Pediatric Rheumatology

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The utility of miR-16, miR-146a and miR-155 in serum and urine for juvenile idiopathic arthritis diagnostics and monitoring

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Abstract

Background Biomarker search for juvenile idiopathic arthritis (JIA) diagnosis and monitoring remain the focus of research worldwide. Several microRNAs (miRNAs) have been identified as relevant in different rheumatic conditions; however, studies in JIA remain limited. Our study aimed to explore the potential of serum and urine-derived miRNAs for JIA diagnostics and longitudinal JIA monitoring.

Methods In this single-center, prospective study, three selected miRNAs (miR-16, -146a and -155) were tested in serial serum and urine samples collected from 31 JIA patients and 22 healthy controls (HC) via quantitative reverse transcription polymerase chain reaction (RT–qPCR). The diagnostic performance of variables for distinguishing JIA patients from HCs was assessed by determining the area under the receiver operating characteristic (ROC) curve (AUC). The prediction of remission was evaluated using Cox regression and Kaplan-Meier analyses. A p-value < 0.05 was considered statistically significant.

Results Lower miR-16 and higher miR-155 levels were detected in serum of JIA patients' vs. HC ($p < 0.01$), whereas the level of miR-146a was lower in urine of JIA patients ($p = 0.032$). In ROC analysis, miR-16 and miR-155 distinguished JIA patients from HC when analyzed in serum (AUC 0.81, 95% CI 0.70–0.93, $p < 0.001$ and AUC 0.73, 95% CI 0.59–0.87, $p = 0.005$, respectively), and miR-146a– in urine (AUC 0.68, 95% CI 0.53–0.82, $p = 0.030$). During 12 months follow-up period increasing miR-16 ($p = 0.021$) and decreasing miR-155 ($p = 0.009$) levels were observed in serum samples. Kaplan-Meier survival analysis revealed that a high level of miR-146a in serum significantly predicts JIA remission (HR = 2.2, 95% CI 0.7–6.9, $p = 0.040$).

Conclusions This study highlights the utility of miRNAs in JIA diagnosis, monitoring and prognosis and demonstrates the feasibility of using urine as a noninvasive source of miRNAs in children with non-systemic JIA.

Keywords Juvenile idiopathic arthritis, MicroRNA, Urine, Serum, Circulating, Non-invasive, Diagnostics, Remission, Children, Biomarkers

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Background

Juvenile idiopathic arthritis (JIA) is a chronic inflammatory joint condition presenting in children with a broad spectrum of clinical phenotypes [1]. Extensive research has established JIA as a multifactorial disease involving diverse pathogenetic pathways, which have led to the development of targeted therapeutic options, such as tumor necrosis factor (TNF) inhibitors, interleukin (IL)-1 and IL-6 inhibitors, among others [2]. However, JIA diagnosis still relies on clinical criteria, and a significant proportion of patients fail to achieve sustained clinical remission [3, 4]. Many patients experience disease relapses in previously treated joints, progressive involvement of additional joints, and/or severe complications [5], highlighting the need for precise disease monitoring and tailored management strategies.

Several disease activity scales are utilized in clinical practice, with the Juvenile Arthritis Disease Activity Score (JADAS) being the most commonly applied [6]. However, inconsistencies among these tools have been reported, e.g., significant discrepancies between different clinical scoring systems were found when they were applied to the same patient cohorts [3]. This underscores the need for more accurate and standardized methods and biomarkers for assessing disease activity, reducing disease burden, and improving the quality of life for children affected by JIA.

Emerging biomarkers for JIA may include small non-coding ribonucleic acids (RNAs), particularly microRNAs (miRNAs). MiRNAs are key post-transcriptional regulators involved in biological processes such as angiogenesis, cell growth, differentiation, inflammation, and the immune response– mechanisms central to the pathogenesis of various chronic pediatric diseases, including JIA [7–9]. These molecules are considered promising biomarkers due to their remarkable stability in different bodily fluids, resistance to RNase digestion, tolerance to varying pH conditions, and ability to withstand multiple freeze–thaw cycles [10, 11]. Some miRNAs have been explored for diagnostic and monitoring, and even have been suggested as potential treatment targets for rheumatoid arthritis in adult patients [12–15].

Most studies exploring miRNAs in JIA have focused on their presence in plasma, serum, or synovial fluid [16–18]. However, a noninvasive and more convenient testing method is preferable for younger children who require frequent follow-up. Urine represents an attractive alternative biofluid for clinical analysis and biomarker research due to its minimally invasive collection, high sample yield, and suitability for repeated measurements. Urine has already been demonstrated as a reliable source of miRNAs in several pediatric diseases, spanning infancy to adolescence [19–21]. Nonetheless, no studies have evaluated urinary miRNAs as potential biomarkers in the context of JIA.

Our study aimed to explore the diagnostic potential of serum and urine miRNAs in JIA. Differences in miRNAs between healthy individuals and JIA patients were analyzed during both the active and remission phases of the disease. Using quantitative reverse transcription PCR (RT–qPCR), we identified miRNAs with significantly altered levels in JIA patients and evaluated their suitability for JIA diagnostics, monitoring, and even remission prediction. Notably, to our knowledge, this is the first study in JIA to analyze longitudinal alterations in miRNA levels over a 12-month follow-up period. Furthermore, our findings underscore the diagnostic potential of serum miRNAs in JIA and represent the first investigation of urine as a novel biofluid for miRNA detection in this disease.

Methods

Study design and study population

A prospective single-center study was conducted at the Hospital of Lithuanian University of Health Sciences Kaunas Clinics. The study included 31 children up to 18 years of age who were diagnosed with JIA by a certified pediatric rheumatologist (A.Sn.), in accordance with the International League of Associations for Rheumatology (ILAR) classification criteria [22]. The exclusion criteria were as follows: (1) a diagnosis of systemic JIA and/or (2) the presence of comorbid chronic diseases. All participants were followed up every 3 months over a 12-month period, with disease activity evaluation by clinical examination and regular laboratory tests at every visit.

Data collection

Demographic data, including age and gender, disease characteristics, and treatment, were collected. Disease-specific information included JIA subtype (classified according to ILAR categories), disease duration, presence/absence of rheumatoid factor (RF), antinuclear antibodies (ANA), and human leucocyte antigen B27 (HLA B27). The most recent ophthalmological examination results were reviewed for signs of uveitis. Clinical evaluation focused on signs of joint inflammation, such as swelling, pain, and morning stiffness. JIA disease activity was assessed using JADAS10 criteria [23]. Patients were categorized into active disease (ACT, $n = 23$) and remission (REM, $n = 8$). At each visit, disease activity parameters were recorded, including signs of joint inflammation, the JADAS10 score, the physician global assessment score (PhGA), and the patient/parent global disease activity score (PaGA). In addition, routine laboratory tests, including complete blood count (CBC), erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) level, were performed.

Blood and urine samples for miRNA analysis were collected at baseline (visit V0) and after 12 months of follow-up (visit V1). Based on the disease progression over the study period, patients were categorized into three groups: (1) patients with active JIA at V0 who achieved remission by V1 (ACT-REM; $n = 14$), (2) patients who remained in an active disease state throughout the follow-up period (ACT-ACT; $n = 7$), and (3) patients who were in remission at V0 and remained in remission throughout the study period (REM-REM; $n = 10$).

