ORIGINAL PAPER
PŮVODNÍ PRÁCE

Contribution of miRNAs and Other Inflammatory Biomarkers to the Differentiation of Inflammatory and Non-Inflammatory Joint Effusions

Příspěvek k odlišení zánětlivých a nezánětlivých kloubních výpotků pomocí miRNA a dalších zánětlivých biomarkerů

P. KUŠNIEROVÁ[1], I. BYSTROŇOVÁ[1,2,5], P. WALDER[3], R. HLUBEK[3], D. STEJSKAL[1,4]

[1] Institute of Laboratory Medicine, Department of Clinical Biochemistry, University Hospital Ostrava
[2] The Department of Epidemiology and Public Health, Faculty of Medicine, University of Ostrava
[3] Orthopedic Department, University Hospital Ostrava
[4] Department of Biomedical Sciences, Faculty of Medicine, University of Ostrava
[5] Department of Epidemiology and Public Health, Faculty of Medicine, University of Ostrava

ABSTRACT

PURPOSE OF THE STUDY

The aim of the study was to determine miR-146a-5p, miR-223-3p and miR-23a-3p by an enzyme immunoassay in patients with inflammatory and non-inflammatory joint effusion and to verify the usefulness of these miRNAs as biomarkers of joint inflammation.

MATERIAL AND METHODS

Synovial fluid (SF) samples were collected from 82 patients. The group consisted of 60 non-inflammatory, 11 inflammatory-non-pyogenic, 11 inflammatory-pyogenic SF. SF miRNA was isolated by RNA Isolation Kit Plasma/Serum. The concentrations of miRNA were determined by enzyme-linked immunosorbent assays (ELISA), C-reactive protein, interleukin-6 and procalcitonin on automatic analyser, presepsin on POCT system, interleukin-1 and human neutrofil defensins 1-3 by ELISA.

RESULTS

A statistically significant negative correlation was found between miR-146-5p and miR-223-3p, WBC, IL-1β, IL-6 and CRP ($P < 0.05$) in all groups; a statistically significant positive correlation was found between miR-223-3p and miR-23a-3p, WBC, PMN, IL-1beta, IL-6 and HNP1-3, as well as a positive correlation of miR-23a-3p with IL-1β, IL-6 and HNP1-3. A statistically significant difference was found between miR-146a-5p, miR-223-3p and miR-23a-3p and individual SF groups, $P = 0.006$, $P < 0.001$, respectively. PMN, WBC, Il-1β, IL-6, HNP 1-3 predicted the inflammatory processes with excellent diagnostic power (AUC > 0.9). The clinical relevance expressed by effect size was the strongest in miR-223-3p, PMN, IL-1 , HNP 1-3 between non-inflammatory and inflammatory-pyogenic group.

CONCLUSIONS

Our study quantified the SF miRNA by ELISA. We have shown that miR-146a-5p, miR-223-3p and miR-23a-3p can be an important group of biomarkers for the detection and monitoring of various pathophysiological conditions in synovial fluid, including inflammatory conditions.

Key words: miRNA, synovial fluid, inflammatory joint disease, enzyme-linked immunosorbent assay.

The study was supported by the Ministry of Health, Czech Republic – conceptual development of research organization (FNOs/2018).

INTRODUCTION

Synovial fluid (SF) can have different etiologies, different appearances and different biochemical and microscopic characteristics. Based on these parameters, the SF can be divided into non-inflammatory, inflammatory and pyogenic (32). If a joint inflammation occurs under the overall change in the patient's condition and with a typical joint puncture there is usually not a diagnostic problem. However, there are also joint infections under a non-specific image, which are difficult to diagnose, mainly due to the absence of specific clinical signs and symptoms, relative lack of accurate laboratory tests, low virulence due to previous treatment and biofilm ability of pathogens. This is especially true for patients treated for non-targeted ATBs, including patients with implanted joint replacements.

Current diagnostics focus on the measurement of standard inflammatory factors such as white blood cell (WBC) levels, determination of serum C-reactive protein (CRP), microbiological analysis of SF and imaging methods such as computed tomography (CT or CAT scanning), magnetic resonance imaging (MRI) and positron emission tomography (PET), or the most modern combination of methods such as SPECT/CT and PET/CT. However, most of these tests (methods) do not show 100% sensitivity and specificity, so a combination with a clinical history and symptoms is neces-

sary. In recent years, attention has been focused on miRNAs.

miRNAs are small non-coding RNA molecules that play an important regulatory role in gene translation through the silencing or degradation of target mRNAs (29). They affect a number of biological processes, including differentiation, proliferation, metabolism, apoptosis or inflammation (20, 30, 37, 39). In the immune system, some miRNAs have been shown to be the key regulators of immune cell development and innate and adaptive immune responses (33). Deregulation of miRNA levels in cells is associated with various diseases, suggesting that they might be used as diagnostic or prognostic biomarkers (47). Accurate detection and quantification of miRNAs is problematic mainly due to the determination of a short section of these molecules (approximately 22 nucleotides), small sequence differences between miRNAs and also their low concentrations in body fluids (6). Many approaches to RNA detection can already be found on the market today, with the focus on enzymatic immunoassays.

There have been studied three miRNAs, miR-146a-5p, miR-23a-3p a miR-223-3p. miR-146a is one of the miRNAs overexpressing in response to LPS and other inflammatory stimuli in the human monocytic cell line THP-1 (43). In addition, miR-146a targets Tumor necrosis factor receptor (TNFR)-associated factor 6 and interleukin-1 receptor associated kinase (IRAK1), two key adaptor molecules in the TLR and IL-1 receptor signalling pathways (2, 43). Furthermore, miR-146a has been found to suppress NF-κB activity (2), as well as the LPS-induced inflammatory response (6, 30, 33, 47), and plays a role in the development of endotoxin tolerance (25, 26). It follows that miR-146a plays a key role in the regulation of inflammatory responses through negative feedback pathway. At the same time, miR-146a has been proven to be differentially expressed in a number of inflammatory autoimmune diseases, such as systemic lupus erythematosus, Sjögren's syndrome (47) and rheumatoid arthritis (34). Nakasa et al. and Stanczyk et al. (27, 41) demonstrated increased expression of miR-146a in synovial tissue and synovial fibroblasts of patients with rheumatoid arthritis compared to normal individuals and patients with osteoarthritis.

