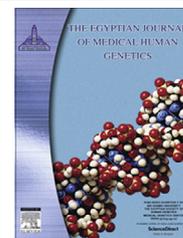




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ORIGINAL ARTICLE

# MicroRNA-146a expression as a potential biomarker for rheumatoid arthritis in Egypt



Heba Mohamed Abdelkader Elsayed<sup>a</sup>, Walaa Shawky Khater<sup>a,\*</sup>,  
Ayman Asaad Ibrahim<sup>a</sup>, Maha Salah El-din Hamdy<sup>a</sup>, Nashwa Aly Morshedy<sup>b</sup>

<sup>a</sup> Medical Microbiology and Immunology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt

<sup>b</sup> Rheumatology and Internal Medicine Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt

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## KEYWORDS

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Autoimmune;  
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Whole blood;  
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Pathogenesis

**Abstract** *Background:* MicroRNAs (miRNAs) are small non-coding RNAs, whose role in regulating diverse immune functions, suggests they might play a role as biomarkers for immune mediated disorders. Studies showed that miRNA-146a (miR-146a) expression is increased by proinflammatory cytokines and is an important modulator of differentiation and function of cells of innate and adaptive immunity.

*Aim of the work:* The current study aimed to evaluate the expression of miR-146a as a potential biomarker for diagnosis of rheumatoid arthritis (RA) and to explore its association with disease activity.

*Subjects and methods:* The study enrolled 50 Egyptian subjects divided into a patient group, which comprised 25 RA patients, and a control group which comprised 25 healthy individuals. The disease activity for the patients' group was determined by simplified disease activity index. Relative quantification of miR-146a expression in whole blood was determined using reverse transcriptase quantitative real time polymerase chain reaction.

*Results:* There were highly significant statistical differences between patients and healthy controls as regards miR-146a relative expression, erythrocyte sedimentation rate (ESR) and anti-cyclic citrullinated peptide (anti-CCP) ( $p < 0.001$ ). Highly significant statistical differences ( $p < 0.001$ ) were also found between different patients' subgroups as regards miR-146a relative expression and ESR. miR-146a levels correlated positively with those of ESR, C-reactive protein and anti-CCP ( $p < 0.001$ ).

miR-146a illustrated best performance in diagnosing RA, showing the highest sensitivity and specificity (96% and 100%, respectively) (AUC: 0.992 at a cut off value of  $\geq 2.16$ ) compared to anti-CCP (sensitivity: 68%, specificity: 100% and AUC: 0.87 at a cut off value of  $\geq 22$  U/ml) and RF (sensitivity: 56%, specificity: 80% and AUC: 0.992 at a cut off value of  $\geq 13$  U/ml).

\* Corresponding author.

E-mail addresses: [walaa\\_khater@med.asu.edu.eg](mailto:walaa_khater@med.asu.edu.eg), [drwalaakhater@gmail.com](mailto:drwalaakhater@gmail.com) (W.S. Khater).

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**Conclusion:** This study demonstrated that miR-146a expression was highly significantly elevated in whole blood of patients with RA. Its diagnostic performance was better than anti-CCP and RF and its level of expression correlates with disease activity.

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## 1. Introduction

Rheumatoid arthritis (RA) is a systemic progressive autoimmune disease characterized by chronic inflammation of the synovial tissue, which leads to irreversible joint destruction and subsequent disability [1]. Considering the variable and heterogeneous clinical presentation of the disease, its diagnosis is primarily based on the 2010 revised American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria which includes the evaluation of several clinical and serum parameters as anti-citrullinated protein/peptide antibodies, rheumatoid factor (RF) and acute phase reactants [2]. Nevertheless, there is still no specific pathognomonic test for the diagnosis and identification of at risk patients, which necessitates defining new biomarkers for RA [3].