For comparison, 22 age- and sex-matched healthy controls (HC) without any signs of inflammation or chronic diseases were included in the study.

Sample collection

Blood samples were collected from all JIA patients into 3.5 mL serum separator vacutainer tubes. Similarly, blood samples of the same volume were collected from the HC group at the time of study enrollment. All serum separation tubes were centrifuged within 4 h of blood collection at 3000 ×g for 10 min at room temperature. The serum layer was then aliquoted into 1 mL portions and stored at -80 °C until further analysis.

Morning urine samples (up to 100 mL) were collected from JIA patients and HC participants in sterile containers. The urine samples were centrifuged within 4 h of collection at 3000 ×g for 15 min at room temperature. The resulting urine sediments were washed with phosphate-buffered saline (PBS), centrifuged under the same conditions twice, resuspended in PBS, and aliquoted into 1 mL portions in cryovials. These samples were stored at -80 °C until further use.

Selection of target MiRNAs

Target miRNAs were selected through a literature review using the National Library of Medicine database (http://www.pub-med.gov) by two researchers (A.Sn. and K.S.) in January 2020. Studies investigating miRNAs in JIA were included. Given the limited and highly variable data on miRNAs in JIA, we also reviewed studies on miRNAs in adult rheumatic diseases. Based on relevance to inflammatory and rheumatic conditions, including JIA, three miRNAs (miR-16, -146a, and -155) were selected for analysis in the prospective longitudinal cohort of JIA patients.

RNA extraction

Total RNA was extracted from 200 µL of serum and urine sediments using miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. During the cell lysis step, 5 µL of synthetic cel-miR-39 (Qiagen) was spiked to each sample as an internal control for RNA extraction and further reactions efficiency. The purified RNA was eluted in 60 µL of RNase-free water, and its concentration and quality were evaluated using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The RNA samples were stored at -80 °C until further analysis.

cDNA synthesis and quantitative reverse transcription–pcr (RT–qPCR)

Copy DNA (cDNA) synthesis of miR-16, -146a, -155, and cel-miR-39 was performed using TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific; assay IDs: 000391, 000468, 467534_mat, and 000200, respectively). cDNA reactions were conducted in a total volume of 7.5 µL, comprising 3.5 µL of reverse transcription mix (100 mM dNTPs with dTTP, 50 U/µL MultiScribe™ Reverse Transcriptase, 10× Reverse Transcription Buffer, and 20 U/µL RNase Inhibitor), 1.5 µL of miRNA-specific stem loop primers, and 2.5 µL of total RNA. The reaction mixes were incubated under the following thermal conditions: 30 min at 16 °C, 30 min at 42 °C, and 5 min at 85 °C. The synthesized cDNA was either used immediately or stored at -20 °C until use.

For miRNA quantification, 1.33 µL of cDNA were further amplified in triplicates using 10 µL final volume RT–qPCR, each consisting of 2× TaqMan Universal PCR Master Mix II, 20× TaqMan™ MicroRNA Assay (both from Applied Biosystems, Thermo Fisher Scientific), and RNase-free water. Reactions were performed on a ViiA7™ Real-Time PCR System, and data were analyzed using ViiA7 Software v1.2 (Applied Biosystems, Thermo Fisher Scientific).

Statistical analysis

Data normality was assessed using the Shapiro–Wilk test. Continuous variables are reported as the mean +/- standard deviation (SD) if the data were normally distributed or as the median and interquartile range (IQR) if not normally distributed. Categorical variables are expressed as counts and percentages (%). To compare normally distributed variables, Student's t-test was applied (paired t-test for paired samples), while the Mann–Whitney U test was used for non-normally distributed data. Fisher's exact test was used for the statistical testing of categorical variables.

RT-qPCR run data preprocessing and statistical analysis were performed using GenEx v.6.0.1 software (MultiD Analyses AB, Göteborg, Sweden). A relative quantification method was used by normalizing Cq values to the spiked-in cel-miR-39 and global miRNA expression levels. The normalized Cq values were then converted into relative quantities, $\log_2$-transformed, and subsequently used for statistical analysis.

Correlations between clinical variables and miRNA levels were analyzed using Spearman's correlation analysis. The diagnostic performance of biomarkers for

distinguishing JIA patients from HC was assessed by determining the area under the receiver operating characteristic (ROC) curve (AUC). Combinations of biomarkers for ROC analysis were generated using logistic regression to enhance diagnostic accuracy. Univariate and multivariate Cox regression analyses were conducted to identify clinico-pathological variables and genetic markers related to remission in ACT JIA. Only the characteristics of ACT JIA cases at baseline (V0) were analyzed, and multivariate analysis was performed with variables with $p \leq 0.1$ in the univariate analysis. Kaplan-Meier analysis was performed to assess remission prediction.

Statistical testing was done using GraphPad Prism 10.2.0 (GraphPad Software, Boston, MA, USA) and IBM SPSS Statistics version 29.0 software (SPSS Inc., Chicago, IL, USA) for Windows. A p-value < 0.05 was considered statistically significant.

Ethical consent

Permission to conduct this study was obtained from the Kaunas Regional Biomedical Research Ethics Committee (No. BE-2-100; 26-Oct-2020). This study was conducted in accordance with the Declaration of Helsinki and the Good Clinical Practice Guidelines.

Results

General characteristics of the participants

In total, 31 JIA patients with a median age of 13.4 years (range 3–17 years) were included in the study, 87% of whom were females. For comparison, 22 gender-matched healthy controls (HC) were enrolled, comprising 18 females (82%) and four males (18%), with a median age of 13.1 years (range 4–17 years). None of the HC participants had signs of inflammation or were diagnosed with chronic diseases. At the time of enrollment, 21 patients (67.7%) had active JIA (ACT) based on the Wallace criteria, while 10 (33.3%) patients were in remission (REM). Among ACT patients, 76.2% exhibited high disease activity according to the JADAS10 criteria, 19.1% had moderate disease activity, and only one patient had low disease activity (Table 1). Compared with ACT patients, REM patients were diagnosed with JIA at a younger age and had a significantly longer disease duration ($p < 0.05$, Table 1). A detailed comparison of the clinical characteristics of ACT and REM patients is provided in Table 1.

The most common JIA subtype was oligoarticular JIA, affecting 41.9% of the patients (8 ACT and 5 REM), followed by enthesitis-related JIA, which was observed in 8 ACT patients. Only one patient in the cohort was diagnosed with RF-positive polyarticular JIA.

Regarding the clinical features, joint pain was reported by all ACT patients (100%), followed by joint swelling (76.2%). Morning stiffness was noted in only six patients (28.6%), and no cases of uveitis were observed in the cohort.

There was no statistically significant difference between the physician global assessment (PhGA) and patient/parent assessment (PaGA) of disease activity using the visual analog scale (VAS) in the ACT group (median value 4 in both, $p = 0.694$).

