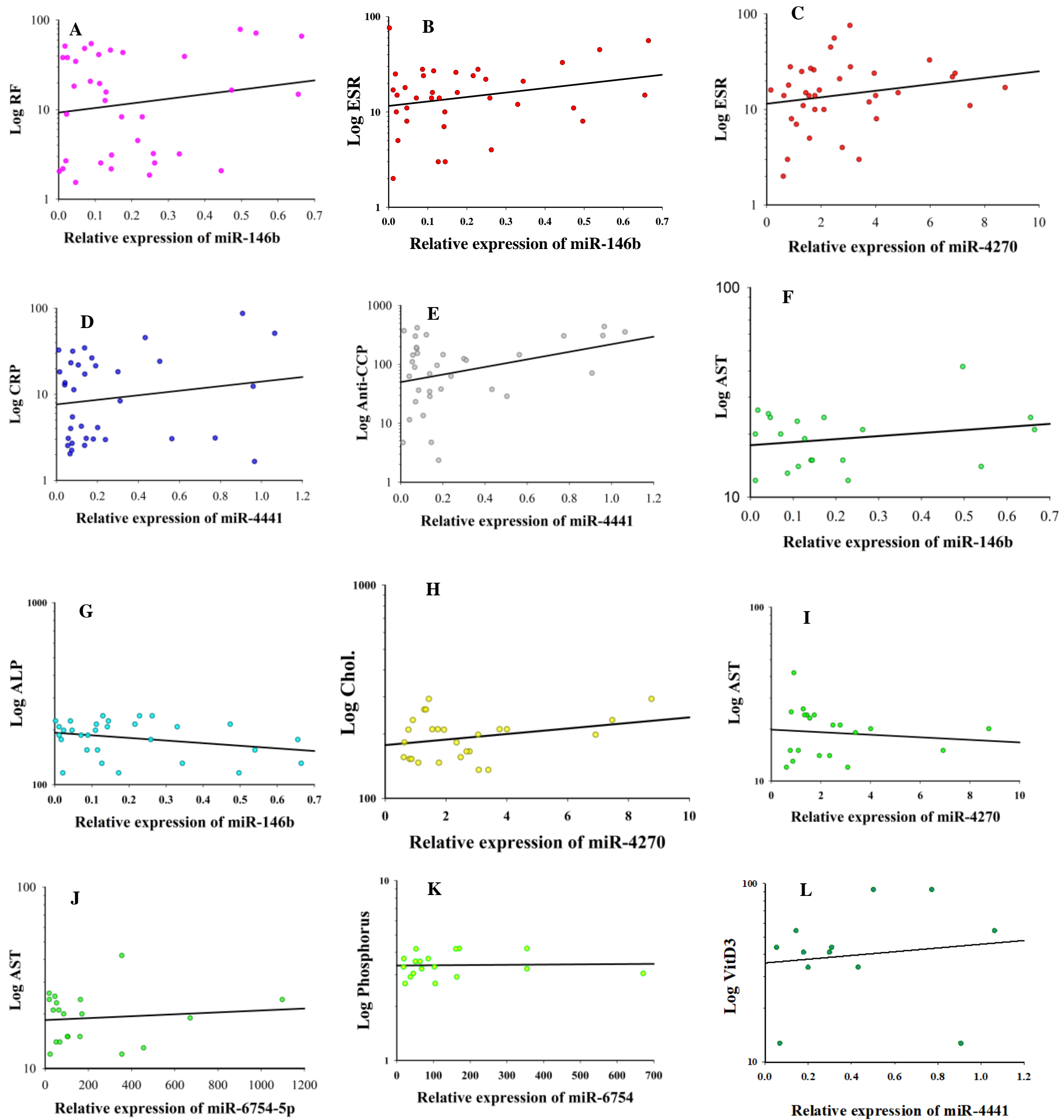

Figure 4. Association of plasma miRNAs expression with clinical characteristics in RA patients. Using Sigma Plot software version 14.0, correlations between plasma microRNA (miRNA) expression and changes in inflammation factors (A to D) and metabolic markers (E to L) were examined. The results are presented based on the significance of *p* values, indicating the correlation between clinical characteristics and miRNA expressions. RF, rheumatoid factor; CRP, C-reactive protein; anti-CCP, anti-cyclic citrullinated peptide; ESR, erythrocyte sedimentation rate; AST, aspartate aminotransferase; ALP, alkaline phosphatase; Chol., cholesterol.