MicroRNAs (miRNAs) are small non-coding RNAs, whose role in regulating diverse immune functions including B and T cell selection and maturation, suggests that they might play a central regulatory role in immunological tolerance and can play a potential role as biomarkers for various immune mediated disorders [4–7]. miRNA-146a (miR-146a) was first described by Taganov et al. who demonstrated its increased expression in response to stimulation with lipopolysaccharides, and its role as a regulator of the Toll-like receptor signaling by targeting tumor necrosis factor (TNF) receptor-associated factor 6 and interleukin-1 receptor-associated kinase 1 [8]. Other studies showed that its expression is increased by proinflammatory cytokines and is considered an important modulator of differentiation and function of cells of innate and adaptive immunity [9,10]. It has been implicated in the pathogenesis of RA via regulation of multiple target genes linked to inflammation and apoptosis [11]. Levels of miR-146a expression have been investigated in synovial tissues, fibroblasts [12–14], T-cells, B-cells, IL-17 producing CD4 cells [15], peripheral blood-derived mononuclear cells (PBMC) [14] and plasma [13] of RA patients. However, There is conflicting evidence regarding the association between the level of miR-146a and the disease course [11].

The current study aimed to evaluate the expression of miR-146a as a potential biomarker for diagnosis of RA using whole blood owing to the feasibility and the minimally required manipulation of the sampling that won't alter the molecules level in samples, and to explore its association with disease activity.

## 2. Subjects and methods

### 2.1. Study design

This observational case-control study was conducted in the period from March to August, 2015.

The work was approved by Ain Shams University (ASU) Ethics Committee. It was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans. Informed consent was obtained from all participants.

### 2.2. Patients and controls

The study involved 50 Egyptian subjects, who were divided into two groups; patients and controls.

**Patient Group:** Included 25 patients recruited from the rheumatology outpatient clinic, ASU Hospitals. Patients were included in this group after being diagnosed with RA according to the ACR/EULAR classification criteria [2]. Patients suffering from any other autoimmune disorders, chronic illnesses or comorbidities were excluded from the study.

**Control Group:** Included 25 apparently healthy individuals whose age and sex were matched with those in the patients group.

### 2.3. Data collection

Relevant sociodemographic and clinical data were collected in a standardized collection form. Results of clinical rheumatology examination including disease duration, number of tender swollen joints, morning stiffness, extra articular manifestations, in addition to the simplified disease activity index (SDAI) score [16] were recorded for each patient.

### 2.4. Laboratory investigations

#### 2.4.1. Sample collection

Adequate blood samples were collected from each subject under complete aseptic condition. The samples were divided into two parts; one part was used for analysis of the erythrocyte sedimentation rate (ESR, mm/h), C-reactive protein (CRP, mg/L), complete blood picture, RF and anti-cyclic citrullinated peptide (anti-CCP). The other part was preserved in tubes containing EDTA at  $-80^{\circ}\text{C}$  for the relative quantification (RQ) of miR-146a expression.

#### 2.4.2. RQ of the miR-146a expression

- (a) **Purification of RNA from blood:** RNA was isolated from blood using the blood MicroRNA extraction kit “miR-Neasy Mini Kit” (Qiagen, Hilden, Germany) according to manufacturer's instruction. Briefly, after thawing the samples, QIAzol Lysis Reagent was added. Chloroform was then added to the lysate to separate the aqueous phase. Samples were then vortexed, incubated at room temperature for 2–3 min, and centrifuged for 15 min at

14,000 rounds per minute at 4 °C. Ethanol was added to the separated aqueous phase to provide appropriate binding conditions for all RNA molecules. The samples were then applied to the RNeasy Mini spin columns, where the total RNA binds to the membrane and phenol and other contaminants were efficiently washed away. Samples were then eluted in the RNase-free water stored at -20 °C until further processed.

- (b) *Reverse transcription*: miR-146a was reversibly-transcribed using miScript II RT Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. Briefly, RNA template was added to the reverse transcription master mix containing 5× miScript HiSpec Buffer, 10× miScript Nucleics Mix, miScript Reverse Transcriptase Mix, nuclease-free water in a total volume of 20 µl. The samples were incubated for 60 min at 37 °C followed by 5 min at 95 °C in conventional polymerase chain reaction (PCR) thermal cycler (Biometra, Germany) to inactivate miScript Reverse Transcriptase Mix. The undiluted complementary DNA (cDNA) was stored at -20 °C till further processing.
- (c) *Quantitative real time PCR*: it was performed following a standard SYBR Green PCR protocol using miScript SYBR Green PCR Kit (Qiagen, Germany) with the StepOne real-time PCR (Applied Biosystems, United States of America). Each reaction mix contained 2× QuantiTect SYBR Green PCR Master Mix, 10× miScript Universal Primer, 10× miScript Primer Assay specific for miR-146a (Qiagen, Germany), template cDNA, and RNase-free water in a total volume of 20 µl. The real-time cycler was programmed as PCR was done as follows; enzyme activation at 95 °C, followed by 40 cycles of denaturation at 94 °C for 15 s annealing at 55 °C for 30 s and extension at 70 °C for 30 s. The expression of the U6B small nuclear RNA (RNU6B) was used as endogenous control for data normalization. The RQ level (fold change) for miR-146a was then calculated using the  $2^{-\Delta\Delta C_t}$  method [17].