Regarding conventional inflammation markers such as ESR and CRP levels, only four patients (12.9%) had elevated CRP during the initial visit, all of whom were in the ACT group. There were no significant differences in ESR between the ACT and REM groups (mean 8.38 vs. 7.7 mm/h, respectively, $p = 0.719$). Analysis of the CBC revealed significantly higher neutrophil counts in ACT JIA than in REM patients ($p = 0.036$). However, all measurements in both groups fell within the normal range for their age. None of the other CBC parameters differed significantly between the groups (Table 1).

Patients were treated according to established treatment protocols for their specific JIA subtypes. Most patients (80.6%) received methotrexate and/or a tumor necrosis factor (TNF) inhibitor (adalimumab). Additionally, 61.9% of ACT patients were treated with nonsteroidal anti-inflammatory drugs (NSAIDs). At the follow-up visit (V1), most children (90.3%) achieved clinical remission on medication, and only three patients displayed signs of active disease (all with low disease activity according to JADAS10).

Associations of MiRNA levels with JIA

MiR-16, -146a, and -155 analysis was performed in 29 serum and 31 urine V0 samples from 31 JIA patients, as well as 21 serum and 22 urine samples from 22 HCs. A comparison of miRNA levels between JIA patients vs. controls revealed significantly lower levels of miR-16 and higher amounts of miR-155 in the serum of JIA patients ($p < 0.001$ and $p = 0.002$, respectively, Fig. 1a). Urinary miRNA analysis revealed a significantly reduced amount of miR-146a in JIA patients ($p = 0.032$, Fig. 1b). Although the serum levels of miR-146a were also lower in JIA patients than in HC, the difference was not statistically significant ($p > 0.05$).

Comparative analysis of ACT and REM JIA patients with HC revealed significantly lower serum levels of miR-16 in both ACT and REM groups compared to HC ($p < 0.001$ and $p = 0.020$, respectively, Fig. 1c). While miR-155 amounts were higher only in the ACT vs. HC ($p = 0.006$, Fig. 1c). However, no significant differences in serum miRNA levels were noted between the ACT and REM groups.

Urinary miRNA levels exhibited distinct patterns between ACT and REM groups. MiR-16 levels were significantly higher, and miR-146a levels were markedly lower in REM patients than in ACT patients ($p = 0.013$

Table 1 Clinical characteristics of JIA patients at inclusion (V0)

Characteristics	ACT (*n* = 21)	REM (*n* = 10)	*p* value	Total (*n* = 31)
Gender:				
Female, n (%)	19 (90.5)	8 (80)	0.577	27 (87.1)
Male, n (%)	2 (9.5)	2 (20)		4 (12.9)
Age at baseline, mean yrs (SD)	13.87 (4.1)	12.47 (4.7)	0.406	13.4 (4.2)
Age of JIA diagnosis, mean yrs (SD)	12.91 (4.1)	8.97 (5.0)	**0.025**	11.8 (4.5)
Disease duration, median months (IQR)	6 (3–11)	23.5 (15.5–75.8)	**0.002**	9 (5–17)
Disease duration:				
<6 months, n (%)	9 (42.9)	0 (0.0)	**< 0.001**	9 (29.0)
6–12 months, n (%)	9 (42.9)	0 (0.0)		9 (29.0)
>12 months, n (%)	5 (23.8)	8 (80)		13 (41.9)
ILAR classification:				
Oligoarthritis, n (%)	8 (38.1)	5 (50)	0.225	13 (41.9)
RF negative polyarthritis, n (%)	3 (14.3)	6 (60)		9 (29.0)
RF positive polyarthritis, n (%)	1 (4.8)	0 (0.0)		1 (3.2)
Enthesitis related arthritis, n (%)	8 (38.1)	0 (0.0)		8 (25.8)
ANA positive, n (%)	14 (66.7)	5 (50)	0.197	19 (61.3)
HLA B27 positive, n (%)	9 (42.9)	1 (10)	0.862	10 (32.3)
Clinical features:				
Joint swelling, n (%)	16 (76.2)	1 (10)	**< 0.001**	17 (54.8)
Joint pain, n (%)	21 (100)	1 (10)	**< 0.001**	22 (70.1)
Morning stiffness, n (%)	6 (28.6)	0 (0.0)	-	6 (19.4)
Uveitis, n (%)	0 (0.0)	0 (0.0)	-	0 (0.0)
Disease activity (DA):				
Low DA, n (%)	1 (4.8)	NA	-	1 (3.2)
Moderate DA, n (%)	4 (19.1)	NA	-	4 (12.9)
High DA, n (%)	16 (76.2)	NA	-	16 (51.6)
JADAS10 median (IQR)	11 (7–13)	0 (0)	**< 0.001**	7 (1–12)
PhGA median (IQR)	4 (3–5)	0 (0–1)	**< 0.001**	3 (1–5)
PaGA median (IQR)	4 (3–6)	0 (0)	**< 0.001**	3 (1–5)
Blood parameters:				
WBC x10^9/L mean (SD)	6.59 (1.7)	5.74 (1.7)	0.202	6.31 (1.7)
Lymph x10^9/L, median (IQR)	2.3 (1.6–2.7)	1.8 (1.6–3.8)	0.639	1.9 (1.6–2.7)
Neu x10^9/L, mean (SD)	3.59 (1.2)	2.65 (0.95)	**0.036**	3.29 (1.2)
Mon x10^9/L, median (IQR)	0.6 (0.4–0.7)	0.5 (0.38–0.6)	0.293	0.5 (0.4–0.7)
CRP mg/L, mean (SD)	7.03 (6.4)	6.23 (3.9)	0.719	6.77 (5.7)
ESR mm/hour, mean (SD)	8.38 (5.6)	7.7 (4.5)	0.719	8.2 (5.2)
Treatment*:				
None, n (%)	2 (9.5)	1 (10)	0.695	3 (9.7)
NSAIDs, n (%)	13 (61.9)	0 (0.0)	-	13 (41.9)
Prednisone, n (%)	1 (4.8)	0 (0.0)	-	1 (3.2)
Intraarticular injections of steroids, n (%)	2 (9.5)	0 (0.0)	-	2 (6.5)
MTX only, n (%)	10 (47.6)	2 (20)	**0.002**	12 (38.7)
Sulfa, n (%)	2 (9.5)	0 (0.0)	-	2 (6.5)
AntiTNF only, n (%)	2 (9.5)	4 (40)	**0.002**	6 (19.4)
MTX + antiTNF, n (%)	4 (19.1)	3 (30)	**< 0.001**	7 (22.6)

*Treatment regimens in each group according to different study time points are provided in Supplemental Table 1

Abbreviations: ACT– active group of JIA; ANA– antinuclear antibodies; antiTNF– tumor necrosis factor inhibitors; CRP– C reactive protein; DA– disease activity; ESR– erythrocyte sedimentation rate; HLA B27– human leucocyte antigen B27; IQR– interquartile range; JADAS10– juvenile arthritis disease activity score 10; Lymph– lymphocytes; Mon– monocytes; MTX– methotrexate; n– number; Neu– neutrophils; NSAIDs– nonsteroidal anti-inflammatory drugs; PhGA– physician global assessment of DA VAS (0: no disease activity– 10: maximum disease activity); PaGA– patient/parent global assessment of DA VAS (0: very good– 10: very poor); REM– remission group of JIA; RF– rheumatoid factor; SD– standard deviation; Sulfa– sulfasalazine; VAS– visual analog scale; WBC– white blood cells. Statistically significant differences between visits are marked in bold ($p < 0.05$ was considered significant)