miR-223-3p is a hematopoietic specific microRNA having a crucial function in the development of the myeloid lineage. It negatively regulates progenitor proliferation and granulocyte differentiation and activation (19, 49). Changes in miR-223-3p expression have been described under various pathological conditions, e.g. in different types of tumors, in patients with rheumatoid arthritis, osteoarthritis and other autoimmune diseases (8). At the same time, the association of miR-223-3p with septic status has been demonstrated, with serum levels of miR-223 and miR-146 being significantly reduced, so both miRNAs can be used as novel biomarkers of sepsis with high specificity and sensitivity (46, 48).

miR-23a-3p is located in the miR-23a~27a~24-2 cluster (18). This cluster encodes a pri-miRNA transcript composed of 3 miRNAs: miR-23a, miR-27a and miR-24. The miR-23a-3p studied has been described in several types of cancer, cardiac hypertrophy and muscle atrophy (9, 10, 17, 14, 23, 38, 45). Previous studies have also shown that miR-23a was down-regulated in sepsis and that miR-23a could modulate the inflammatory response by targeting autophagy mediated by the ubiquitin-like protein ATG12 (40). It has also been found that miR-23a can reduce the expression of IL-17-mediated pro-inflammatory cytokines (16, 22).

The present study investigated miR-146a-5p, miR-223-3p and miR-23a-3p expression in patients with inflammatory and non-inflammatory joint disease.

MATERIAL AND METHODS

Patients

This study includes 82 patients from Orthopedic Department whose SF and serum (S) samples were sent for analysis to the Institute of Laboratory Diagnostics, Department of Clinical Biochemistry, University Hospital Ostrava. Clinical criteria for inclusion: age >18 years, signed informed consent, sufficient amount of SF obtained during a joint or burst puncture; clinical criteria for exclusion: age < 18 years, insufficient SF (less than 5 ml), suspected aspiration of venous or arterial blood, patients treated with an oncology program for systemic chemotherapy, patients at risk of post-puncture bleeding (full anticoagulant therapy at the time of puncture, or congenital or acquired hypo-coagulation conditions with insufficient substitution), pregnancy in women. Approval of informed consent was obtained by the Ethics Committee of the University Hospital Ostrava, the Czech Republic (reference number of the project: 322/2018) in accordance with the Declaration of Helsinki 1975 revised in 2000. Patients were divided into individual groups of the MSIS criteria: (1) non-inflammatory (Non-Inf; CRP < 10 $mg.L^{-1}$, WBC < $3*10^9.L^{-1}$, PMN < 65 %, negative cultivation, absence of pus; n = 60; 30 women, average age 67.8 ± 15.4 years; 30 men, average age 55.5 ± 15.6 years), (2) inflammatory - pyogenic (Inf-P; CRP > 10 $mg.L^{-1}$, WBC > $3*10^9.L^{-1}$, PMN > 65 %, positive cultivation, presence of pus; n = 11; 3 women, average age 68.0 ± 8.2 years; 8 men, average age 63.5 ± 16.6 years) and (3) inflammatory - non-pyogenic (Inf-NP; CRP > 10 $mg.L^{-1}$, WBC > $3*10^9.L^{-1}$, PMN > 65 %, negative cultivation, absence of pus; n = 11, 6 women, average age 56.5 ± 13.0 years; 5 men, average age 51.8 ± 8.2 years). The diagnoses in Inf-P comprised prosthetic joint infection (n = 6) and septic arthritis which was caused by Staphylococcus aureus (n = 4) and Streptococcus dysgalactiae (n = 1). The Inf-NP included patients with rheumatoid arthritis (n = 5), gouty arthritis (n = 4), Lyme arthritis (n = 1) and Haemophilic arthritis (n = 1). The Non-Inf included patients with osteoarthritis (n = 55) and meniscopathy (n = 5). All individuals were observed for at least half a year after the tests. The duration of the problem, comorbidity (diabetes mellitus, high blood pressure, cancer, depressive disorder, etc.) and associated medication were monitored, however, no effects on the tested methods were found, which is consistent with the general use of these types of tests.

Samples

SF samples were collected into a polypropylene tube (Sarstedt, Nümbrecht, Germany) and S samples into a Serum Gel with Clotting Activator tube (Sarstedt). Both the SF and the S were collected on the same day. A portion of the uncentrifuged SF sample was used for cytological analysis. The remain of the SF and whole S samples were centrifuged at 2,500 × g for 6 minutes at 4°C, and the supernatants were then aliquoted into at least 3–5 vials (0.5 ml per vial) and stored at -70° C until analysed. Some samples of non-inflammatory SF were very viscous. To reduce the viscosity, hyaluronidase (5.5U) was added to the SF sample before miRNA isolation, some samples were also heated to 37 °C, however even these procedures did not always lead to a positive result and the sample could not be isolated (n = 5).

RNA extraction

The RNA Isolation Kit Plasma/Serum (REF RIK002, Bio-Vendor Laboratorni medicina a.s., Czech Republic) was used for purification of small and large RNA including miRNA from SF. 300 µl of SF was added to 90 µl of Lysis Buffer and vortex for 5 s. Then was added 30 µl of Protein Precipitation Buffer, vortexed for 5 s and incubated for 1 min at the room temperature (18–25 °C) and centrifuged for 3 min at 11,000 x g to pellet the protein. After, the clear supernatant was transferred into a new Collection Tube and 400 µl of isopropanol was added and vortexed for 5 s. Next, an isolation column was placed in the collection tube and the sample loaded onto the column. Incubated for 2 min at room temperature, centrifuged for 30 s at 11,000 x g and discarded the flow-through and the column was placed back into the collection tube. Then, 700 µl of Wash Buffer (WB2P) was added to the isolation column, centrifuged for 30 s at 11,000 x g, discarded flow through and placed the column back into the collection tube. 250 µl of (WB2P) was added to the isolation column, centrifuged for 2 min at 11,000 x g. In the next step, 50 µl of rDNase was added directly onto the silica membrane of the isolation column, the lid was closed and incubated at room temperature for 15 min. Then, 100 µl of Wash Buffer (WB1P) was added to the isolation column, centrifuged for 30 s at 11,000 x g, discarded flow-through and the