## 2.5. Statistical analysis

Analysis of data was using SPSS (Statistical Program for Social Science) version 20. Quantitative variables were described as mean, standard Deviation (SD) and range. Qualitative variables were described as number and percentage. Chi-square test was used to compare qualitative variables between groups. An independent (unpaired) *t*-test was used to compare quantitative variables, in parametric data

(SD < 50% mean). One-way ANOVA (analysis of variance) test was used to compare more than two groups as regards quantitative variables (least significant difference). Pearson's correlation co-efficient test is a measure of the linear correlation between two variables *X* and *Y*, giving a value between +1 and -1 inclusive, where 1 is a total positive correlation, 0 is no correlation, and -1 is a total negative correlation. To assess the diagnostic accuracy of miR-146a, a receiver operating characteristic (ROC) curve analysis was performed. The combined ROC Curve is calculated using binary logistic regression. The area under the ROC curve (AUC) and 95% confidence interval (CI) were then estimated to determine the specificity and sensitivity of RA diagnosis.

Probability (*p*) value: *p* value > 0.05 was considered non-significant, *p* value < 0.05 was considered significant and *p* value < 0.001 was considered highly significant.

## 3. Results

The age of participants in the patients group that ranged from 26 to 63 years ( $41.88 \pm 11.46$ ). There were 24 females (96%) and one male (4%), whereas the age of controls ranged from 25 to 60 years ( $41.72 \pm 10.93$ ). There were also 24 females (96%) and one male (4%). There was no statistically significant difference between both groups as regards age or sex (Table 1).

Relevant clinical data of the patient group is summarized in Table 2. The patient group was further subdivided according to the disease activity by SDAI into:

- (1) Remission [*n* = 4 (16%)].
- (2) Low disease activity [*n* = 7 (28%)].
- (3) Moderate disease activity [*n* = 8 (32%)].
- (4) High disease activity [*n* = 6 (24%)].

### 3.1. Comparison between results of study participants as regards miR-146a, ESR, RF, anti-CCP

There were highly significant statistical differences between patients and healthy controls as regards miR-146a relative expression, ESR and anti-CCP (*p* < 0.001) (Table 3).

One-way ANOVA test was used to compare between the subgroups of patients (divided according to disease activity) regarding the same parameters. Highly significant statistical differences (*p* < 0.001) were found between different patients' subgroups as regards miR-146a relative expression and ESR, while levels of RF showed no statistical significant difference between the subgroups (*p* = 0.042) (Table 4).

**Table 1** Demographic data of the studied participants.

Characteristics	Patient group ( <i>n</i> = 25)	Control group ( <i>n</i> = 25)	Chi-square test $X^2/t^*$	<i>p</i> -value
Age (years)	41.88 ± 11.46	41.72 ± 10.93	0.051	0.960 (NS)
Sex				
Female	24 (96%)	24 (96%)	0.000	1.000 (NS)
Male	1 (4%)	1 (4%)		

NS, non-significant.

Data are presented as mean ± S.D. for continuous variables and as number (percentage) for categorical variables.

\* Chi-square test.

