Identification of putative miRNA biomarkers in early Rheumatoid Arthritis by genome-wide microarray profiling: A pilot study

Short title: MicroRNA profiling in rheumatoid arthritis

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1. ABSTRACT

Despite the existing research, the etiology of rheumatoid arthritis (RA), an autoimmune disease remains poorly understood with early and accurate diagnosis difficult to achieve. MicroRNAs (miRNAs) play an important role in biological processes as modulators of transcription and translation. Previous studies have demonstrated a downregulation of several genes in early RA stages and in addition, miRNAs may serve as early biomarkers of subclinical changes in early RA.

When comparing the four groups (ANOVA $P<0.01$, fold change >4), we found 253 differentially expressed miRNAs. Of these, 97 miRNAs were identified as overexpressed in early rheumatoid arthritis. The validation of miRNA microarray expression was performed in a set by RT-qPCR and showed strong agreement with microarray expression data. The putative targets of overexpressed microRNAs in early RA were significantly enriched in apoptosis, tolerance loss and Wnt pathways. Moreover, ROC analysis showed values of AUC 0.76 and $p<0.05$ for miR361-5p, identifying this miRNA as a potential biomarker of disease.

We identified specific microRNAs associated with early rheumatoid arthritis and proposed them as early biomarkers of disease. Our results provide novel insight into immune disease physiopathology and describe unreported microRNAs in RA with potential for clinical use.

Keywords: Early Rheumatoid Arthritis, Biomarker, miRNA, CCP, Microarray Expression.

2. INTRODUCTION

Rheumatoid arthritis (RA) is an immune mediated disease characterized by a symmetrical appearance of symptoms like swelling, joint tenderness and destruction of synovial joints. The prevalence of rheumatoid arthritis worldwide is estimated to be 0.5 to 1.1% [1]. RA affects women three times more than men [2] and incidence peaks in the 30 to 55 year-old age group, leading to disability and significant loss of productivity. Cyclic citrullinated peptide antibodies (CCP) are detectable months to years prior to RA clinical manifestations. A strong association has been found between the production of CCPs and the presence of the HLA-DRB1 risk alleles [3–5]. For RA diagnosis, CCP antibodies have a sensitivity of 72% and a specificity of 92% [6,7]. However, these antibodies are not exclusive to rheumatoid arthritis and in some cases; titers do not reach diagnostic thresholds. Early detection of RA may influence progression and prognosis. Recent reports suggest that remission-oriented treatment in early stages of the disease leads to normal function without disability in 40% to 60% of patients [8]. Therefore, there is an urgent need for the identification of novel biomarkers for early stages of RA. Previous work done by our research team identified a wide signature of downregulated genes in early RA [9]. This transcriptional arrest could be due to an overexpression of microRNAs which have been reported as important modulators of transcription and translation.

MicroRNAs (miRNAs) are small non-coding RNA molecules of about 22 nucleotides in length. They act as post-transcriptional regulators primarily by inhibiting gene expression through different mechanisms that impair the translation of mRNA (accelerating the deadenylation, binding to the 3 UTR region or binding regulatory proteins such as translation factors). The biogenesis of microRNAs begins in the nucleus with the transcription by RNA pol II or III synthesizing a double-stranded RNA. A pri-miRNA is processed by the Drosha endonuclease, forming a pre-miRNA which is exported to the cytoplasm. In the cytoplasm, the pre-miRNA is processed by Dicer to form a double-stranded RNA fragment that is recruited into the RNA-induced silencing complex (RISC

by AGO2). While only one of the two single-stranded fragments, the so called guide strand, is base paired to target mRNA, the other strand is generally degraded [10]. The miRNAs are highly stable in several fluids like saliva or serum [11–14], making them ideal as markers of disease. Because one of the functions of the miRNAs is to promote mRNA degradation, it is likely that an overexpression of miRNAs could be involved in the transcriptional arrest observed at the early stages of RA, This could provide an insight into the regulatory mechanisms involved in RA pathogenesis and making miRNAs potentially useful as disease biomarkers.

The interest in miRNAs is increasing due to their recently discovered participation in RA development [16,20,24,25]. Among the most studied miRNAs in RA to date are miR-155 and miR-146a [15–20], but recently an increasing number of miRNAs have been associated with RA [21]. For example, in a study using peripheral blood samples from RA patients and healthy subjects, 26 miRNAs showed significant differences in expression in the RA group compared to controls [22,23]. Other miRNAs can also serve as predictors of treatment response such as miR-125b, which is elevated in bDMARD treated RA patients, and is involved in B cell differentiation, TNF production and apoptosis, therefore acting as a biomarker for response to rituximab [26]. Another example is miR-432-5p which has been associated with remission and relapse with tofacitinib [27] or for determination of cardiovascular risk in RA patients [28].

Nevertheless, despite the well-known participation of several miRNAs in RA, there is little information regarding miRNA expression in early rheumatoid arthritis and in preclinical autoimmune stages of the disease (CCP+) [29,30]. We performed genome-wide profiling and differential expression of miRNA in RA patients and identified a set of candidate biomarkers that discriminate between disease stages.

3.- MATERIALS AND METHODS

3.1 Ethics statement

All study protocols conformed to the relevant ethical guidelines of the General Health Law on Health Research and with the Helsinki Declaration of 1975 and its amendments, as well as with the current international codes and norms for good practices in clinical research. The study was approved by the Ethics Committee under the register number CNCI R-2013_785_009. Clinical data was obtained from subjects during the interview and clinical examination and all subjects signed an informed consent to participate in the study.