Table 1. Descriptive characteristics; values of mean, medians and 2.5th resp. 97.5th percentiles estimated in individual groups of patients

	Categories of diagnostic groups								
	Inf - NP			Inf - P			Non-Inf		
	N	Mean	Median 2.5 to 97.5 P	N	Mean	Median 2.5 to 97.5 P	N	Mean	Median 2.5 to 97.5 P
Age	11	54.4	59.0 35.0 to 73.0	11	64.7	64.0 39.0 to 94.0	60	61.8	63.0 18.0 to 90.0
miR-146a-5p (amol.µl^{-1})	8	8.58	6.65 4.50 to 22.6	9	4.44	4.80 2.50 to 6.30	49	7.96	7.10 1.92 to 18.0
miR-223-3p (amol.µl^{-1})	7	2.33	2.50 0.40 to 5.70	10	9.63	4.75 0.30 to 31.2	38	0.45	0.40 0.30 to 1.80
miR-23a-3p (amol.µl^{-1})	8	2.25	0.80 0.80 to 8.00	8	19.0	7.75 0.80 to 52.7	44	1.49	0.80 0.70 to 10.3
WBC *10^{9}.L^{-1}	11	13.0	11.7 3.19 to 33.7	11	32.9	22.9 12.0 to 68.4	60	0.50	0.24 0.05 to 2.20
PMN (%)	11	76.0	72.0 65.5 to 95.4	11	88.5	88.9 62.1 to 96.3	60	27.4	25.7 4.60 to 67.8
IL-1β (ng.L^{-1})	11	11.4	1.89 1.25 to 107	11	44.0	17.5 1.90 to 224	60	6.13	1.2 0.400 to 50.0
IL-6 (ng.L^{-1})	11	2392	787 30.0 to 13906	10	44686	55000 309 to 55000	60	2866	488 2.70 to 36465
CRP (mg.L^{-1})	11	22.3	13.0 10.0 to 95.0	11	39.5	39.0 10.0 to 91.0	60	2.05	1.00 1.00 to 9.00
PCT (µg.L^{-1})	10	0.53	0.61 0.01 to 0.80	10	0.71	0.55 0.50 to 1.40	58	0.67	0.70 0.50 to 0.97
PRES (ng.L^{-1})	10	5977	2090 589 to 21390	10	3610	2300 712 to 13310	57	3804	2540 558 to 15387
HNP1-3 (µg.L^{-1})	11	1689	1260 0.156 to 6270	11	613	7200 116 to 8910	57	652	90.3 0.156 to 9536

WBC, white blood cell; PMN, polymorphonucleocytes; IL-1β, Interleukin-1β; IL-6, Interleukin-6; CRP, Creactive protein; PCT, procalcitonin; PRES, presepsin; HNP1-3, human neutrofil defensins 1-3; HLF2, lactoferrin; NGAL, neutrophil gelatinase-associated lipocalin; COMP, cartilage oligomeric matrix protein; PMNE, polymorphonuclear elastase; N, number of patients; * 2.5th and 97.5th percentile values; Inf-NP, inflammatory-non-pyogenic group; Inf-P, inflammatory-pyogenic group; Non-Inf, non-inflammatory group