**Table 2** Clinical data of the patients group ( $n = 25$ ).

Parameters	Mean $\pm$ SD	Range
Disease duration (years)	6.62 $\pm$ 5.24	1–25
SDAI	20.98 $\pm$ 14.77	2–52
CRP (mg/dl)	6.22 $\pm$ 3.64	0.5–10
ESR (mm/h)	42.80 $\pm$ 22.42	11–95
RF (U/ml)	49.88 $\pm$ 43.16	9–125
Anti-CCP (U/ml)	62 $\pm$ 54.4	10–200

SDAI, simplified disease activity index; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; RF, rheumatoid factor; CCP, cyclic citrullinated peptide.

miR-146a levels correlated positively with those of ESR, CRP and anti-CCP ( $p < 0.001$ ) (Fig. 1). On the other hand, it did not correlate with either RF or disease duration (data not shown).

### 3.2. Accuracy of miR-146a in diagnosing RA compared to anti-CCP and RF

Combined ROC curve was plotted to compare the performance of miR-146a relative expression, anti-CCP and RF in the diagnosis of RA (Fig. 2). miR-146a illustrated best performance characteristics showing the highest sensitivity and specificity (96% and 100%, respectively) (AUC: 0.992 at a cut off value of  $\geq 2.16$ ) followed by anti-CCP (sensitivity: 68%, specificity: 100% and AUC: 0.87 at a cut off value of  $\geq 22$  U/ml) and RF (sensitivity: 56%, specificity: 80% and AUC: 0.992 at a cut off value of  $\geq 13$  U/ml) (Table 5).

## 4. Discussion

Several reports considered miR-146a an important modulator of differentiation and function of cells of innate and adaptive immunity, and associated it with the development and pathogenesis of autoimmune diseases [18,10,19]. In the current study, we evaluated its expression in RA. Statistically significant differences ( $p < 0.001$ ) were observed between patients and healthy controls as regards miR-146a relative expression. Other researchers had studied the levels of miR-146a expression in patients with RA in different body samples. The most

commonly studied samples were the synovial fluid, tissues and fibroblasts as reported by Stanczyk et al. [12], Murata et al. [13], Niimoto et al. [15] and Kriegsmann et al. [20]. In all of these studies elevated levels of expression of miR-146a were documented, suggesting its significance as a potential biomarker for RA diagnosis. However, the invasiveness of the synovectomy procedure encouraged researchers to investigate the usefulness of other less invasive samples as indicators for the levels of miRNA-146a expression. Pauley et al. [5] and Abou-zeid et al. [21] reported upregulated miRNA-146a expression in PBMCs from patients with RA than in healthy controls, and in patients suffering from other autoimmune disorders. They concluded that in patients with RA, PBMCs exhibit elevated miRNA expression in a pattern similar to that observed in RA synovial tissue. Ormseth et al. found miR-146a levels of expression elevated in the plasma of patients with RA [22]. In the current study we used whole blood and so did Mookherjee and El-Gabalawy [4] who stated that whole blood samples could accurately reflect miRNA levels in PBMC and would be useful in monitoring the expression of miRNAs as biomarkers.

Conflicting results were observed as regards the association between the level of miR-146a expression and the course of the disease. Our results showed a positive correlation between levels of miR-146a and disease activity as demonstrated by the highly significant statistical difference in the levels of miR-146a expression among different patients' subgroups ( $p < 0.001$ ) and the highly significant positive correlations between miR-146a and ESR ( $r = 0.965$ ,  $p < 0.005$ ), CRP ( $r = 0.946$ ,  $p < 0.005$ ) and anti-CCP ( $r = 0.715$ ,  $p < 0.005$ ). Similarly, Nakasa et al. [14] reported a higher expression of primary and mature mi-146a in synovial tissue of patients with RA with a high disease activity. Pauley et al. [5] found an association between high miR-146a expression levels in PBMCs and disease activity. Abou-zeid et al. [21] documented positive correlations of miR-146a expression and disease activity represented by the values of the Disease Activity Score 28 and ESR. On the other hand, in the study conducted by Li et al. [23] miR-146a expression was not found to be associated with DAI but correlated positively with tumor necrosis factor- $\alpha$  in both peripheral blood and synovial fluid. It is important to note that all of these studies are limited by a relatively small number of patients, and were conducted on patients from different races and ethnicities.