3.2 Sample collection

The sample of the study consisted of individuals recruited in rheumatology clinics and their healthy relatives and healthy volunteers from clinics located in the cities of Guadalajara and Zacatecas between January 2013 and November 2017. Samples of 4 to 5 ml of whole blood were collected in EDTA tubes and frozen at minus 70°C within the first 30 minutes until processed for RNA extraction. As a preventive step, 1ml of RNA later (Invitrogen, USA), which can preserve RNA integrity at room temperature for 24 hr [11,31], was added immediately after sample collection according to previous protocols used in our group [9,32,33]. The patients and volunteers were classified in groups according to classification criteria by ACR/EULAR 2010, by certified clinical rheumatologists. Participants were classified in healthy controls with no clinical symptoms and negative to CCP (CTRL), subjects with no clinical symptoms and CCP positive (CCP+), early RA patients, naïve to RA treatment like DMARDs or prednisolone (ERA) and established RA patients (CRA) [34,35]. For the stratification of early and established RA, the main criterion was symptoms persistent for less or more than one year, respectively. Healthy controls were considered as individuals with no relevant medical history, normal results on the COPCORD questionnaire, CCP levels <25U/ml and a normal ESR. The criteria for the CCP+ group was CCP values >25U/ml and no RA clinical symptoms as evaluated by a rheumatologist. CCP antibodies were determined by 2nd generation CCP (Euro-Diagnostica, Sweden). A total of 5 subjects per group were randomly selected for comprehensive analysis of whole blood miRNA expression by microarrays. To validate the findings of the microarray experiment, the expression of 5 miRNAs was assessed for

validation by RT-qPCR in 12 CTRL, 6 CCP+, 13 ERA and 9 CRA (including subjects recruited for microarray). All individuals signed the informed consent letter.

3.3 Sample size calculation

A power analysis was used to determine the minimum sample size needed for accurate classification of subjects. The procedures were followed as described by Hwang D. [36]. Discriminatory genes were based on the calculation of median for each group and the pooled SD was calculated and used to determinate sample size, resulting in a power of 80% for a total sample size of 20 (5 samples per group). This criterion was considered for the microarray experiments.

3.4 RNA extraction

A sample of 500 µl of whole blood was mixed with 700 µl of Tri-Pure reagent (ROCHE, USA) and centrifuged at 13,000xg for 15 minutes at 4°C. The aqueous phase was then transferred to a pre-filter column of Absolutely-miRNA RNA kit (Agilent) following the manufacturer's instructions. RNA was stored at -70°C until use.

3.5 Microarray hybridization and data analysis

For the microarray, 100 ng of total RNA was dephosphorylated, desalted, labeled with Cy-3 and hybridized to the Agilent Human miRNA Microarray (Agilent, cat #. G4870A). This microarray includes probes for 1205 human and 144 human viral miRNAs. After hybridization, arrays were scanned using an Agilent Scanner according to the manufacturer's instructions (Agilent Technologies). The generated image files were analyzed using Agilent Feature Extraction Software (v 10.7.3.1). Files of microarray data are available on the Gene Expression Omnibus (**GEO**) database repository with identifier number GSE115885 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE115885]. Further analyses were performed using GeneSpring GX version 14.5 software (Agilent Technologies). Data files for each

array (GSE115885) were preprocessed using default settings as follows: threshold raw signals to 1.0 followed by 95 percentile shift normalization and further exclusion of control probes. We also excluded miRNAs detected in less than 50% of samples (10 out of 20 samples). Differential expression analysis across all subjects was performed using an ANOVA with Benjamini-Hochberg correction. Probes with p-value < 0.05 and a base 2 logarithmic fold difference >4 in at least one of the comparisons between the subsets were considered for further analysis. For a graphical illustration of miRNA expression profiles per group, Venn diagrams were constructed using an online tool (http://www.bioinfogp.cnb.csic.es/tools/venny).

3.6 RT-qPCR

RNA was retrotranscribed into cDNA using a stem-loop primer and U6 RT primer [37,38]. Reverse transcription started with 150 ng total RNA in a 10 µl RT reaction mixture containing 1 µl of stem-loop primer (5 mM) and 1 µl of U6 RT primer (5 mM), 1 µl of 10 mM dNTP Mix, 0.5 µl of RNase inhibitor RNaseOUT™ Recombinant Ribonuclease Inhibitor (20 U/ml) (Invitrogen, USA), 0.5 µl SuperScript™ II Reverse Transcriptase (200 U/ml), 2 µl of 5X first-strand buffer, and 1 µl of DTT (Invitrogen, USA). The mixture was incubated in a GeneAmp 9700 PF thermal cycler (Applied Biosystems) at 25° C for 5 min, then at 42° C for 60 min., and finally inactivated by heating at 70°C for 5 min. The remaining RNA was removed with the addition of 0.5 µl of RNase H (2U/µl) (Invitrogen, USA).

Real-time qPCR was performed using SsoFast-Eva green (Bio-Rad) on a Real-time quantitative PCR instrument Aria Mx (Agilent). The 10 µl reaction volume included 1 µl of RT product, 5 µl of SsoFast Eva green PCR Master Mix, and 1 µl of primers (forward and reverse, 1 mM each). Each sample was analyzed in triplicate. The reactions were incubated on an ARIA MX thermal cycler (Agilent, USA) at 95°C for 3 min, followed by 40 cycles of 95°C for 5 sec., 62°C for 35 sec. [37]. The level of miRNA expression was measured using the Ct (threshold cycle) value and miRNAs expression assay was quantified by the comparative $2^{-\Delta\Delta Ct}$ method and normalized to U6

expression. Primer sequences for retro-transcription were for U6 *5´- GCG TGT CAT CCT TGC GCA GGG-3´* and for miRNAs the stem-loop *5´- GAA AGA AGG CGA GGA GCA CGA GGA AGA AGA CGG AAG AAT GTG CGT CTC GCC TTC TTT CNN NNN NNN-3´*. For qPCR, they were Foward U6 *5'-GCT TCG GCA GCA CAT ATA CTA AA-3´*, Reverse U6 *5'- CGC TTC ACG AAT TTG CGT GTC-3´*, miR- 4634 *5´- CGG CGC GAG CGG CCC-3´*, miR-23a-3p *5´-CTG TCA GTT TGT CAA ATA CCC CA-3´*, miR-223-3p *5´- CTG TCA GTT TGT CAA ATA CCC CA-3´*, miR-361-5p *5´-ATT ATC AGA ATC TCC AGG GGT AC-3´,* and the Universal Reverse primer for miRNAs was *5´-ACG AGG AAG AAG ACG GAA GAA T-3´*.