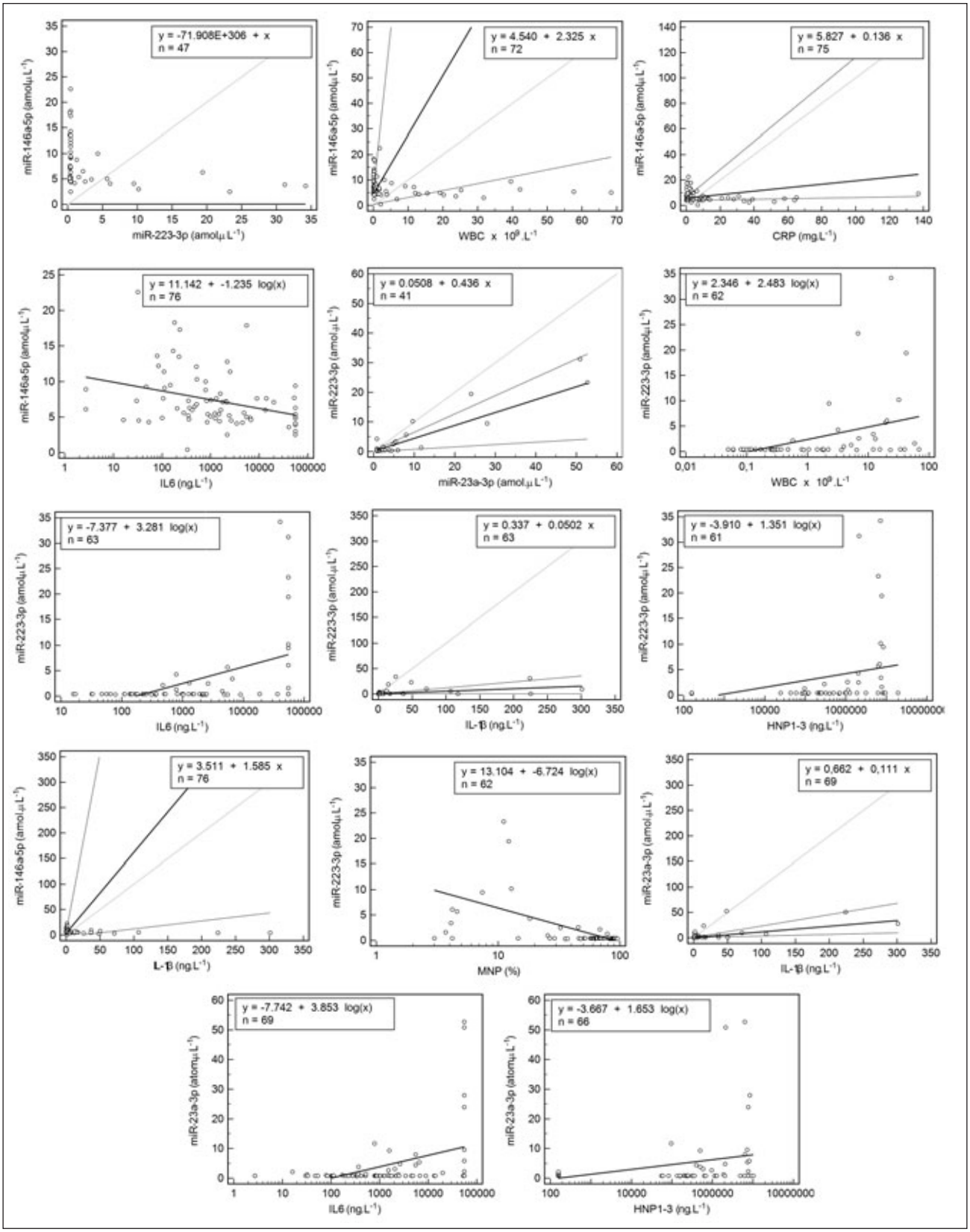

Fig. 1. Passing-Bablok regression analysis of specific miRNAs and selected biomarkers in all groups.

column was placed back into the column tube. In the following two steps, the silicone membrane was repeatedly washed and dried with 700 µl and afterwards with 250 µl WB2P, centrifuged for 30 s, resp. 2 min at 11,000 x g. In the last step, the isolation column was placed in a new collection column (1.5 ml), 30 µl of RNase-free H_2O was added directly to the silicone membrane, incubated for 1 min at room temperature and then centrifuged

for 1 min at 11,000 x g. The isolate thus obtained was used directly for miRNA analysis or frozen at –70 °C.

Enzyme immunoassay of human miRNA

The concentration of miRNAs, specifically miR-146a-5p, miR-23a-3p a miR-223-3p (hsa-miR-146a-5p miREIA, REF RDM0024H; hsa-miR-23a-3p miREIA, REF RDM0009H; hsa-miR-223-3p miREIA, REF RDM0015H, Bio-Vendor Laboratorni medicina a.s., Czech Republic) were determined by Enzyme-Linked ImmunoSorbent Assay (ELISA). These assays for miRNA quantification involve hybridization of miRNA isolate to complementary biotinylated DNA probe for specific miRNA. The DNA/RNA hybrids were then transferred into microplate wells pre-coated with monoclonal antibody specific to perfectly matched DNA/miRNA hybrids. After washing, the solid phase was incubated with streptavidin-HRP conjugate and after another washing step, the resulting complexes were visualized by chromogenic substrate. The absorbance was proportional to the concentration of specific miRNA.

Twice Diluted RNA samples with RNase Inhibitor working solution was used for determination of miR-223-3p and miR-23a-3p and for miR-146a-5p the sample was diluted three times.

The quality control samples supplied by the manufacturers of the diagnostic kits were used for accurate measurement. Kit manufacturers reported that analytical sensitivity was 0.033 amol.μL^{-1} for miR-146a-5p, 0.13 amol.μL^{-1} for miR-23a-3p and miR-223-3p. All samples were measured in duplicate. The average coefficients of variation for miR-146a-5p, miR-23a-3p a miR-223-3p were 7.1%, 5.7%, and 9.1%, respectively.

Other analytical methods

The concentrations of other biomarkers, specifically C-reactive protein (CRP, REF OSR6147, Beckman Coulter, Inc., Brea, CA, USA) were determined on an AU 5800 automated analyzer; haematological parameters, relative and absolute numbers of leukocytes (WBC), erythrocytes (RBC), mononuclear (MNP) and polymorphonuclear (PMN) leukocyte counts on a Sysmex XN-9000 Automated in Body Fluid mode; Interleukin-6 (IL-6, ADVIA Centaur IL-6, REF 10995080, Siemens) and procalcitonin (PCT, ADVIA Centaur BRAHMS Procalcitonin, REF 10378883, Siemens) on Advia Centaur XP automated analyzer and presepsin (PRES, Pathfast Presepsin, REF 1110-4000, Mitsubishi Kagaku Iatron, Inc.) on Pathfast system. Interleukin-1β (IL-1β, Human Interleukin-1 beta ELISA, REF RD194559200R, BioVendor Research and Diagnostic Products) and human neutrofil defensins 1-3 (HNP1-3, Human HNP1-3 ELISA KIT, REF HK317, Hycult-Biotech, Inc. United States) were determined by Enzyme-Linked ImmunoSorbent Assay (ELISA).

Undiluted SF were used for determination of routine and inflammatory biomarkers on the automated analyser, for PRES were used 1/10 diluted samples, for IL-1β 1/3 diluted samples and for HNP1-3 1/1000 diluted samples.

Table 2. Correlations between selected biochemical markers in synovial fluid in all groups

		miR-146a-5p	miR-223-3p	miR-23a-3p
miR-146a-5p	r_s		-0.415	-0.141
	P		0.008	0.281
	n		40	60
miR-223-3p	r_s	-0.415		0.519
	P	0.008		<0.001
	n	40		35
miR-23a-3p	r_s	-0.141	0.519	
	P	0.281	<0.001	
	n	60	35	
WBC	r_s	-0.305	0.441	0.359
	P	0.013	<0.001	0.005
	n	66	55	60
PMN	r_s	-0.215	0.567	0.350
	P	0.083	<0.001	0.006
	n	66	55	60
IL-1β	r_s	-0.318	0.502	0.371
	P	0.009	<0.001	0.004
	n	66	55	60
IL-6	r_s	-0.354	0.320	0.391
	P	0.004	0.017	0.002
	n	66	55	60
CRP	r_s	-0.361	0.369	0.311
	P	0.003	0.006	0.015
	n	66	55	60
PCT	r_s	0.140	-0.133	-0.114
	P	0.269	0.347	0.393
	n	64	52	58
PRES	r_s	0.036	0.033	-0.047
	P	0.783	0.815	0.733
	n	62	54	56
HNP1-3	r_s	-0.188	0.607	0.373
	P	0.139	<0.001	0.004
	n	63	53	57

WBC, white blood cell; PMN, polymorphonucleocytes; IL-1β, Interleukin-1β; IL-6, Interleukin-6; CRP, C-reactive protein; PCT, procalcitonin; PRES, presepsin; HNP1-3, human neutrofil defensins 1-3; r_s, Spearman's correlation coefficient; P values < 0.05 were considered significant; N, number of values; the values in bold are the P values that indicated significance.