**Table 3** Comparison between patient and healthy controls as regards miR-146a, ESR, RF, anti-CCP.

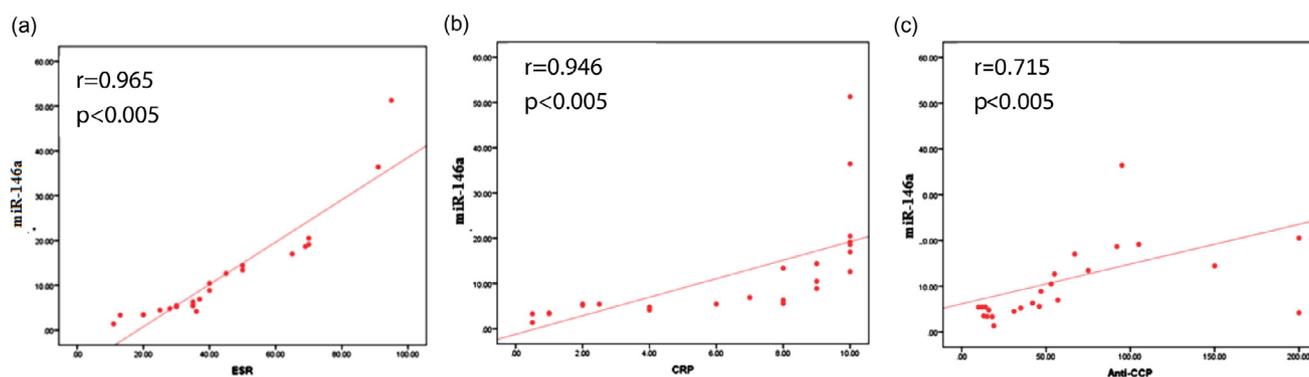
Parameters		Patients group ( $n = 25$ )	Control group ( $n = 25$ )	Independent <i>t</i> -test	
				<i>t</i>	<i>p</i> -value
miR-146a	Mean $\pm$ SD	11.52 $\pm$ 11.43	1.01 $\pm$ 0.48	4.592	<0.001 (HS)
	Range	1.36–51.33	0.42–2.17		
ESR	Mean $\pm$ SD	42.80 $\pm$ 22.42	12.88 $\pm$ 2.44	6.635	<0.001 (HS)
	Range	11–95	9–17		
RF	Mean $\pm$ SD	49.88 $\pm$ 43.16	21.72 $\pm$ 24.11	2.848	0.006 (S)
	Range	8–125	7–85		
Anti-CCP	Mean $\pm$ SD	62.00 $\pm$ 54.41	13.60 $\pm$ 3.65	4.438	<0.001 (HS)
	Range	10–200	8–22		

ESR, erythrocyte sedimentation rate; RF, rheumatoid factor; CCP, cyclic citrullinated peptide; HS, highly significant; S, significant.

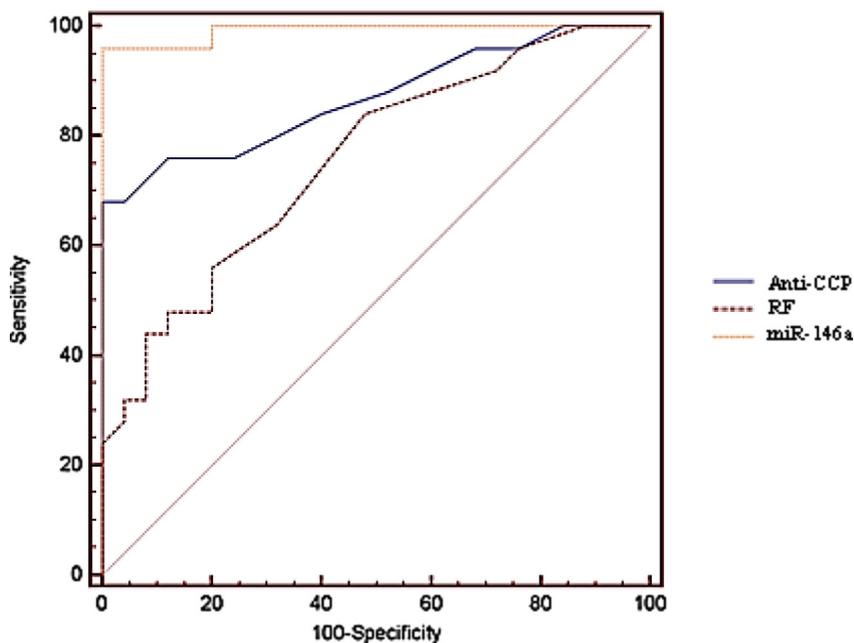
**Table 4** Comparison between patient subgroups as regards miR-146a, ESR, RF, anti-CCP.