3.7 MicroRNA target gene prediction, functional enrichment analysis and miRNA network construction

Differentially expressed miRNAs were submitted to bioinformatics analysis to identify their experimentally validated miRNA target genes using miRNA Enrichment Analysis and Annotation Tool (MIEAA), which uses the algorithms and data of the HMDD, miRBase, miRTAR, Targetscan and MiRanda databases. The resulting target genes were further analyzed using a gene set enrichment analysis with FDR multiple test correction (total genome as background) on the PANTHER online tool (http://pantherdb.org/) [39] to classify the genes in pathways and molecular functions. The significance level was set to 0.05 (p-value < 0.05, threshold level= 2). Genes under the G.O. immune process term were selected to build an interaction network. The Gene-miRNA and Gene-Gene interaction networks were constructed with Cytoscape software [40] based on interaction scores obtained from the GENE MANIA online tool (http://genemania.org/).

3.8 Statistical analysis

Differences in miRNA expression detected by microarray were evaluated by ANOVA with Benjamini-Hochberg correction with the GeneSpring Software. RT-qPCR data were analyzed by Kruskal-Wallis and Dunn's post-test. Differences between groups for variables such as age and CCP levels were determined by Kruskal-Wallis and ANOVA, respectively. For gender, a proportion analysis was performed by a Yates corrected, Chi-square test. To determine the potential of miRNAs as biomarkers of early RA, receiver-operating characteristic (ROC) curves were performed and the area under the curve (AUC) was calculated for 4 miRNAs. Early RA group (ERA) was compared to the CCP+/CTRL reference groups. The predicted probability for early RA diagnosis was plotted in a ROC curve and specificity and sensitivity were calculated. $P<0.05$ was considered as statistically significant. Unless otherwise stated, all analyses were carried out in GraphPad Prism v5.0 (GraphPad Software, USA).

4.-RESULTS

4.1 Study participants

For a comprehensive miRNA analysis by microarrays, 5 subjects per group were selected (**Table 1**). As expected, we observed a higher number of women. We analyzed the statistical differences among demographic and clinical characteristics to identify those variables that could have any influence on the results (**Table 1**). We next validated miRNA expression profile by RT-qPCR in a group of 13 in CRA, 16 in ERA, 9 in CCP+ and 14 for CTRL, including RNA samples from the subjects of the microarray analysis. The characteristics of these subjects are summarized in **S1 Table**.

Table 1. Data of selected subjects for microarray analysis

Group	CRA	ERA	CCP+	CTRL	*p*-value
n	5	5	5	5	
Women (%)	80%	100%	100%	60%	*0.22*
Age (years)	54.5 ±14	46 ±17.3	39 ±12.2	45 ±13.2	*0.34*
ACPA (U/ml)	1727.10 *(1011-1901.9*)	231.1 *(24-2613.5)*	25.7 *(24.5-26.3)*	14 *(11.5-17.9)*	*0.008*
Time with disease	11 ±6.7 years	3 ± 2.1 months	ND	ND	
Treatment	None	DMARDs	None	None	

Characteristics of subjects whose RNA samples were analyzed on microarray. CTRL: Healthy Controls, CCP+: Positives to Cyclic citrullinated peptide antibodies, ERA: Early Rheumatoid Arthritis CRA: Established Rheumatoid Arthritis, ND: not determined. The statistical difference for age between groups was determined by ANOVA, median and S.D. are shown. Statistical difference in CCP levels was determined by Kruskal-Wallis, mean and IQR are shown. Statistical difference for gender between groups was determined by analysis of significance of proportions.

4.2 Expression signature of shared miRNAs identify candidate biomarkers for early Rheumatoid Arthritis

Following quality control, we identified 253 human miRNAs whose expression was significantly differential among disease and control groups ($p<0.05$) (**S2 Table**). The top ten miRNAs with higher significance and their fold change are shown in **Table 2**. Unsupervised hierarchical clustering of the 253 miRNAs was performed on the four different groups to visualize the changes in miRNA expression. We observed a shared profile within each group but not amonggroups (**Fig. 1A**). This expression is more evident in the subjects of the ERA group, which presented an overexpression of most of the miRNAs as shown in clusters at the left side of the heatmap, classifying miRNAs based on similar expression profiles (**Fig. 1A)**. One of the miRNA clusters was overexpressed only in ERA. A Venn diagram was created to identify expressed miRNAs for each group (**Fig. 1B**). 97 miRNAs were overexpressed in ERA and showed a specific pattern of an increased expression during preclinical stages (CCP+), increasing in ERA individuals and decreasing in CRA (but not at the basal levels observed in the CTRL group (**Fig. 1C**).