Statistical methods

The software Excel, Stata version 13, MedCal version 17.9.7., R and NCSS 2007 were used for statistical analysis (42).

Basic descriptive statistics, including frequency tables, medians, arithmetic means and percentiles, were used to describe the results. The normality hypothesis was rejected; therefore, non-parametric tests were used, including the Kruskal-Wallis rank test.

The relationship between the parameters was assessed by Spearman's correlation coefficient. Statistical tests were evaluated using a significance level of 5%. The effect size of correlation coefficient was evaluated according to Cohen (5). The diagnostics value of SF biomarkers was evaluated by receiver operating characteristics (ROC) curve analysis. When calculating the sensitivity and specificity (and 95% confidence

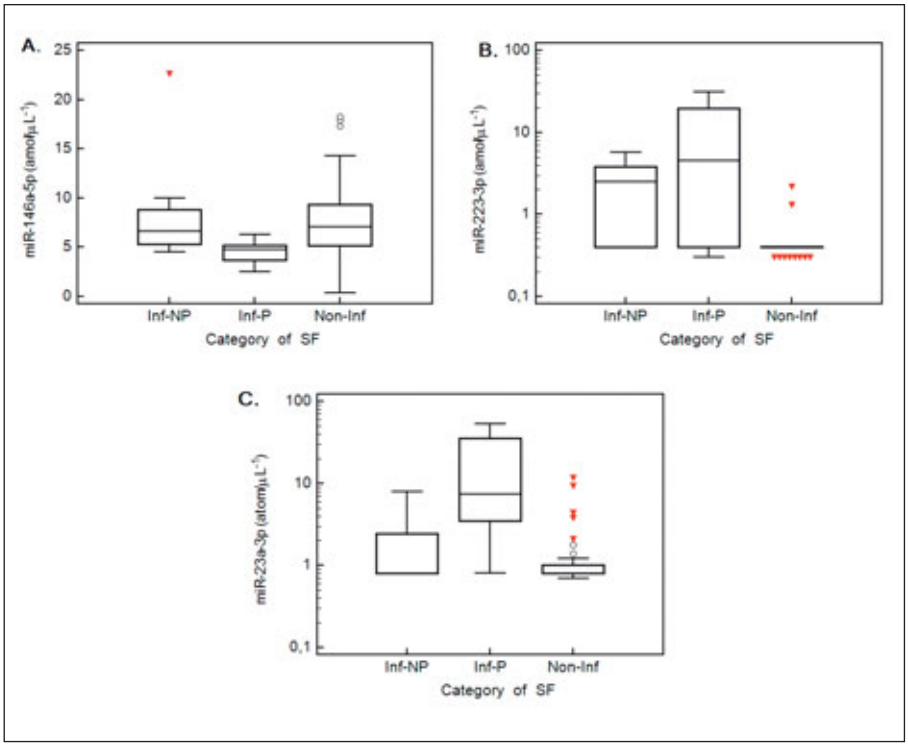

Fig. 2. Box-plot of SF miR-146a-5p (A), SF miR-223-3p (B) and SF miR-23a-3p (C) in individual groups according to type of synovial fluid (SF). Inf-NP, Inflammatory-non-pyogenic group; Inf-P; Inflammatory-pyogenic group; Non-Inf, Non-inflammatory group

interval – CI) of each SF biomarker, the results of the non-inflammatory group were considered against the group involving any inflammatory process. A biomarker with an AUC ≤ 0.5 represented a biomarker without diagnostic power. The significance level for statistical tests was set at P = 0.05.

Clinical relevance was assessed using Cohen's d, which was expressed as the absolute difference of weighted averages between two groups to the standard deviation units. Results with a Cohen´s d of 0.2–0.5 represents a small effect size, 0.5–0.8 a medium effect size, and values greater than 0.8 as a large effect size (4).

RESULTS

A total of 82 patient samples were included in the study. The development of their disease allowed us to place them into specific groups with almost 100% probability, e.g. subsequent cultivation of the pathogen in the Inf-P group, healing of exudates in the Non-Inf group without ATB treatment such as application of corticoids, surgical treatment with mechanical or degenerative changes-arthroscopically. And for the Inf-NP group effusions, an anamnestic finding of another etiological cause and at the same time repeatedly negation of the detection of a microorganism in the monitored effusion. Characteristics of the studied groups are presented in Table 1.

The correlation was evaluated between miR-223-3p, miR-23a-3p and miR-146a-5p levels and several other laboratory markers. There was a statistically significant negative correlation between miR-146-5p and other biomarkers (P <0.05) such as miR-223-3p, WBC, IL-1β, IL-6 and CRP in all groups. A statistically significant positive correlation was found between miR-223-3p and other studied biomarkers such as miR-23a-3p, WBC, PMN, IL-1β, IL-6, CRP and HNP1-3, as well as a positive correlation of miR-23a-3p with WBC, PMN, CRP, IL-1β, IL-6 and HNP1-3 (Table 2). At the same time, according to Cohen's, the large correlation effect size was between miR-223-3p and miR-23a-3p, PMN, Il-1β, HNP 1-3, the medium effect size between miR-146a-5p and miR-223-3p, WBC, CRP, Il-1β, Il-6, between miR-223-3p and WBC, IL-6, and between miR-23a-3p and WBC, PMN, CRP, IL-1β, Il-6, HNP 1-3.

The regression dependence between the studied parameters were graphically evaluated (Fig. 1).