DISCUSSION

This study investigated whether plasma expressions of miR-4270, miR-153-5p, miR-4441, and miR-6754-5p correlate with RA patients and metabolic parameters in this disease condition. We found significant differences between RA and healthy subjects in plasma miR-4270, miR-4441, and miR-146b profiles. miR-6754-5p expression was not significantly different in RA plasma and healthy samples. Nevertheless, this miR was significantly associated with AST enzyme and serum phosphorus. Hence, it can be assumed that miR-6754-5p is probably unrelated to RA disease. It may be involved in regulating biochemical pathways due to metabolic diseases. More studies should be done to get more definitive results. As mentioned, it was the first time miR-153-P was evaluated in RA disease. Our results showed the absence of miR-153-p in plasma. Thus, we could not establish the relationship between miR-153-5p and RA and its relationship with biochemical factors. Past studies on miR-6754-5p and miR-153-5p have mainly focused on their roles as potential tumor biomarkers in cancers[11,12] but have not established the relationship between them and RA. Therefore, the role of miR-153-5p in RA remains to be determined once it is re-examined in the future in other parts of the body, including inside blood cells, inside exosomes, or in joint fluids and cells.

MiR-146 plays an essential role in the nuclear factor kappa light chain enhancer of activated B cells (NF-κB) signaling pathway in innate immunity. It downregulates interleukin 1 receptor-associated kinase 1 (IRAK1) and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), decreasing apoptosis.[12,13] Also, miR-146 regulates the immune system by affecting autophagy, the signal transducer, and activator of transcription (STAT) family pathways.[13-15] Against most previous literature reports,[7,16-19] and consistent with some research,[20] we found that miR-146b expression decreases in the plasma of patients compared to healthy controls. The inconsistency is likely due to the instability of miRNAs in plasma. Because the studies mentioned above have primarily examined the expression of miR-146 inside the cells, some miRNAs are secreted as exosomes.[21] It may be speculated that long-term inflammation without treatment in patients may lead to secondary events affecting the miRNA expression profile. On the other hand, we cannot completely rule out that glucocorticoid or anti-rheumatic treatments may have at least partially influenced the results of our study.

We observed that miR-4270 expression in RA plasma was significantly higher than in normal control subjects. MiR-4270 expression changes have been primarily investigated in cancer diseases, including breast cancer,[22] gastric cancer,[23] and helicobacter pylori (Hp) gastric infection.[24,25] It has been reported that changes in the expression miR-4270 in breast cancer patients are significantly increased compared to healthy individuals.[22] This miRNA also plays an essential role in brain metastasis associated with lung adenocarcinoma.[26] PCR results of current research determined that miR-4441 expression was significantly decreased in RA patients compared to normal individuals. Limited studies have been conducted on miR-4441; in one study, miR-4441 was identified along with miR-4270 as a new biomarker of Alzheimer's disease (AD).[27] The best AUC results from ROC analysis were for miR-4270 and miR-4441 (Figure 3D), suggesting that miR-4270 and miR-4441 could be biomarkers in RA disease.

Our results revealed that miR-4270 and miR-4441 expressions were related to inflammation indexes (Table 2 and Figures 3C-E). In agreement with previous studies, we observed significant associations between inflammation markers, including RF and ESR, with miR-146. It is suggested that the dysregulation of the expression of miR-4270, miR-4441, and miR-146 may be closely related to RA activity. Ciechomska et al. identified circulating miR-146b as a reliable marker of disease activity with improved specificity. Increased miR-146b serum concentration is associated with lower the disease activity score-28 for rheumatoid arthritis with CRP (DAS28-CRP) and ESR (DAS28-ESR).[28]

In the past, researchers showed the levels of RA antibodies (CCP-Ab or RF) are increased in patients compared to healthy individuals, and we showed the same result in this study.[29] Anti-CCP should be preferred over RF as it is associated competently with disease activity.[30] Positive correlations have been observed between ESR, CRP, anti-CCP, and miR-146a levels.[31]

A raised white blood cell (WBC) count implies inflammation, while a lower count suggests a mechanical condition or osteoarthritis.[32] Concerning the relationship between WBCs and microRNAs, it has been remarked that some microRNAs, including miR-146, in RA synovial fluids (SF), leukocytes, and peripheral blood mononuclear cells (PBMCs) had higher expression than blood.[33,34] However, we did not observe any noteworthy correlation between WBCs and analyzed microRNAs in plasma.