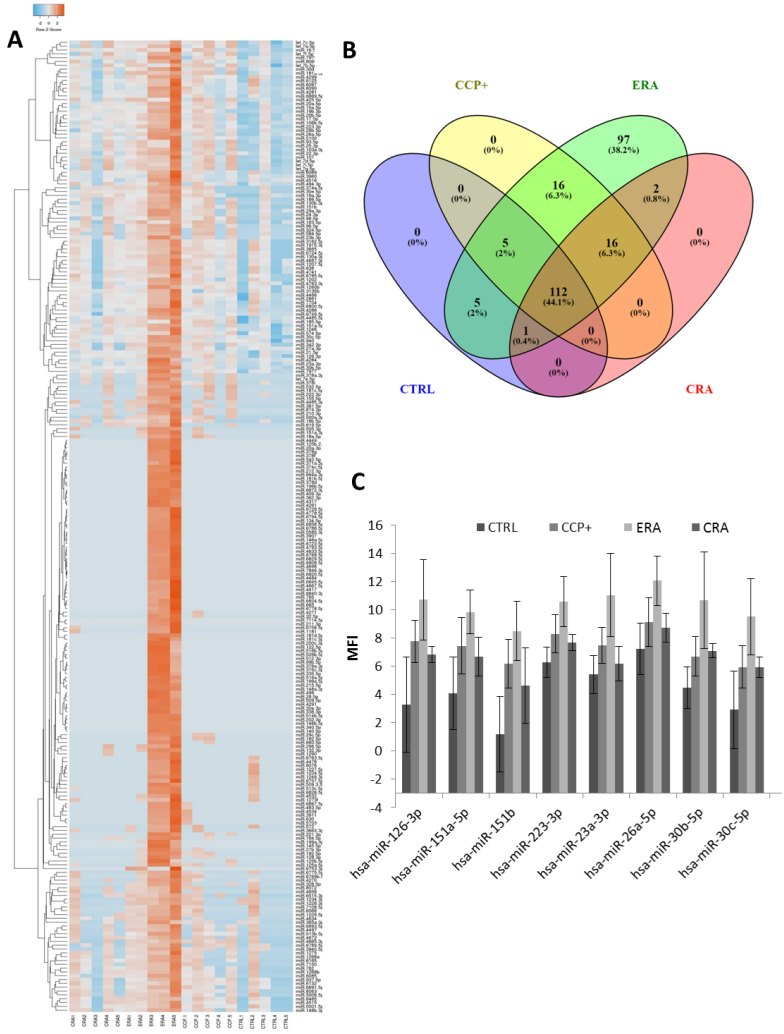

Figure 1. Unsupervised hierarchical clustering of globally expressed miRNAs.

(A) A heatmap of 253 expression signatures characterizing 4 groups was assessed in 20 patients and controls using genome-wide miRNA profiling. Top legend: range of expression values (blue = low, orange = high, white = below detection limit). **(B)** Venn diagram depicting miRNAs fingerprint per group. miRNAs specific to each group were selected from those differentially expressed. **(C)** Average expression measured on median intensity of fluorescence (MFI) per group of miRNAs sharing the same expression pattern. Bars of S.D. are shown. CTRL: Healthy controls, CCP+: Positives to CCP, ERA: Early rheumatoid arthritis, CRA: Established rheumatoid arthritis.

Table 2. Differentially expressed miRNAs

		Logarithmic Fold Change					
microRNA	***p* -value**	**CRA vs ERA**	**CRA vs CCP+**	**CRA vs CTRL**	**ERA vs CCP+**	**ERA vs CTRL**	**CCP+ vs CTRL**
hsa-miR-361-5p	0.00026	-5.693	-1.220	1.581	4.473	7.274	2.801
hsa-miR-223-3p	0.00041	-2.884	-0.596	1.403	2.289	4.287	1.998
hsa-miR-23a-3p	0.00092	-4.850	-1.319	0.764	3.532	5.615	2.083
hsa-miR-4634	0.00093	-4.286	2.945	2.873	7.231	7.159	-0.072
hsa-miR-151b	0.00107	-3.868	-1.563	3.429	2.305	7.297	4.992
hsa-miR-199a-3p	0.00109	-7.226	0.000	0.000	7.226	7.226	0.000
hsa-miR-126-3p	0.00118	-3.901	-0.954	3.514	2.947	7.415	4.469
hsa-miR-151a-3p	0.00129	-5.743	-0.771	0.000	4.972	5.743	0.771
hsa-miR-132-3p	0.00132	-4.249	0.819	0.819	5.068	5.068	0.000
hsa-miR-30b-5p	0.00140	-3.584	0.408	2.620	3.992	6.204	2.212

Results of one way ANOVA analysis with Tukey post-hoc test realized whit Gene Spring Software. miRNAs are ranked by p-value. The base 2 logarithm fold change of miRNA expression between groups is shown. CTRL: Healthy Controls, CCP+: Positives to Cyclic citrullinated peptide antibodies, ERA: Early Rheumatoid Arthritis CRA: Established Rheumatoid Arthritis.

4.3 Predicted pathways associated with miRNAs overexpressed in early RA

Many of the 97 miRNAs in the transcriptional fingerprint of ERA (**Fig. 1B**) have not been previously reported to be associated with RA and their function is not known. A pathway and gene ontology analysis of the early rheumatoid arthritis overexpressed miRNAs was performed in order to identify possible key processes using the MIEAA software. Those miRNAs were related to 118 significant pathways (p-value < 0.05, FDR 5%) (**Table S3)**. Several pathways have been previously described in RA, such as the signaling pathways of Wnt, p53, angiogenesis, TGF-β, inflammation mediated by chemokine and cytokine, and PI3 kinase pathways (**Table 3**). These pathways have been described to have a central role in synovial inflammation through IL-6 [41].

Table 3. Significant pathways in early rheumatoid arthritis

Pathway	***p*-value**	**Observed miRNAs**	**Expected miRNAs**
Wnt signaling pathway	1.42E-20	71	43.8
p53 pathway	4.35E-19	70	42.7
Apoptosis signaling pathway	4.98E-19	69	39.6
Huntington disease	3.04E-18	58	36.2
Angiogenesis	6.53E-18	64	41.4

TGF beta signaling pathway	5.04E-17	55	31.8
Inflammation mediated by chemokine and cytokine signaling pathway	7.61E-17	67	39.4
PI3 kinase pathway	1.85E-16	57	31
Parkinson disease	3.91E-16	56	30.5
PDGF signaling pathway	5.90E-16	58	33.7
p53 pathway feedback loops 2	9.29E-16	58	36.4
Integrin signalling pathway	1.09E-15	62	39.6
FGF signaling pathway	2.34E-15	55	33.7
EGF receptor signaling pathway	3.46E-15	57	34.5
Interleukin signaling pathway	3.46E-15	57	36
Alzheimer disease presenilin pathway	7.81E-15	51	32.9
Oxidative stress response	2.36E-13	46	21.7
Heterotrimeric G protein signaling pathway Gi and Gs alpha mediated pathway	1.06E-12	44	23.6
Ras Pathway	1.07E-12	49	28.7
Cytoskeletal regulation by Rho GTPase	1.82E-12	49	28.5
T cell activation	6.36E-12	47	28
VEGF signaling pathway	6.36E-12	47	29.1

22 of 116 significant pathways identified through enrichment analysis of 97 miRNAs overexpressed in early rheumatoid arthritis.. Expected miRNAs were determined by an overrepresentation analysis with MIEAA software.