A statistically significant dependence was found by the nonparametric Kruskal-Wallis test between miR-146a-5p, miR-223-3p and miR-23a-3p and individual SF groups, P = 0.006, P < 0.001, respectively. For miR-146a-5p and miR-23a-3p, a statistically significant dependence between Inf-P and Inf-NP and Non-Inf groups was demonstrated; in the case of miR-223-3p, a statistically significant difference between Non-Inf and Inf-NP and Inf-P groups was demonstrated (Fig. 2).

Table 3. Sensitivity and specificity of synovial fluid biomarkers

	AUC	P	Sensitivity (%)	Specificity (%)	Cut-off
PMN	0.976	<0.001	90.0	98.6	84.8 %
WBC	0.970	<0.001	100.0	88.6	6.44x10^9 L^{-1}
IL-1β	0.935	<0.001	90.9	93.0	4.73 ng.L^{-1}
IL-6	0.932	<0.001	90.0	91.5	5.5 µg.L^{-1}
HNP1-3	0.913	<0.001	90.9	92.6	2050 µg.L^{-1}
miR-23a-3p	0.888	<0.001	87.5	88.5	2.1 amol.µl^{-1}
CRP	0.862	<0.001	90.9	83.1	5.0 mg.L^{-1}
miR-146a-5p	0.836	<0.001	100.0	57.91	6.3 amol.µl^{-1}
miR-223-3p	0.798	0.003	70.0	88.9	1.3 amol.µl^{-1}
PCT	0.556	0.647	50.0	77.9	0.50 µg.L^{-1}
PRES	0.534	0.730	40.0	80.6	1240 ng.L^{-1}

AUC, area under the curve; PMN, polymorphonuclear; MNP, mononuclear; IL, interleukin; WBC, leukocytes; CRP, C-reactive protein; HNP1-3, human neutrophil defensin 1-3; PCT, procalcitonin; PRES, presepsin;

Table 4. Clinical relevance of studied biomarkers of synovial fluid expressed by Cohen's d

	Effect size (Cohen's d)		
	Non-Inf vs. Inf-NP	Non-Inf vs. Inf-P	Inf-NP vs. Inf-P
PMN	**1.72**	**3.81**	**1.36**
WBC	**2.93**	**4.11**	**1.26**
IL-1β	0.18	**1.05**	0.65
IL-6	0.06	**3.78**	**2.76**
HNP1-3	0.53	**2.60**	**1.93**
miR-23a-3p	0.34	**2.11**	**1.09**
CRP	**1.37**	**2.77**	0.61
miR-146a-5p	0.14	**0.97**	**1.00**
miR-223-3p	**2.23**	**1.88**	**0.83**
PCT	**0.93**	0.25	0.62
PRES	0.50	0.04	0.40

PMN, polymorphonuclear; MNP, mononuclear; IL, interleukin; WBC, leukocytes; CRP, C-reactive protein; HNP1-3, human neutrophil defensin 1-3; PCT, procalcitonin; PRES, presepsin; Inf-NP, inflammatory-non-pyogenic group; Inf-P; inflammatory-pyogenic group; Non-Inf, non-inflammatory group; small effect size, d = 0.2 to 0.5; medium effect size, d = 0.5 to 0.8; large effect size, d ≥ 0.8

All of the studied biomarkers showed a diagnostic power predicting the inflammatory process greater than ≥ 0.5. Five biomarkers in the SF (PMN, WBC, Il-1β, IL-6, HNP 1-3) predicted the inflammatory processes with excellent diagnostic power, AUC > 0.9 (Table 3, Fig. 3A). Three additional biomarkers (miR-23a-3p, miR-146a-5p, CRP) demonstrated AUC values between 0.8 and 0.9 %, and one biomarker (miR-223-3p) AUC value between 0.8 and 0.7 % (Fig. 3B).

The clinical relevance of the studied biomarkers was assessed by using Cohen's statistics ("d") too. The biomarkers, such as WBC, PMN, IL-6, CRP, HNP 1-3, miR-23a-3p and miR-223-3p show the large effect size between non-inflammatory and inflammatory-pyogenic group (Table 4).

DISCUSSION

In this study has been tested miRNAs as a marker of inflammatory joint diseases. For their determination, unlike other authors, the enzyme immunoassay method was used involving hybridization of miRNAs isolated from SF to complementary biotinylated DNA probe for a specific miRNA, making this work unique. There was compared the concentration of specific miRNAs with other biochemical and hematological inflammatory markers in all groups and in individual groups of the SF type. At the same time, the group of patients were divided by the inflammatory type of SF into the inflammatory category - non-pyogenic with the absence of a pyogenic form of inflammation and inflammatory pyogenic with a proven bacterial or other infectious agent. The first of the miRNAs studied was miR-146a-5p. The results show a statistically significant correlation between miR-146a-5p and IL-1β, IL-6 and other inflammatory biomarkers in all groups of SF type, but when this parameter was compared with other inflammatory biomarkers in the individual groups, a statistically significant dependence was not demonstrated. Similar issue was published by Perry et al. (35), who dealt with the post-transcriptional regulation of microRNA gene expression under physiological and chronic pathological conditions. They have shown that only the simulation with high concentrations of IL-1β results in a rapid time- and concentration-dependent increase in miRNA-146a. This may explain our results as well. They have also found that increased expression of miRNA-146a negatively regulates the release of pro-inflammatory chemokines, IL-8 and RANTES (Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted), and that the effect