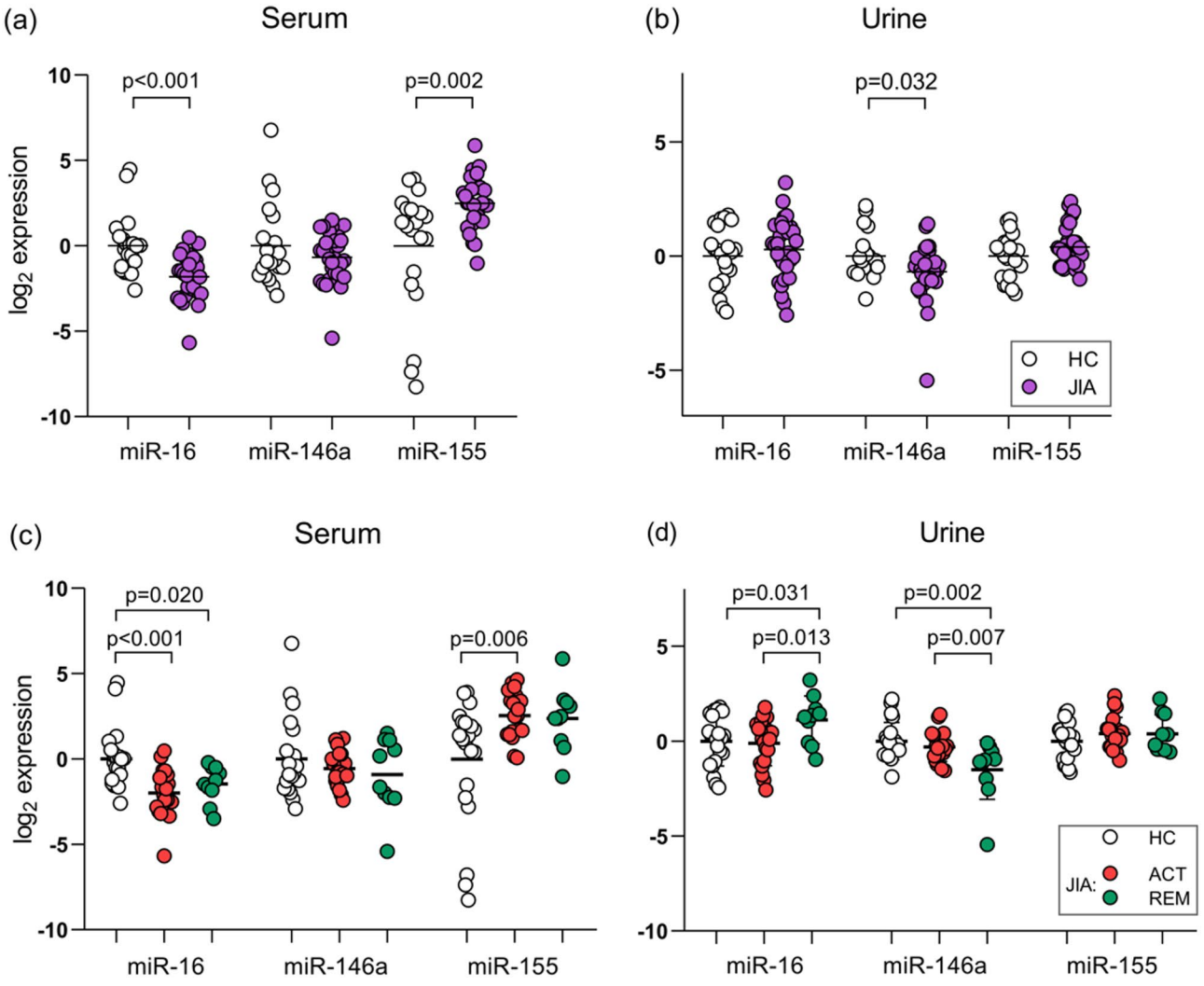

Fig. 1 Comparison of miRNA levels: (**a**) serum and (**b**) urine samples collected from juvenile idiopathic arthritis (JIA) patients and healthy controls (HCs); (**c**) serum and (**d**) urine miRNA levels according to disease activity at the baseline visit. White dots represent HC group, purple dots– total JIA sample group, red dots– active (ACT) disease group, and the green dots– remission (REM) group. Lines depict the average miRNA values

and $p = 0.007$, respectively, Fig. 1d). Moreover, REM patients presented elevated miR-16 levels ($p = 0.031$) and reduced miR-146a levels ($p = 0.002$) compared with HC (Fig. 1d). No significant miRNA changes were noted between the ACT JIA patients and HC subjects.

Additionally, an analysis of miRNA levels in different JIA clinical forms was performed. None of the analyzed miRNAs could distinguish between oligoarthritis, polyarthritis, or enthesitis-associated clinical courses of JIA in either serum or urine samples ($p > 0.05$). Similarly, no differences in miRNA levels were detected when comparing ANA or HLA-B27 positivity or treatment regimens ($p > 0.05$) (data not shown).

Correlation analysis of MiRNA levels with JIA clinical variables

Correlation analysis between selected serum and urinary miRNAs and clinical or laboratory parameters yielded diverse but predominantly weak associations (Fig. 2). Serum miR-16 statistically significantly correlated with white blood cell (WBC) count (r_s=0.316, $p = 0.007$), lymphocyte count (r_s=0.369, $p = 0.001$), PCT (r_s=-0.281, $p = 0.016$) and MPV (r_s=0.272, $p = 0.020$). MiR-146a exhibited a significant albeit weak correlation with hemoglobin (Hgb) (r_s=-0.300, $p = 0.01$), platelet count (r_s=0.241, $p = 0.04$), and platelet biomarkers (PDW r_s=-0.258, $p = 0.027$; PCT r_s=0.278, $p = 0.017$), and CRP (r_s=-0.276, $p = 0.048$). Furthermore, miR-155 in serum was weakly positively correlated with PaGA (r_s=0.307, $p = 0.027$), although no significant correlations were noted between miR-155 and laboratory markers. Additionally,