4.4 miRNA target genes involved in immune response in early and preclinical RA stages are associated with loss of tolerance

Given that many of the identified pathways were related to immune processes, we investigated the possible role of these 97 miRNAs in ERA by performing a gene ontology analysis (MIEEA and PANTHER software) of the miRNA target genes and classified them in biological processes (**Fig. 2A**). We selected miRNA target genes related to immune system process (GO: 0002376) and identified the following 12 genes: EIF2AK3, ZFHX3, CSF1, NFKBIA, PCOLCE2, PTGS2, NFKB1, MAPK9, NLK, SESTD1, TNFSF9 and RUNX3 (see details in **Table 4**). This last gene has an important role in T cell development [42,43]. These genes were considered for further analysis of miRNA-Gene interaction on the GeneMANIA platform in order to identify the relationship between the miRNAs overexpressed in ERA and their possible role on gene expression. The interaction network shows co-expression between ZFHX3, PCOLCE2, PTGS2, NFKB1, MAPK9, NLK, SESTD1 and RUNX3 and physical interaction between NFKBIA and NFKB1, which are NFKB

inhibitors [44,45] and are validated targets of miR-335-5p, miR-16-5p, miR-15a-5p and miR-25b-5p (**Fig. 2B**). This network schematizes the closer relationship between Gene-miRNAs involved in the immune response in ERA and because the majority of identified genes are co-expressed It is thus feasible that a downregulation of one of these genes can affect other gene expressions. These identified genes were different from those associated with immune system processes identified in CRA (WEE1, TNFAIP3, HSP90AA1, PAG1, MAP3K12, PRKAB2) and in CCP+ (PXDN, PPP2R2A, ZFHX3, PSME3, HSPA1B, TRAF1, BNIP3L and PAG1) (**data not shown**), with the exception of ZFHX3 which was identified in CCP+ and in ERA, suggesting a link between preclinical and clinical RA stages (this gene was not identified as a miRNA target gene in established disease).

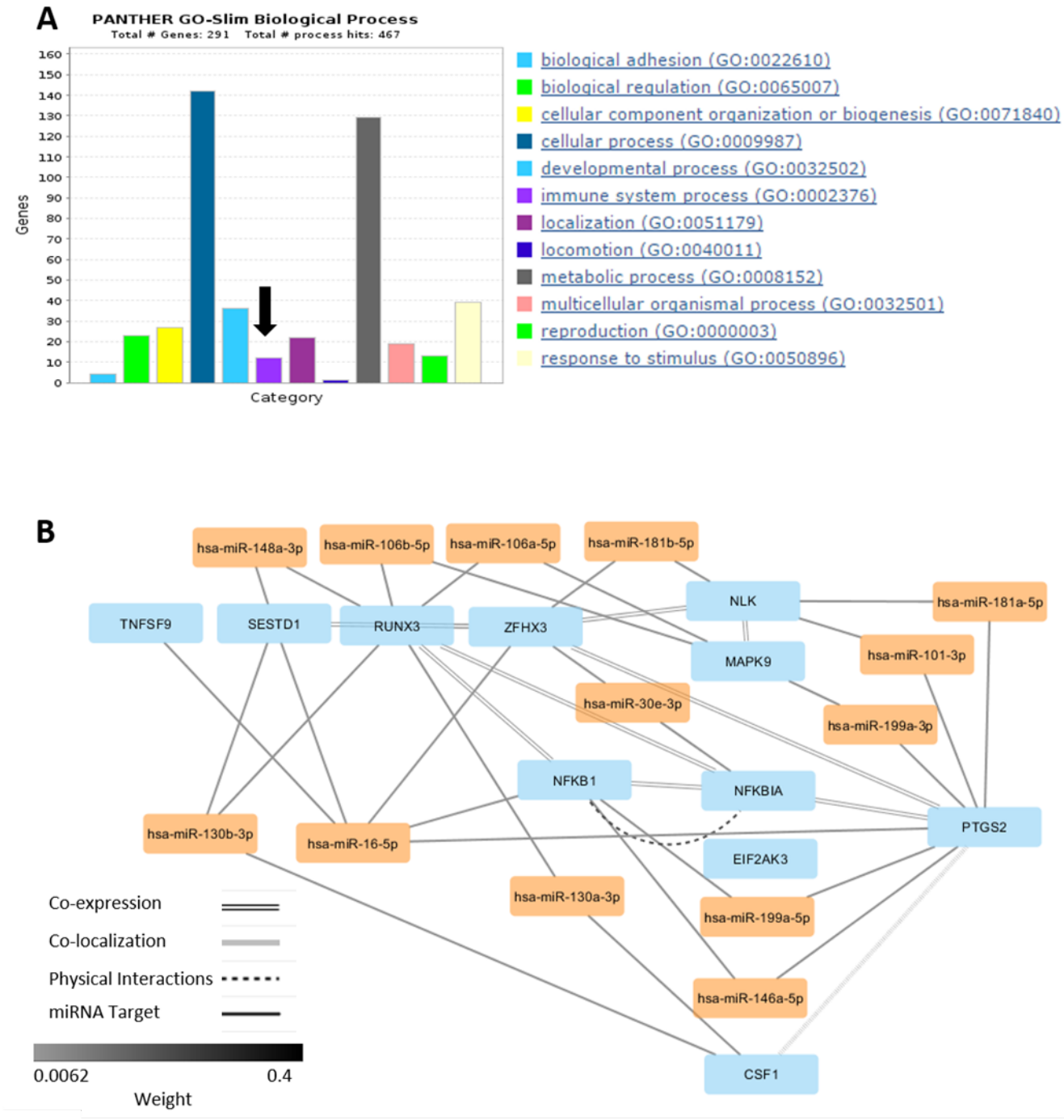

Figure 2. Comparative analysis of differentially expressed genes in ERA.