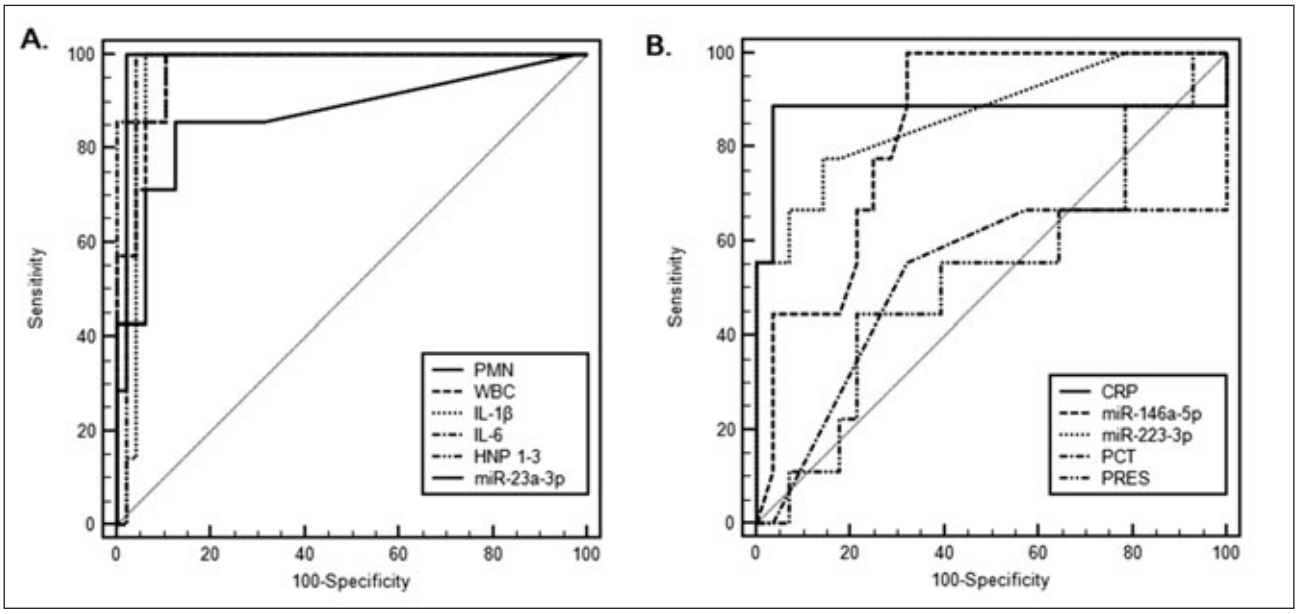

Fig. 3. ROC analysis of selected biomarkers in the synovial fluid.

of miRNA-146a is mediated at the translational level and not through down-regulation of proteins involved in the IL-1β signalling pathway or transcription or chemokine secretion. They have deduced a new mechanism for the negative regulation of severe inflammation during the innate immune response.

Also, Nziza N. et al. (28) studied miRNAs in patients with inflammatory disease, this time with juvenile idiopathic arthritis (JIA) compared with septic arthritis (SA). They identified 19 miRNAs in SF, of which 80% of miRNAs in S samples showed comparable expression in both types of disease, 10% miRNAs differing in expression, whereas in SF there was a significant reduction in expression in more than 20% of detectable miRNAs. At the same time, two of these miRNAs, miR-146a-5p, miR-155, were overexpressed in the SF of children with JIA versus SA. Thus, the authors consider these miRNAs to be useful negative regulators of inflammation, the early measurement of which could be beneficial for the rapid initiation of optimal treatment and care.

Nakase et al. reported that miR-146 expression is induced by stimulation with inflammatory cytokines such as TNFα and IL-1β (27). Goldring (12) demonstrated that inflammatory cytokines play an important role as catabolic factors in osteoarthritis (OA) in cartilage. Ying-Jie Guan et al. (15) further found that miR-146a may be a central mediator of several critical factors in the pathogenesis of osteoarthritis (OA), including aging, inflammation, and mechanical overload. miR-146a apparently functions through the Notch regulatory pathway of the inflammatory cytokines IL-6 and IL-1β and their catabolic effects on joint tissues. The content of miR-146a in articular cartilage appears to be crucial for maintaining cartilage homeostasis and preventing OA and progression. Recovery of miR-146a in chondrogenic cells in miR-146a was sufficient to rescue joint degeneration due to meniscus injury. This suggests that topical supplementation of miR-146a in the joint may serve as a potential therapeutic for the treatment of OA to inhibit joint inflammation and degeneration and shift homeostasis out of catabolism during ageing and injury.

Another miRNA studied was miR-223-3p. A statistically significant correlation was demonstrated between this miRNA and other standardly studied biomarkers of inflammation, namely WBC, PMN, IL-1β, IL-6 and HNP1-3. HNP1-3 is used as a small antimicrobial peptide with bactericidal effects against gram-positive and gram-negative bacteria to diagnose patients with prosthetic joint infection (3).

At the same time, according to Cohen's, the large effect size was between miR-223-3p and some biochemical markers such as PMN, IL-IB, HNP 1-3 that meet validated criteria from 2018 for the evaluation of knee infections (31).

Also, Murata et al. (24) measured the concentrations of miR-146a, miR-223 and other in the SF and plasma. The study was focused on patients with rheumatoid arthritis (RA) and osteoarthritis (OA) versus healthy controls and for this purpose there was used transcription-polymerase chain reaction. Concentrations of miR-146a, miR-223 in synovial fluid were significantly higher in RA than in OA, so they can be considered as diagnostic biomarkers for distinguishing among these diagnoses. These authors emphasize that specific plasma miRNAs can distinguish a group of healthy volunteers from patients with rheumatoid disease, whereas synovial fluid miRNAs distinguish different types of rheumatism.

In this study, miR-23a-3p showed a statistically significant correlation with IL-1β, IL-6, and HNP1-3. This miRNA has also been described in connection with markers of the inflammatory response. Xiang Si et al. (40) showed that miR-23a was down-regulated during the initial septic insult. There was demonstrated significantly different concentrations of miR-23a in patients with sepsis compared with patients with non-infectious systemic inflammatory response syndrome. As well as, it was shown that miR-23a is negatively associated with autophagic activities after septic insult, its down-regulation alleviates the inhibition of autophagy with subsequent suppression of inflammatory mediators.

Li et al. (21) study miRNA in knee synovial fluid from early-stage and late-stage knee osteoarthritis patients. They found that IL-1β contributes to the release of miR-23a-3p and miR-27b-3p into the SF from synovium. Also Tavallaee et al. (44) describe the increased levels of miR-23a-3p in the synovial fluid of OA patients with late-stage compared to early-stage radiographic knee OA.