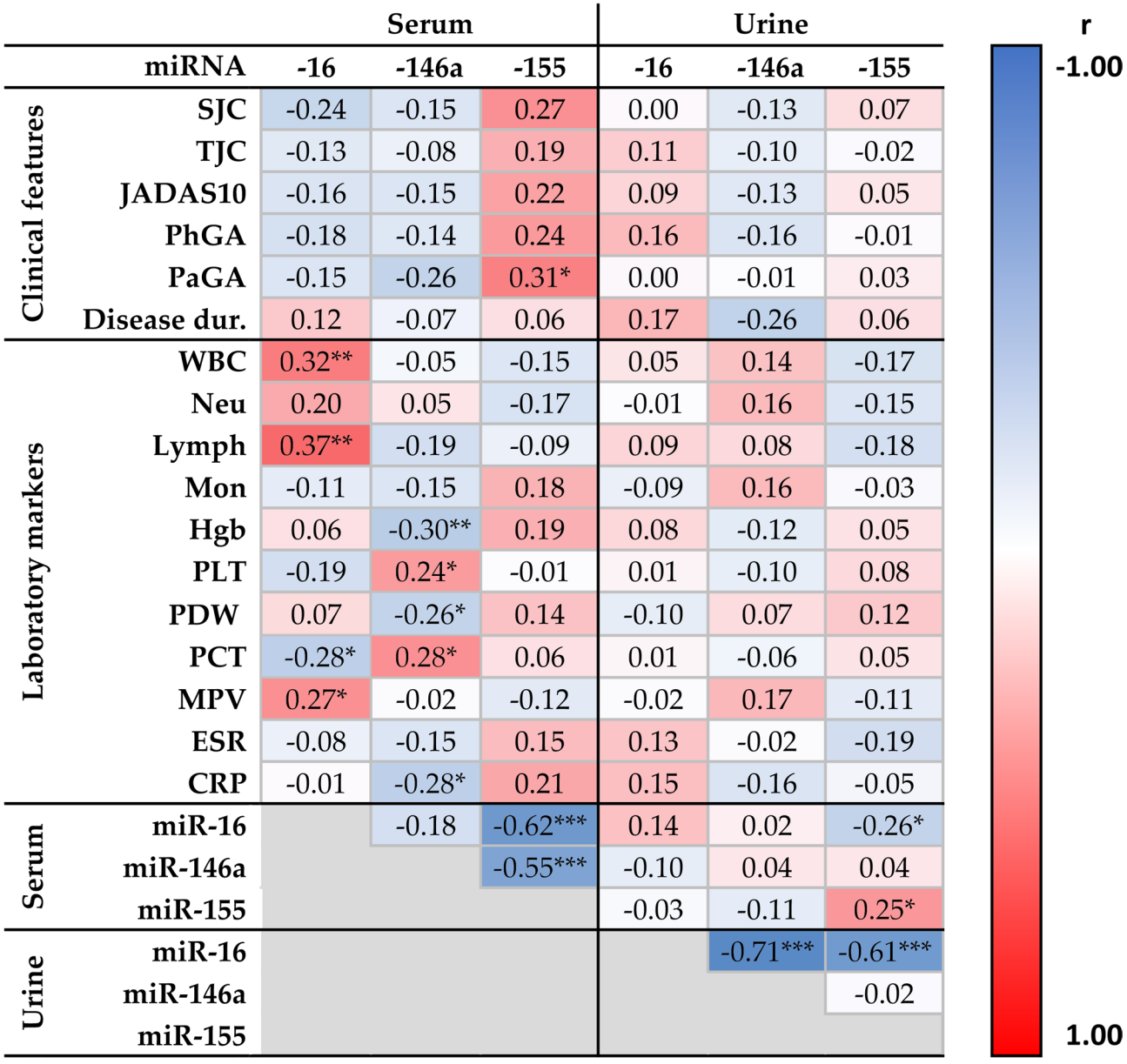

Fig. 2 Correlation of clinical symptoms, inflammation markers and patient- and physician-reported measures with miRNAs in serum and urine. The Spearman correlation coefficients are color-coded according to the scale bar, with red signifying a positive correlation, blue indicating a negative correlation, and white indicating no correlation. The results showing significant correlations are indicated as follows: * $p \leq 0.050$, ** $p \leq 0.010$, *** $p \leq 0.001$. Abbreviations: CRP– C reactive protein; dur.– duration; ESR– erythrocyte sedimentation rate; Hgb– hemoglobin; JADAS10– juvenile arthritis disease activity scale of 10 joints; Lymph– lymphocytes; Mon– monocytes; Neu– neutrophils; PaGA– patient/parent global disease activity; PCT– plateletcrit; PDW– platelet distribution volume; PhGA– physician's global disease activity; PLT– platelet count; r– correlation coefficient; SJC– swollen joint count; TJC– tender joint count; WBC– white blood cells

a moderate negative correlation was identified between miR-155 and miR-146a (r_s=-0.550, $p < 0.001$), and a strong negative correlation was observed between miR-155 and miR-16 (r_s=-0.619, $p < 0.001$). Finally, none of the serum miRNAs were significantly related to disease duration.

No urinary miRNA levels correlated with any of the clinical or laboratory parameters. However, strong negative correlations were identified between urinary miR-16 and other miRNAs (miR-146a r_s=-0.711, $p = 0.001$, and miR-155 r_s=-0.609, $p < 0.001$).

Correlation analysis of miRNAs between different bodily fluids revealed a weak positive correlation between miR-155 levels in serum and urine (r_s=0.245, $p = 0.040$) and a weak negative correlation between serum miR-16 and urine miR-155 (r_s=-0.259, $p = 0.029$) (Fig. 2). A statistical comparison of the levels of analyzed miRNAs between bodily fluids revealed significant differences in

some cases. JIA patients showed increased levels of urinary miR-16 ($p < 0.001$) and decreased levels of miR-155 ($p < 0.001$) compared to serum. However, the differences in miR-146a levels between bodily fluids in JIA patients were not statistically significant ($p > 0.05$). In healthy controls, no significant differences in miRNA levels were detected between different bodily fluids (data not shown).

Diagnostic potential of MiRNAs

The diagnostic performance of the selected miRNAs was evaluated using receiver operating characteristic (ROC) curves and by calculating the area under the curve (AUC). Serum miR-16 was identified as a potential biomarker for distinguishing JIA patients from HC, showing a sensitivity of 72% and a specificity of 71% (AUC 0.81, 95% CI 0.70–0.93, $p < 0.001$, Fig. 3a). Serum miR-155 also demonstrated significant diagnostic ability for identifying JIA, with a sensitivity of 62% and specificity of 71% (AUC 0.73, 95% CI 0.59–0.87, $p = 0.005$). Combining serum miR-16 and miR-155 slightly improved diagnostic accuracy, increasing specificity and yielding a marginally higher AUC (0.82 vs. 0.81, $p = 0.002$, Fig. 3a). In urine, only miR-146a could distinguish JIA patients from HC with a sensitivity of 65% and a specificity of 64% (AUC 0.68, 95% CI 0.53–0.82, $p = 0.030$, Fig. 3b). The other analyzed urinary miRNAs or their combinations did not demonstrate statistically significant diagnostic capabilities ($p > 0.05$).

Changes in MiRNA levels during the follow-up period

To evaluate miRNA changes during the follow-up period, JIA patients were grouped into three groups based on their disease course, as described in the Methods section. Paired V0 and V1 samples for longitudinal miRNA level analysis were available for 23 serum and 23 urine samples (74%, 23/31). Patients transitioning from active disease to remission (ACT-REM group) showed significantly lower levels of serum miR-16 ($p = 0.021$) and higher levels of miR-155 ($p = 0.009$) during the active phase of the disease (Fig. 4a). In contrast, no significant differences in serum miRNA levels were detected between V0 and V1 in the ACT-ACT group (Fig. 4b). Notably, patients in remission at both V0 and V1 (REM-REM group), exhibited significantly 6-fold lower levels of miR-146a in serum collected at V0 vs. V1 visit ($p = 0.004$, Fig. 4c). No significant changes in selected miRNA levels were observed across all groups in the paired urine sample miRNA analysis.