(A) PANTHER GO-Slim biological process analysis, identified 11 functional categories associated with the 284 validated miRNA target genes of the ART miRNA fingerprint. Cellular and metabolic processes were the major categories. The arrow points to the selected category for identification of miRNA target genes associated with immune response. (**B)** Network of interactions between Gene-Gene and miRNA–Gene, functional interaction network including integrin pathway-

associated genes extracted from the meta-analysis. Key (grey to black) shows the weight of each interaction.

Table 4. Genes involved in early rheumatoid arthritis immune process

Gene	Protein	Class
EIF2AK3	TRANSLATION INITIATION FACTOR 2-ALPHA KINASE 3	non-receptor serine/threonine protein kinase
ZFHX3	ZINC FINGER HOMEOBOX PROTEIN 3	RNA binding protein, actin family cytoskeletal protein
CSF1	MACROPHAGE COLONY-STIMULATING FACTOR 1	cytokine
NFKBIA	NF-KAPPA-B INHIBITOR ALPHA	enzyme modulator, phospholipase
PCOLCE2	PROCOLLAGEN C-ENDOPEPTIDASE ENHANCER 2	cell adhesion, molecule, extracellular protein, signaling molecule, metalloprotease
PTGS2	PROSTAGLANDIN G/H SYNTHASE 2	oxygenase
NFKB1	NUCLEAR FACTOR NF-KAPPA-B P105 SUBUNIT	nucleic acid binding, transcription factor
MAPK9	MITOGEN-ACTIVATED PROTEIN KINASE 9	non-receptor serine/threonine protein kinase
NLK	SERINE/THREONINE-PROTEIN KINASE NLK	non-receptor serine/threonine protein kinase
SESTD1	SEC14 DOMAIN AND SPECTRIN REPEAT-CONTAINING PROTEIN 1	guanyl-nucleotide exchange factor, signaling molecule
TNFSF9	TUMOR NECROSIS FACTOR LIGAND SUPERFAMILY MEMBER 9	-
RUNX3	RUNT-RELATED TRANSCRIPTION)	nucleic acid binding, transcription factor

Function and class of genes corresponding to the GO term 0002376 derived from of the GO PANTHER analysis.

4.5 Validation of miRNA expression by RT-qPCR and biomarker capacity

In order to validate the microarray results data and miRNA expression levels, we selected 4 miRNAs for expression level measurement by RT-qPCR. These 4 miRNAs were selected based on the lowest *p*-value and highest fold change obtained from microarray expression analysis. Consistent with microarray data, the four miRNAs showed a tendency to be overexpressed in ERA and two of these miRNAs showed statistical differences compared to other groups. The miR-361-5p presented statistical differences between CTRL and ERA ($p<0.001$) and between CTRL and CRA ($p<0.05$, **Fig. 3A**) while miR-223-3p showed relative expression differences in the CTRL group compared to ERA (**Fig. 3C**, $p<0.05$). For miR-4634 (**Fig. 3B**,) and miR-23a-3p (**Fig. 3D**), no significant differences in expression were found.