		Remission ( <i>n</i> = 4)	Low ( <i>n</i> = 7)	Moderate ( <i>n</i> = 8)	High ( <i>n</i> = 6)	One Way ANOVA test	
						<i>F</i>	<i>p</i> -value
miR-146a	Mean ± SD	2.87 ± 1.01	4.98 ± 0.52	9.82 ± 3.43	27.17 ± 13.81	14.068	< 0.001 (HS)
	Range	1.36–3.46	4.15–5.43	5.56–14.41	17–51.33		
ESR	Mean ± SD	16.00 ± 4.69	30.57 ± 3.82	41.50 ± 6.16	76.67 ± 12.85	60.805	< 0.001 (HS)
	Range	11–20	25–36	35–50	65–95		
RF	Mean ± SD	68.25 ± 43.87	32.29 ± 34.55	53.13 ± 47.73	53.83 ± 49.26	0.631	0.603 (NS)
	Range	12–118	12–93	9–125	8–120		
Anti-CCP	Mean ± SD	16.25 ± 2.75	45.43 ± 68.84	65.63 ± 35.54	107.00 ± 47.32	3.255	0.042 (S)
	Range	13–19	10–200	42–150	67–200		

ESR, erythrocyte sedimentation rate; RF, rheumatoid factor; CCP, cyclic citrullinated peptide; HS, highly significant; S, significant; NS, non-significant.



**Figure 1** Scatter diagrams showing the positive correlation between miR-146a and (a) ESR (b) CRP and (c) anti-CCP in RA patients., (*r*; Spearman correlation coefficient).



**Figure 2** Combined ROC curve analysis of miR-146a, RF and CRP. miR-146a showed highest area under the curve (AUC = 0.992) followed by anti-CCP (AUC = 0.87) then RF (AUC = 0.756).

**Table 5** Performance characteristics of miR-146a expression, RF and anti-CCP in diagnosing RA.

Variables	Cut off point	AUC	95% CI	SE	Sensitivity	Specificity	PPV	NPV	Accuracy
miR-146a	≥2.1676	0.992	0.914–1.000	0.0129	96%	100%	100%	96.2%	99.2%
RF (U/ml)	≥13	0.756	0.614–0.866	0.0688	56%	80%	73.7%	64.5%	75.6%
Anti-CCP (U/ml)	≥22	0.870	0.744–0.948	0.0520	68%	100%	100%	75.8%	87%

RA, rheumatoid arthritis; RF, rheumatoid factor; CCP, cyclic citrullinated peptide; AUC, area under ROC (Receiver operating characteristic) curve; CI, confidence interval; SE, standard error; PPV, positive predictive value; NPV, negative predictive value.

Despite that Churov et al. [11] concluded in a review article that the specificity and sensitivity of a single miRNA as biomarker of RA is generally low, and it is preferable to use a set of several miRNAs or the combination of miRNAs with other parameters to get an effective diagnostic tool. In the current study, the accuracy of miR-146a was superior to that of RF and anti-CCP in the diagnosis of patients with RA with an AUC of 0.992 at a cut off value of ≥2.16 folds with a sensitivity and specificity of 96% and 100%, respectively. Kriegsmann et al. [20] reported that miR-146a had an AUC of 0.83. Yet in the latter study, they were comparing RA patients with those with osteoarthritis, not healthy controls.

A total of fifty participants were included in the study, which is considered a limitation due to their small number. However, our findings, that proved the value of miR-146a as a biomarker for RA, could be a guide for further wider scale studies to help reach a better understanding of the exact role of miRNAs in rheumatic disorders.

In this study, we intended to use and evaluate easier and less invasive sampling procedures for better compliance.

## 5. Conclusion

This study demonstrated that miR-146a expression was highly significantly elevated in whole blood of patients with RA compared to healthy controls. Its diagnostic performance was better than anti-CCP and RF and its level of expression correlated with disease activity.

## Conflict of Interest

None.

## Source of Funding

None.

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