Univariate and multivariate Cox proportional hazards ratio analyses were used to assess associations between demographic, clinico-pathological variables, genetic markers, and remission of ACT JIA cases at V0. In the univariate analysis, lower PhGA and JADAS scores and higher serum miR-146a levels tended to be associated with REM, but none of the variables reached statistical significance ($p > 0.05$; Supplemental Table 2). In multivariate analysis (run by using variables with $p \leq 0.1$, highlighted in bold), none of the variables retained statistical significance in the remission prediction (Supplemental Table 2). However, Kaplan-Meier survival analysis of the same variables– specifically JADAS10, PhGA, and serum miR-146a– indicated that higher serum miR-146a levels were significantly associated with JIA remission ($p = 0.040$, Fig. 4d).

Discussion

Early diagnostics and longitudinal monitoring in JIA remain a clinical challenge. Discrepancies in several clinical disease activity evaluation scales [3] emphasize the

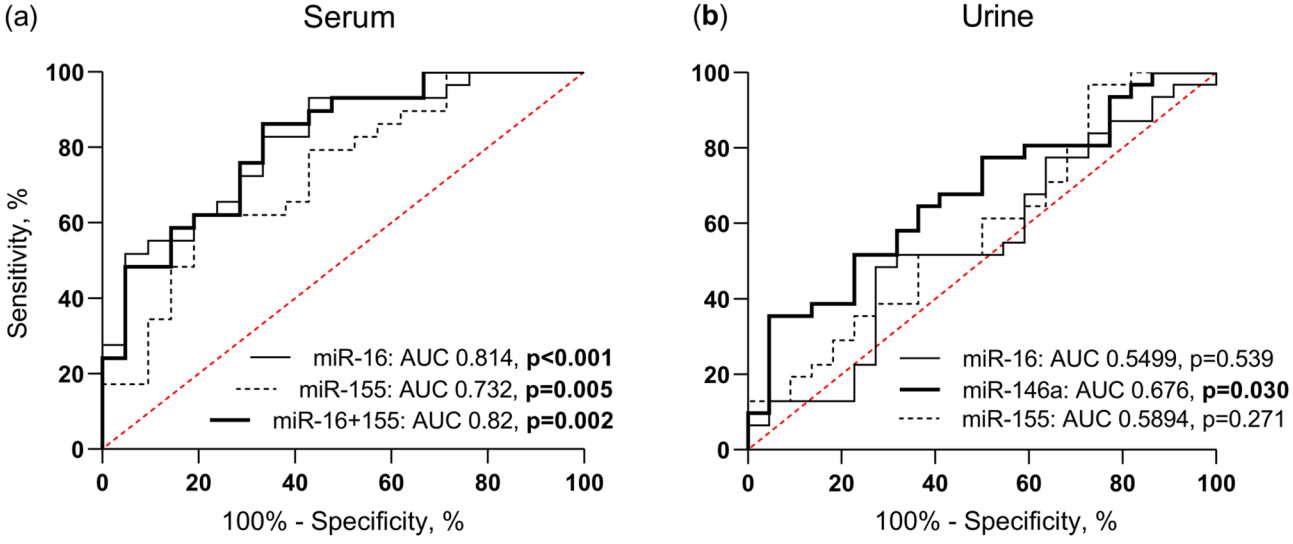

Fig. 3 Receiver operating characteristic (ROC) curve analysis of circulating miRNAs for distinguishing JIA patients from healthy controls (HC): (**a**) miR-16, miR-155, and their combination in serum; (**b**) miR-16, miR-146a, and miR-155 in urine. AUC– area under the ROC curve

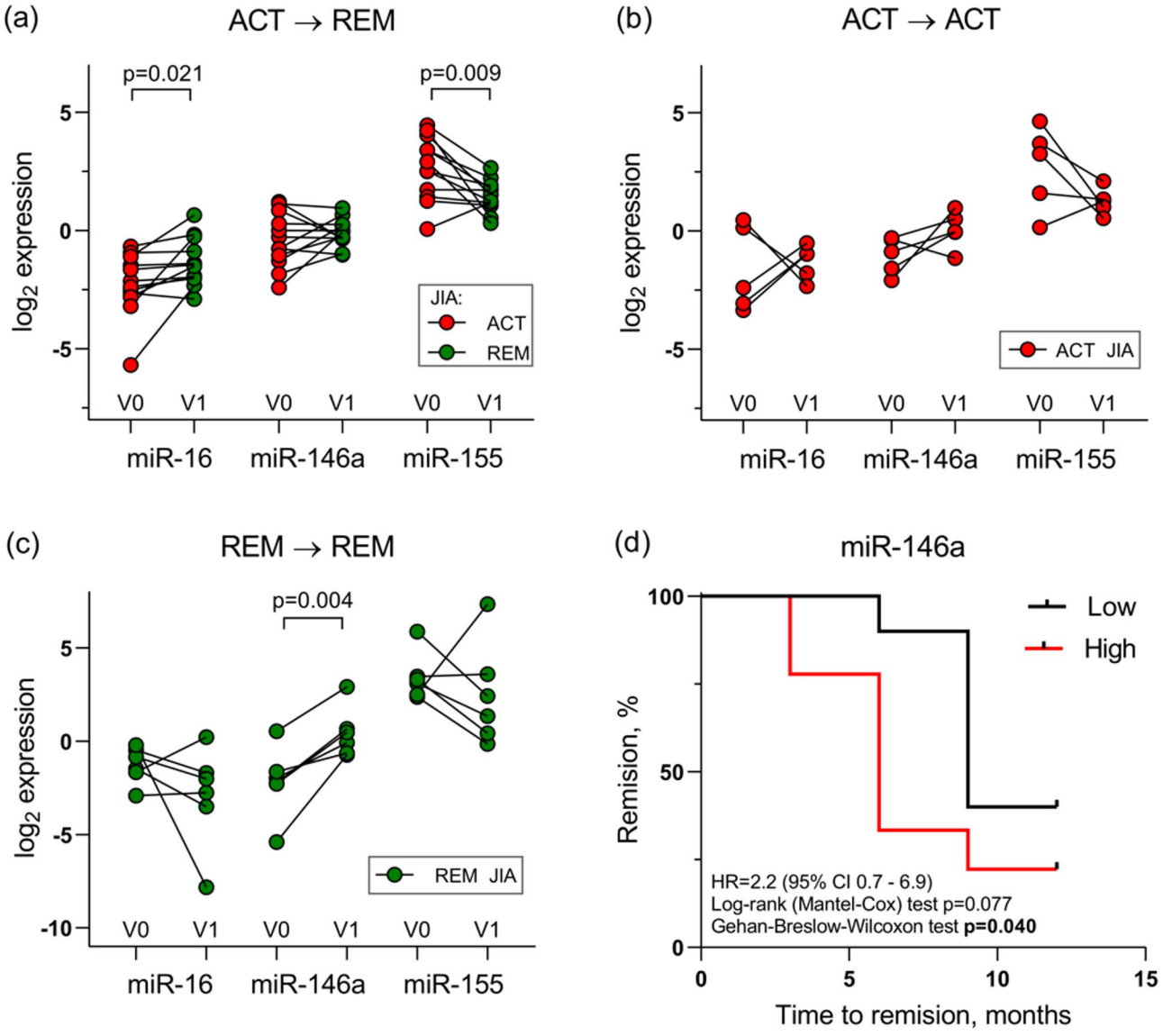

Fig. 4 Comparison of miRNA levels between paired serum samples of JIA patients' in different groups according to the course of the disease: (**a**) ACT patients in V0 who reached REM at V1, (**b**) patients who stayed ACT during the entire study period, (**c**) patients who were in REM during the entire study period. Red dots represent patients with active (ACT) disease, green dots– patients in remission (REM). (**d**) Kaplan-Meier survival analysis of remission prediction for JIA patients according to serum miR-146a values. HR– hazard ratio