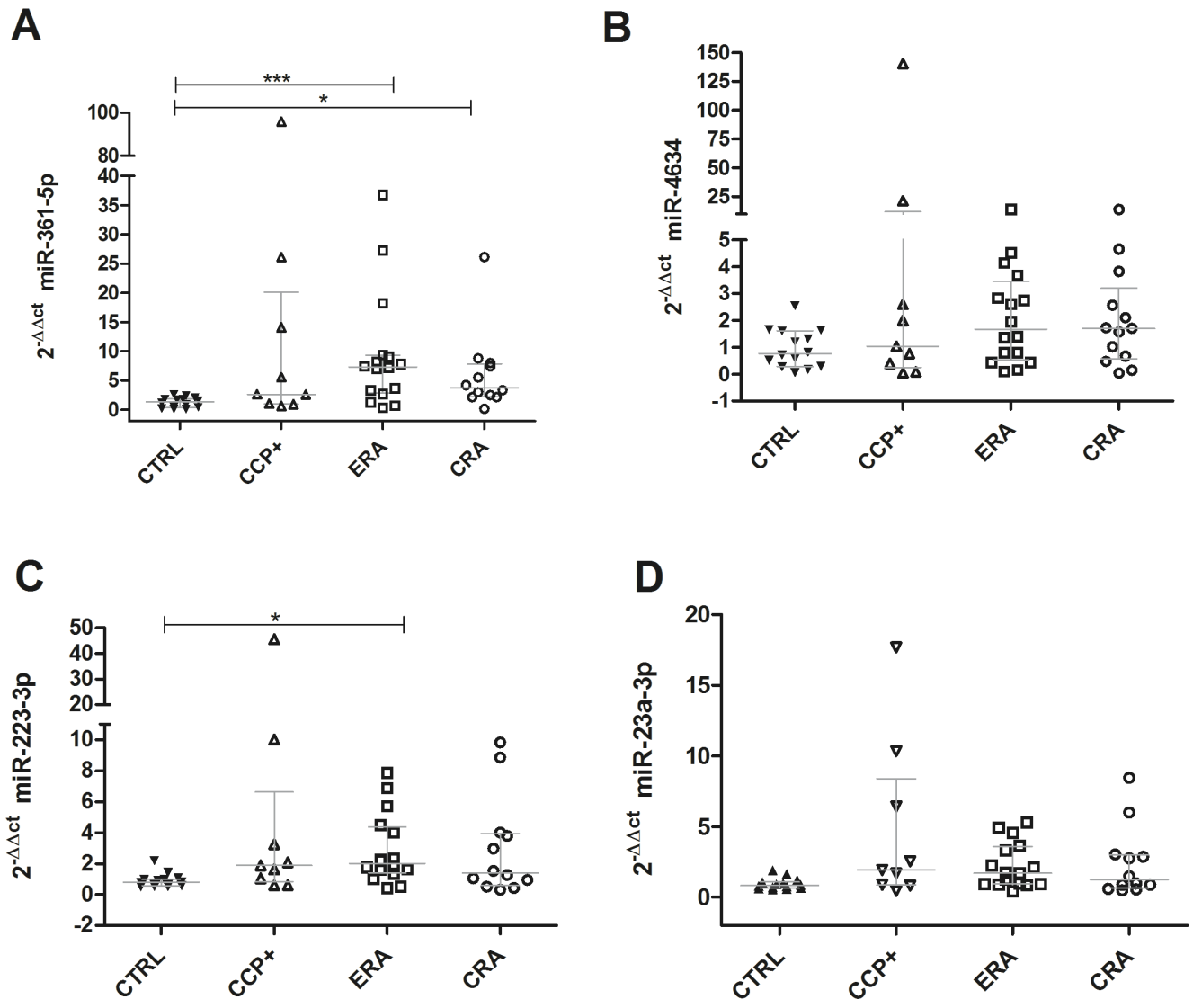

Figure 3. Relative expression of microRNAs

Relative expression of microRNAs in whole blood from CTRL: Healthy controls, CCP+: CCP Positive subjects, ERA: Early rheumatoid arthritis, CRA: Established rheumatoid arthritis. Fold change of miRNAs relative expression in whole blood of CTRL (filled triangles), CRA (open circles), CCP+ (open triangles) and ERA (open squares), median values analyzed by Kruskal-Wallis test with Dunn's test or multiple comparisons for (**A)** miR-361-5p, (**B)** miR- 4634, (**C)** miR-223-3p, (**D)** miR-23-3p and. Error bars are the first and third quartiles (* $p<0.05$, **$p<0.01$ and *** $P< 0.001$).

Finally, as the CCP+ individuals have an increased risk of developing RA, but these autoantibodies are not exclusive to RA, we decided to evaluate the potential of miR-361-5p and miR-223-3p as biomarkers in early RA group (ERA) since we wanted to differentiate them from subjects at risk (CCP+) and healthy controls (CTRL). ROC curves were performed in order to evaluate the sensitivity and specificity of miRNAs (**Fig. 4, A-B**). The sensitivity, specificity and *p*-value for each miRNA are summarized in **Table 5.** Only the results of miR-361-5p expression **(Fig. 4A**) were significant with specificity of 82.6% and sensitivity of 81.25 %.

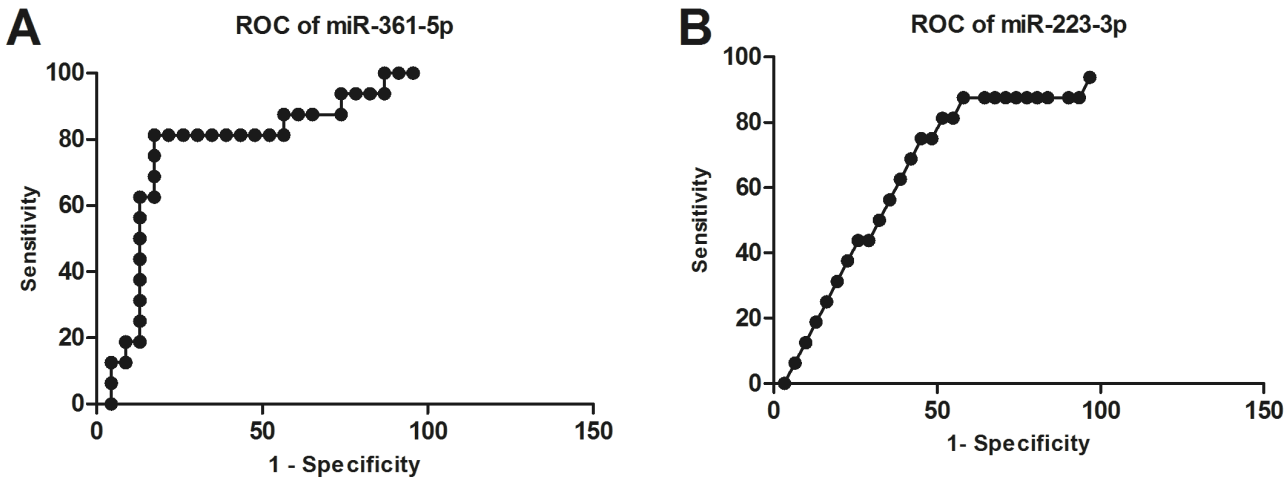

Figure 4. Receiver Operating Characteristic Curves

ROC curves (representing 1-specificity vs. sensitivity values) for ERA patients vs. non-RA group (CTRL and CCP+).

Table 5. Receiver operating characteristic curve of miRNAs overexpressed in ERA

miRNA	Specificity	Sensitivity	Cut-off	A.U.C.	*p*-value
miR-361-5p	82.61	81.25	2.6	0.76	***0.005***
miR-223-3p	61.29	62.5	1.7	0.63	*0.130*

Specificity and sensitivity of miRNA expression determined from comparison between early rheumatoid arthritis miRNA expression vs CCP+/controls. ROC Curves were calculated using $2^{-\Delta\Delta CT}$ miRNA values AUC = area under the curve

5.- DISCUSSION

We show a comprehensive whole blood miRNA analysis in RA and provide evidence of the existence of a specific transcriptional profile of miRNAs, some of which have not been identified before as deregulated in early rheumatoid arthritis. This miRNA expression profile could provide possible candidate miRNA biomarkers for early RA.

The miRNA expression detected by qPCR was consistent with the expression profile of the microarray data. To identify miRNA biomarker potential for early RA, preference was established for specificity rather than sensitivity for the cut-off points selection, because there is no golden standard for RA diagnostics and CCP positivity is the most accepted disease predictor, we decided to use CCP sensitivity and specificity values as a reference to compare our results.