It has been reported that several plasma miRNAs in inflammatory disorders have specific patterns of association with inflammatory factors, obesity, and metabolic disorders.[5] Among the metabolic factors we studied, miR-4270 positively correlated with cholesterol (Table 2). In line with this finding, higher cholesterol, cholesterol esters, and changes in phospholipid composition have been reported in RA. Potent anti-inflammatory treatments for rheumatoid arthritis are associated with low-density lipoprotein (LDL) increases.[35] Further prior data have exhibited that lipid levels are somewhat related to inflammatory elements, including hsCRP and ESR. Lipids, as membrane components, affect cellular signaling pathways, and on the other hand, they can serve directly as pro-inflammatory signals. Most clinical trials have indicated that n-3 polyunsaturated fatty acid (PUFA) supplementation for RA patients can improve muscle metabolism and limit muscle atrophy in obese and insulin-resistant subjects, thereby preventing cardiovascular diseases (CVDs), deficient activity and risk of death in RA patients.[36,37]

Our results revealed that three miRNAs, miR-6754, miR-146b, and miR-4270, had a significant positive relationship with liver enzyme AST, and miR-146b was inversely associated with ALP. Previous research showed that serum aminotransferases (AST and ALT) and ALP markedly increased in RA patients compared to controls.[38] These results confirm the metabolic relationships of miR-4270, miR-4441, and miR-146 with RA (Figure 5). However, more research is needed to elucidate these connections fully.

To further elucidate the relationship between RA and hormones, we surveyed associations of TSH as a potential metabolic hormone with studied miRNAs in RA. While there was no relationship between TSH and any of miR-4270, miR-153-5p, miR-4441, miR-6754-5p, and miR-146b in our experiments (Table 2), a study on mice established dysregulation of miR-146b-5p related to dietary obesity via hepatic thyroid hormone receptor β (THR-β) and type 1 deiodinase (Dio1) genes.[39] miR-146b associated with papillary thyroid carcinoma (PTC) was reported in another study.[40] Consistent with us, prior studies did not find a significant difference between thyroid dysfunction and RA. Some drugs, including glucocorticoids and Leflunomide, may also increase the risk of thyroid dysfunction. In addition, it has been reported that thyroid disorders in RA patients are often autoimmune innately and are associated with increases in thyroid autoantibody titers.[41] Thyroid hormones have many normal pro-inflammatory functions, but there is still no definitive conclusion, and results are conflicting about the relationship between RA and thyroid dysfunction.

Limited studies have investigated the relationship between serum levels of vitamin D, calcium, phosphorus, and disease severity in arthritis patients. We observed significant associations of miR-4441 with vitamin D3 and miR-6754-5p with serum phosphorus. Many studies have proven the relationship between vitamin D3 deficiency or dysfunction with RA.[42] Other studies have reported no significant relationship between the activity of rheumatoid arthritis and the serum level of vitamin D.[43] Vitamin D metabolic disorder in patients with rheumatoid arthritis may be associated with RA-related osteoporosis.[44] Therefore, studies should be conducted to investigate the definitive effect of this vitamin and the effective dose in improving the severity of the disease in these patients.

This research had limitations: first, we measured miRNA expressions in plasma alone without evaluating them in other sites such as tissues. Moreover, we did not measure the miRNAs into microvesicles or exosomes or attach circulating proteins. Knowing the miRNA expression site, packaging mode, and target tissue or cellular site of mRNA regulating function may help understand complex miRNA pathways. Second, we did not perform a complete analysis because of patients' heterogeneity and various stages of RA disease. Therefore, we could not distinguish the complementary role of these circulating miRNAs about RA, and our data need to be confirmed in more extensive studies. Besides, specific investigations are still required on mechanisms underlying the altered expression of these miRNAs after using RA drugs. Finally, because in the results of our bioinformatics investigations, miR-153-5p was introduced as one of the four possible miRs involved in RA, and on the other hand, the analysis of that test was not performed in this complete study. We suggest that the expression of miR-153-5p be re-examined in future studies. Solving these ambiguities is hoped to advance science to better diagnose and treat this disease for future generations.