need for additional biomarkers in managing patients with JIA. The biological features of miRNAs and their proven involvement in diverse biological processes make them promising molecules for describing the state of chronic inflammation [7, 9]. This single-center, prospective JIA study evaluated the diagnostic and disease monitoring potential of serum and urine miRNAs, specifically miR-16, -146a, and -155. Using RT-qPCR, we demonstrated that circulating miRNAs effectively distinguish JIA patients from healthy controls with high sensitivity and specificity. Moreover, longitudinal analysis revealed that miRNAs were significantly associated with the disease activity and remission, highlighting their potential as biomarkers for monitoring JIA. Notably, this is the first study to demonstrate that urine can serve as an additional biofluid for miRNA research in JIA patients.

Analysis of serum miRNAs revealed the significant diagnostic potential of serum miR-16 with meaningful sensitivity and specificity. In addition, lower levels of serum miR-16 were observed in our cohort of JIA patients, both in the ACT and REM states of the disease, compared to healthy children. Serum miR-16 levels were also significantly lower in clinically active JIA patients than in REM patients. Furthermore, paired analysis of serum samples over the follow-up period identified an increase in miR-16 levels, suggesting its potential

as a dynamic and individualized biomarker for JIA. These results are supported by studies performed in adults with rheumatoid arthritis (RA), which have shown lower levels of miR-16 in the early stages of the disease [24]. Similarly, Raggi et al. found that miR-16 was downregulated in the synovia and plasma of JIA patients [8]. In contrast, studies by Ma et al. [25] and Mc Alpine et al. [26] reported an opposite fluctuations of miR-16 in plasma samples from JIA patients compared with HC, whereas Demir et al. found no differences in miR-16 levels between JIA patients in ACT and REM states compared with HC [27]. Discrepancies between the results could be influenced by the different biofluids explored or the distinct methods used for miRNA detection. However, extensive cancer research has demonstrated that miR-16 significantly impacts cell proliferation and invasion, promotes cell apoptosis and suppresses cell cycle progression [28, 29]. An important mechanism through which miRNAs influence immune function is their impact on T-cell metabolism and activation [30]. Marcais et al. identified that T-cell activation can be triggered by the absence of miR-16, which stimulates the nuclear factor-kappa B (NF-κB) pathway [31], a crucial regulator of the inflammatory response [32, 33]. Furthermore, Zhou et al. demonstrated that miR-16 targets a silencing mediator for retinoid and thyroid hormone receptor (SMRT), modulating NF-κB-regulated inflammatory responses in human monocytes [34]. Although we did not find a correlation between miR-16 and total lymphocyte count, further investigations exploring lymphocyte phenotyping might enhance the understanding of specific pathways in which miR-16 is involved. Considering previously published data, changes in miR-16 levels may reflect a restored balance in T-cell regulation, contributing to reduced inflammation and supporting the potential use of miR-16 in clinical practice for monitoring JIA disease progression.

Another inflammation-related miRNA in our study was miR-155. Our findings of increased miR-155 levels in the serum of JIA patients compared to HC are in line with previous studies on JIA, which identified increased levels of miR-155 in plasma of JIA patients, and this elevation persisted during the inactive phase of the disease [27, 35]. In a study by Raggi et al., which focused on the oligoarthritis form of JIA, increased miR-155 expression was observed in both synovial fluid and plasma compared to HC [8]. Similarly, Nziza et al. demonstrated the ability of miR-155 to differentiate between JIA synovial fluid and septic arthritis [16]. All of those studies characterize miR-155 as a potent pro-inflammatory miRNA that amplifies inflammation. Several mechanisms of miR-155 in inflammation have been described, including interactions with molecules such as the Treg-specific transcription factor FoxP3 [36], AID-mediated myc IgH [37], and Src homology 2-containing inositol phosphatase-1 (SHIP-1), among others [36, 38–40]. In RA inhibition of miR-155 in synovial CD14 + cells reduced TNF-α production [41], and its overexpression interferes with monocyte polarization into M2-like macrophages, leading to increased cytokine production [42]. Although similar mechanisms have not been directly studied in JIA, macrophages—key targets of miR-155—are central to JIA pathogenesis [43, 44], supporting its biological relevance in our study. Notably, although miR-155 levels were elevated in JIA patients compared to HC, no significant cross-sectional difference was observed between the active and remission groups. In contrast, paired longitudinal analysis showed a significant decrease in miR-155 expression from the active phase to remission within the same individuals. This suggests that miR-155, similar to miR-16, may function more effectively as a dynamic biomarker for monitoring intra-individual changes in disease activity over time, rather than as a static marker for distinguishing disease state across patients. Collectively, these findings for miR-155 and miR-16 highlight the potential utility of certain circulating miRNAs for individualized, longitudinal assessment of disease activity rather than for cross-sectional disease classification. However, further validation is required through long-term studies involving serial sample collection.

MiR-146a– the most frequently studied miRNA in rheumatic diseases [45]– was found to increase in serum during the follow-up period in JIA patients who remained in remission throughout the entire study, suggesting potential long-term treatment efficacy. Furthermore, higher serum miR-146a levels at baseline in ACT JIA patients predicted JIA remission. However, we did not observe a significant difference in serum miR-146a levels between JIA patients and HC. Similarly, a study by Kamiya et al. showed no significant difference in miR-146a levels between the JIA and HC groups [46]. In contrast, a study by Ma et al. [25] reported elevated miR-146a levels in plasma and demonstrated high sensitivity in distinguishing JIA patients from HC. Interestingly, we detected lower miR-146a levels in urine samples from JIA patients compared to HC, while significantly elevated levels were observed during active disease relative to remission. These findings suggest a potential value for miR-146a as a marker of disease activity. However, the absence of significant differences between healthy controls and the active JIA group, along with the lack of other disease control groups, limits the ability to draw conclusions regarding its specificity to JIA. Although we explored potential treatment-related influences, no significant associations were identified. Nonetheless, further investigations in larger, treatment-stratified cohorts that include additional disease controls will be necessary to determine whether changes in urinary miR-146a reflect JIA-specific immunological processes or more general

responses to immune-modulating therapies. Despite conflicting data concerning miR-146a in blood, several experimental models indicate that miR-146a functions as a negative-feedback regulator through the Toll-like receptor (TLR) in the NF-κB inflammatory pathway [47]. Animal studies have shown that miR-146a can prevent joint destruction and bone damage in arthritis models when used as a therapeutic agent [14, 48]. Furthermore, Mann et al. reported cross-regulation of miR-146a and miR-155 in macrophage inflammatory responses via the NF-κB pathway, emphasizing the dominant function of miR-155 in promoting inflammation [49]. In support of these findings, our study revealed a negative correlation of miR-155 with both miR-16 and miR-146a. Taken together, our findings across different bodily fluids support the potential role of miR-146a as a biomarker of disease dynamics in JIA. However, its disease specificity remains to be confirmed in extended cohorts.