In our study group, we detected a novel miRNA, has-miR-361-5p, associated with RA. There are no other reports of this miRNA in the physiopathology of rheumatoid arthritis to our knowledge. The miR-361-5p had the highest specificity and sensitivity (82.61 and 81.25, respectively). This suggests that this miRNA could be important for RA pathogenesis apart from having potential as a biomarker for early stages of the disease.

Our findings agree with previous reports where miR-223-3p was found overexpressed in RA synovial cells, plasma and serum [28,46]. Filková et al. found this miRNA overexpressed in early RA sera but not in synovial fibroblasts. They determined that the miRNA sera expression correlated with C reactive protein (p=0.008), DAS28 (p=0.031) [47], but didn't find changes in expression among early and established RA patients as we report.

The evidence for miR-23a-3p is centered on its role as a biomarker of treatment response and therapy effectiveness [25,48]. Although the expression of this miRNA hasn't been measured before in early RA, it has been detected at high concentrations in ostheoarthritis (OA) tissue [49]. Thus, a comparison between OA and early RA is necessary for validation of this miRNA as a specific biomarker for RA.

For the case of miR-4634 there is only one study where it was found overexpressed in RA patients compared to controls in a Chinese population [50]. This is in agreement with our data when comparing CRA vs. CTRL miRNA expression (p= 0.007) and provides further evidence that the expression of this miRNA is even higher in early RA.

It is of interest to remark that an elevation of several miRNAs occurs in the CCP+ state. These antibodies generally have an increase in serum concentration long before disease onset .Our results suggest that the identified miRNAs are good candidates to be used as biomarkers for disease onset prediction.

The principal pathways identified by gene ontology in early RA were the Wnt, p53 and the Apoptosis signaling pathway. These pathways have been described previously in synovial tissue. For example, apoptosis was lower in longstanding RA than in the patients with early RA [52]. The p53 pathway was also identified in other works that described miRNA expression in risk RA subjects [30][41]. Negative regulators of Wnt pathway like RANKL was overexpressed in early RA synovia[51]. Other identified pathway was VEGF, related processes to this pathway like angiogenesis have been identified in early RA patients with less than one year with symptoms [53–55].

The miRNA target genes identified by MIEAA software are related to 3 main processes: inflammation, apoptosis and tolerance loss. The principal process apparently involved in RA development is tolerance loss. According to our GO analysis, the miRNA target gene related to this process was RUNX3, which is able to mediate inflammation by interacting physically with Foxp3 protein [56,57]. RUNX3 is required for T cell development and its inhibition reduces the number of CD8 T cells. A subset of such cells maintains an expression of double positive $CD4^+CD8^+$, cells which have been described with higher frequencies in RA patients [42,43].Our finding of RUNX3 as a possible target of overexpressed miRNAs needs further corroboration by RUNX3 protein detection in early RA in order to describe the importance of its gene expression. For the CCP+ group, we identified PP2A as a target of several miRNAs. Evidence suggests that

the T regulatory cells lacking PP2A develop severe autoimmunity in mice models with increased CD4 + and CD8 + T cell counts, thus leading to an overproduction of IL-2 and IL-17 and IFN-γ [58]. ZFHX3 was a miRNA target gene detected in ERA as well as in CCP+ groups by gene target analysis. However, there are no reports of this gene expression in whole blood of RA patients and it is only in RA patient synovial macrophages where its expression is diminished [59] (accession GPL8300, dataset). As we mentioned, these findings determined by gene ontology in blood are similar to those detected in synovial tissue and could suggest that some joint processes are mirrored in peripheral blood. Further study of miRNA expression in early RA and their target genes in the synovium is needed to corroborate the relationship between whole blood data and the intended target genes in the tissues of interest.

This is the first comprehensive whole blood miRNA analysis by microarrays in preclinical and RA stages, although there are previous reports using PCR arrays showing a set of differentially expressed miRNAs in these disease stages [29,30]. Thus, our study has detected a set of miRNAs (miR-23a-3p, miR-4634 and miR-361-5p) that haven't been described before in early RA. We have presented here the differentially expressed miRNAs in groups representing the RA stages from the preclinical to the clinical phase as well as the miRNA target genes by GO analysis and the possible processes involved during RA onset. We identify two miRNAs which have a potential use as biomarkers; nevertheless, further investigation with a more robust sample size as validation of other differentially expressed miRNAs is needed.

6.- CONCLUSION

We have identified a distinctive miRNA profile that is overexpressed in early RA and described its relationship to key pathways/genes associated with RA immune deregulation and the potential of miRNAs to be used as biomarkers for early RA.

7.-LIST OF ABBREVIATIONS

RA: Rheumatoid arthritis

CCP: Cyclic citrullinated peptide antibodies

MiRNAs: MicroRNAs

CTRL: Healthy controls

CCP+: Subjects positive to cyclic citrullinated peptide antibodies

ERA: Early rheumatoid arthritis patients

CRA: Established rheumatoid arthritis patients

8.-DECLARATIONS

8.1.- Consent to publish

All authors agree with the consent to publish the data in this article.

8.2 .- Funding

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8.3.- Availability of data and materials

The authors' original submitted files of microarray data are available on the Gene Expression Omnibus (**GEO**) database repository with the identification number GSE115885 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE115885].

8.4.- Competing interests

None

8.5.- Authors' Contributions

Romo-García MF made the first draft and performed most determinations. Enciso-Moreno JA, Castañeda-Delgado JE, conceived the original idea and Castillo-Ortiz JD, Zapata-Zuñiga M, Ramos-Remus C participated in the clinical classification of subjects and obtaining samples also participated in the draft. Romo-García MF, Lara-Ramírez EE and Fernández-Ruíz JC acquired, analyzed and interpreted the data. Bastian Y, Macías-Segura N Berlanga-Taylor AJ, González-Amaro R, and Castañeda-Delgado J participated in the final drafting and revision. Funding was awarded to Castañeda-Delgado J. All authors approved the final manuscript.

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Supporting information

Supplementary Table 1: Data of selected subjects for RT-qPCR

Supplementary Table 2: Differentially expressed miRNAs

Supplementary Table 3: Significant pathways in early rheumatoid arthritis