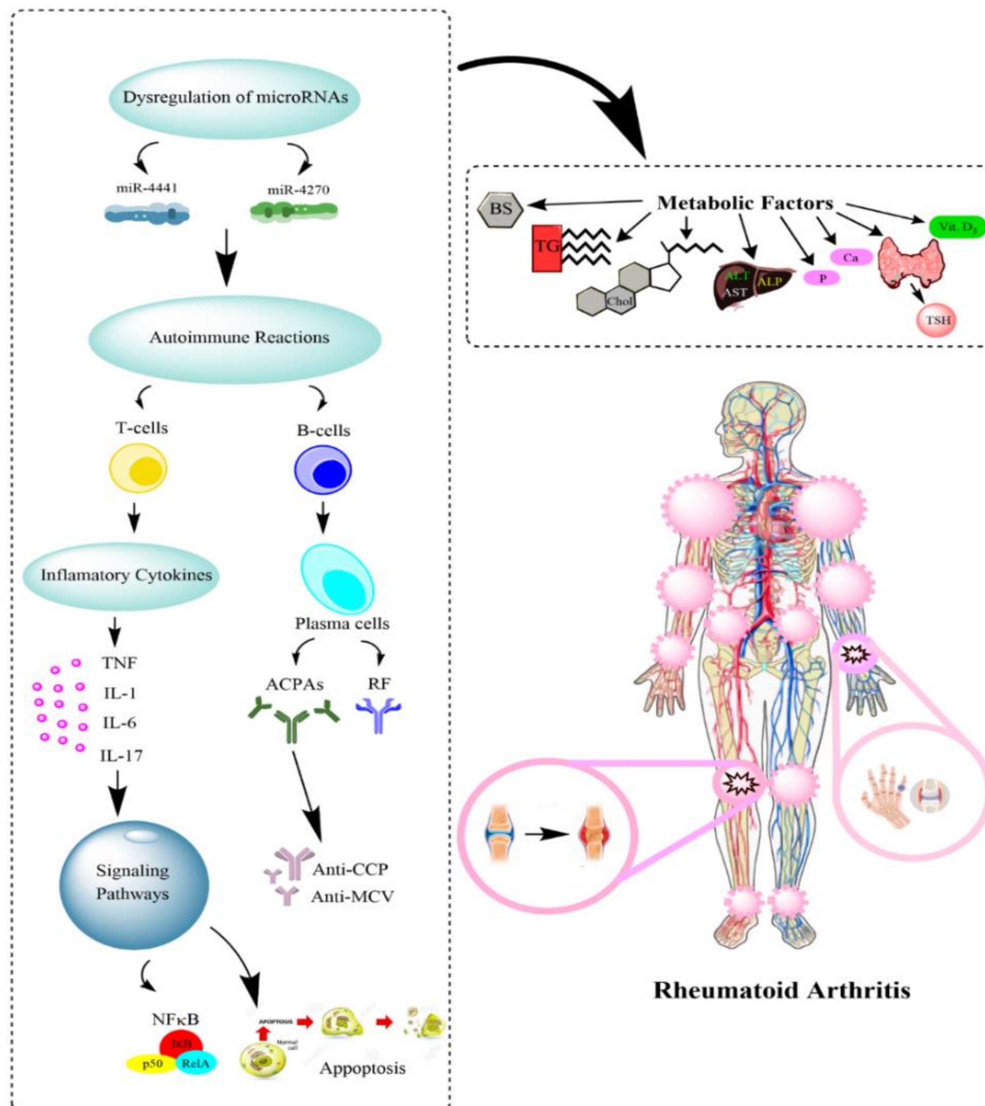

Figure 5. Schematic representation illustrates the regulatory role of microRNAs (miR-4270 and miR-4441) in rheumatoid arthritis, highlighting their impact on metabolic factors. Dysregulation of these miRs, particularly affecting T cells, may produce inflammatory cytokines such as IL-1, TNF, IL-6, and IL-17. These cytokines, in turn, influence crucial signaling pathways like NFκB and apoptosis, contributing to the gradual onset of the disease. Additionally, B and plasma cells further exacerbate RA by generating antibodies, including RF, anti-CCP, and anti-MCV. Cumulatively, these factors can adversely affect an individual's health by influencing metabolic processes. IL-1: Interleukin-1; TNF: Tumor necrosis factor; IL-6: Interleukin 6; IL-17: Interleukin 17; NFκB: Nuclear factor kappa-light-chain-enhancer of activated B cells; RA: Rheumatoid arthritis; RF: Rheumatoid Factor; anti-CCP: Anti-cyclic citrullinated peptide; anti-MCV: anti-mutated citrullinated vimentin.

In conclusion, the present study identified miR-4270 and miR-4441 as likely RA biomarkers affecting inflammation and biochemical pathways. However, there was no significant difference in expression profiles of other studied miRNAs, such as miR-6754-5p, in plasma from patients and healthy subjects. All four miRNAs had correlations with some inflammatory markers such as RF, ESR, CRP, and anti-CCP, as well as some biochemical factors, including liver enzymes, cholesterol, phosphorus, and vitamin D_3. It confirms the critical role of these miRNAs in maintaining the balance of regulating the immune and metabolic systems. Further well-designed

studies in larger patient cohorts seem necessary to investigate detailed mechanisms regarding miR-4441 and miR-4270 and their effects on multiple inflammatory and metabolic pathways.

STATEMENT OF ETHICS

Written consent was obtained from all participants, and the Ethics Committee of the University of Tabriz (52.407666.1) has approved the study.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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