Notably, the majority of the studies on JIA have focused on miRNA changes in blood [8, 25, 27, 41, 46]. To the best of our knowledge, this is the first study of urinary miRNAs in both JIA and even in RA patients to date. The use of urine as a source of miRNAs has been extensively studied in cancer patients and is gaining interest in pediatric population, particularly those with renal conditions such as nephrotic syndrome [50] and lupus nephritis [21, 51]. However, there are limited data on urinary miRNA levels in primarily non-renal conditions [19, 52]. Other molecules, such as bone and cartilage degradation proteins [53] or glycosaminoglycans [54], have been successfully studied in the urine of JIA patients. Research in RA patients' urine has predominantly focused on proteomic studies [55]. Further validation of urinary miRNAs in independent cohorts is essential to expand the understanding of their roles in chronic inflammation and to explore their clinical utility.

This study has some limitations. One limitation is the relatively small sample size; however, given the rarity of JIA and the 12-month follow-up period, our cohort is comparable to, or even larger than some previous single-center studies [8, 16, 26, 27, 46]. Another limitation is the inclusion of various JIA clinical subgroups, which reduced the homogeneity of the cohort. As discussed, results may vary across the studies, possibly due to factors such as different JIA subgroups included (e.g., polyarticular JIA [46] vs. mainly oligoarthritis [8]), different biomaterials used (e.g., plasma [25] vs. extracellular vesicles [8] vs. serum [27]), and variations in miRNA detection methods. Additionally, discrepancies may arise from different scales for evaluating JIA disease activity used, such as the JADAS [25] or active joint count [26]. We used the validated JADAS10 tool, with separate cut-offs for oligo- and polyarticular JIA, ensuring representation of all forms of non-systemic JIA. Furthermore, we did not include samples from joint aspiration, which would have provided insight into miRNA changes at the site of inflammation. However, not all JIA patients require this invasive procedure, and comparisons with HC would have been excluded because of ethical concerns.

Further investigations should prioritize minimally invasive or non-invasive sample collection methods, such as urine, to reduce patient stress during regular visits to pediatric rheumatologist. Non-invasive sampling would also facilitate longitudinal studies and improve patient compliance, ultimately enhancing the understanding of miRNA dynamics in JIA. Moreover, assessing miRNA expression differences between autoimmune and non-autoimmune conditions associated with joint injury, such as pediatric orthopedic diseases, could help to identify miRNAs specific to autoimmune processes. The consistency of future studies could be improved by including larger JIA cohorts, standardizing technical approaches for miRNA detection, and analyzing additional biomarkers involved in miRNA pathways.

Conclusions

In conclusion, our study demonstrated that the serum levels of miR-16 and miR-155 can distinguish JIA patients from healthy controls with high specificity and sensitivity. Additionally, we identified significant changes in miRNA levels throughout the disease course, particularly between the clinically active and remission states of JIA. Furthermore, we established the potential of miR-146a as a predictor of remission in JIA patients. Notably, this study is the first to highlight the utility of urine as a noninvasive biofluid for miRNA analysis in children with JIA, with miR-146a in urine showing promise as a diagnostic biomarker. However, further research is essential to enhance our understanding of the role of miRNAs in JIA, to examine the prognostic value of miRNA alterations in the long term and the influence of treatment on miRNA dynamics in JIA, and to validate these findings in more extensive, independent cohorts.

Abbreviations

ACT	active disease
ANA	antinuclear antibodies
antiTNF	tumor necrosis factor inhibitors
AUC	area under the ROC curve
CBC	complete blood count
CI	confidence intervals
CRP	C-reactive protein
DA	disease activity
ESR	erythrocyte sedimentation rate
HC	healthy controls
Hgb	hemoglobin
HLA B27	human leucocyte antigen B27
HR	hazard ratio
JADAS	Juvenile Arthritis Disease Activity Score
JIA	juvenile idiopathic arthritis
IL	interleukin
ILAR	International League of Associations for Rheumatology
IQR	interquartile range

Lymph	lymphocytes
miRNA	microribonucleic acid
Mon	monocytes
MPV	mean platelet volume
mTOR	target of rapamycin
MTX	methotrexate
N	number
Neu	neutrophils
NF-κB	nuclear factor-kappaB
NSAIDs	nonsteroidal anti-inflammatory drugs
PaGA	patient/parent global disease activity score
PBS	phosphate-buffered saline
PCT	plateletcrit
PDW	platelet distribution volume
PhGA	physician global assessment score
PLT	platelet count
RA	rheumatoid arthritis
REM	remission
RF	rheumatoid factor
RNAs	ribonucleic acids
ROC	receiver operating characteristic
RT	reverse transcription
RT-qPCR	quantitative reverse transcription polymerase chain reaction
SD	standard deviation
SHIP-1	Src homology 2-containing inositol phosphatase-1
SMRT	silencing mediator for retinoid and thyroid hormone receptor
Sulfa	sulfasalazine
TGF	transforming growth factor
TNF	tumor necrosis factor
VAS	visual analog scale
WBC	white blood cells

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12969-025-01136-w.

Supplementary Material 1

Acknowledgements

We kindly thank Ieva Juskeviciute and Brigita Skerbaite for their extensive work in preparing and testing the samples. We kindly thank all the patients and their families, as well as our hospital nursing staff.

Author contributions

ASn was a major contributor to the study design, conceptualization, methodology, analysis, data curation, funding acquisition, writing and editing of the manuscript, and literature review of the miRNA studies done in JIA.KS contributed to the methodology of the study, performed the literature review of the miRNA studies done in JIA, investigated the miRNA results, analyzed the data, and reviewed and edited the manuscript.ASl contributed to data and funding acquisition and reviewed and edited the manuscript.BBS investigated and validated the results of miRNAs and contributed to data acquisition. AB contributed to the conceptualization and supervision of the study and substantively edited and reviewed the manuscript.SJ supervised the study and substantively edited and reviewed the manuscript.LJ contributed to the conceptualization and supervision of the study and substantively edited and reviewed the manuscript.All the authors read and approved the final manuscript.

Funding

This research was funded by LITHUANIAN UNIVERSITY OF HEALTH SCIENCES, grant number 2022-MC3-0003, and the APC was funded by LITHUANIAN UNIVERSITY OF HEALTH SCIENCES. The funder had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Data availability

All the data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate

Permission to conduct this study was obtained from the Kaunas Regional Biomedical Research Ethics Committee (No. BE-2-100; 26-Oct-2020). Informed consent was obtained from all the subjects involved in the study. This study was conducted in accordance with the Declaration of Helsinki and the Good Clinical Practice Guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 7 May 2025 / Accepted: 18 July 2025
Published online: 28 July 2025

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