Another aim of this study was to correlate specific miRNA with rapid laboratory response tests, procalcitonin and point of care determination of presepsin. Presepsin is a biomarker of sepsis, a tool for diagnosing severe conditions caused by G- and G+ bacteria as well as fungi. Presepsin is a product of the direct reaction of CD14 protein and lipopolysaccharides, while the increase in CRP and PCT levels is indirectly influenced, mostly by interleukin-6 (1, 7, 11, 13, 36). Therefore, increased presepsin levels are considered as useful tool that specifically reflects the presence of infection as opposed to CRP, which may be elevated for non-infectious reasons, such as after trauma. However, the results have shown no correlation between these parameters in all groups and in individual groups.

Limitations of the study

Unfortunately, there might be also weaknesses found in this study. Although usually three to five aliquots of SF were frozen for each patient in the study, there was not always enough material for all assays, especially for miRNA isolation and repeated determination to adjust the sample dilution. At the same time, some samples of synovial fluid were so viscous that they were not usable for analysis.

CONCLUSIONS

Our study quantified the synovial fluid miRNA by Enzyme-Linked ImmunoSorbent Assay which involves hybridization of miRNA to complementary biotinylated DNA probe for specific miRNA. The data showed large

effect size between miR-223-3p and miR-23a-3p, PMN, Il-1β, HNP 1-3. The clinical relevance was the strongest in miR-223-3p, PMN, IL-1β, HNP 1-3 between non-inflammatory and inflammatory-pyogenic group. We have shown that miR-146a-5p, miR-223-3p and miR-23a-3p can be an important group of biomarkers for the detection and monitoring of various pathophysiological conditions in synovial fluid, including inflammatory conditions.

Acknowledgments

We wish to thank to Barbora Dvořáková from BioVendor – Laboratorní medicína a.s., Research & Diagnostic Products Division for valuable advice and comments. We thank Ing. F. Všianský for help with the statistical analysis.

References

1. Berbari E, Mabry T, Tsaras G, Spangehl M, Erwin PJ, Murad MH, Steckelberg J, Osmon D. Inflammatory blood laboratory levels as markers of prosthetic joint infection: a systematic review and meta-analysis. J Bone Joint Surg Am. 2010;92:2102–2109.
2. Bhaumik D, Scott GK, Schokrpur S, Patil CK, Orjalo AV, Rodier F, Lithgow GJ, Campisi J. MicroRNAs miR-146a/b negatively modulate the senescence-associated inflammatory mediators IL-6 and IL-8. Aging (Albany NY). 2009;1:402–411.
3. Bonanzinga T, Ferrari MC, Tanzi G, Vandenbulcke F, Zahar A, Marcacci M. The role of alpha defensin in prosthetic joint infection (PJI) diagnosis: a literature review. EFORT Open Rev. 2019;4:10–13.
4. Citrome L. Quantifying clinical relevance. Innov Clin Neurosci. 2014;11:26–30.
5. Cohen J. A power primer. Psychol Bull. 1992;112:155–159.
6. Dellett M, Simpson DA. Considerations for optimization of microRNA PCR assays for molecular diagnosis. Expert Rev Mol Diagn. 2016;16:407–414.
7. Ettinger M, Calliess T, Kielstein JT, Sibai J, Brückner T, Lichtinghagen R, Windhagen H, Lukasz A. Circulating biomarkers for discrimination between aseptic joint failure, low-grade infection, and high-grade septic failure. Clin Infect Dis. 2015;61:332–341.
8. Filková M, Aradi B, Senolt L, Ospelt C, Vettori S, Mann H, Filer A, Raza K, Buckley CD, Snow M, Vencovský J, Pavelka K, Michel BA, Gay RE, Gay S, Jüngel A. Association of circulating miR-223 and miR-16 with disease activity in patients with early rheumatoid arthritis. Ann Rheum Dis. 2014;73:1898–1904.
9. Frey N, Olson EN. Cardiac hypertrophy: the good, the bad, and the ugly. Annu Rev Physiol. 2003;65:45–79.
10. Gao P, Tchernyshyov I, Chang TC, Lee YS, Kita K, Ochi T, Zeller KI, De Marzo AM, Van Eyk JE, Mendell JT, Dang CV. c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. Nature. 2009;458:762–765.
11. Glehr M, Friesenbichler J, Hofmann G, Bernhardt GA, Zacherl M, Avian A, Windhager R, Leithner A. Novel biomarkers to detect infection in revision hip and knee arthroplasties. Clin Orthop Relat Res. 2013;471:2621–2628.
12. Goldring MB. Osteoarthritis and cartilage: the role of cytokines. Curr Rheumatol Rep. 2000;2:459–465.
13. Gollwitzer H, Dombrowski Y, Prodinger PM, Peric M, Summer B, Hapfelmeier A, Saldamli B, Pankow F, von Eisenhart-Rothe R, Imhoff AB, Schauber J, Thomas P, Burgkart R, Banke IJ. Antimicrobial peptides and proinflammatory cytokines in periprosthetic joint infection. J Bone Joint Surg Am. 2013;95:644-651.
14. Gottardo F, Liu CG, Ferracin M, Calin GA, Fassan M, Bassi P, Sevignani C, Byrne D, Negrini M, Pagano F, Gomella LG, Croce CM, Baffa R.Micro-RNA profiling in kidney and bladder cancers. Urol Oncol. 2007;25:387–392.
15. Guan YJ, Li J, Yang X, Du S, Ding J, Gao Y, Zhang Y, Yang K, Chen Q. Evidence that miR-146a attenuates aging- and trauma-induced osteoarthritis by inhibiting Notch1, IL-6, and IL-1 mediated catabolism. Aging Cell. 2018;17:e12752.
16. Hu J, Zhai C, Hu J, Li Z, Fei H, Wang Z, Fan W. MiR-23a inhibited IL-17-mediated proinflammatory mediators expression via targeting IKKalpha in articular chondrocytes. Int Immunopharmacol. 2017;43:1–6.
17. Hunter JJ, Chien KR. Signaling pathways for cardiac hypertrophy and failure. N Engl J Med. 1999;341:1276–1283.
18. Chhabra R, Dubey R, Saini N. Cooperative and individualistic functions of the microRNAs in the miR-23a~27a~24-2 cluster and its implication in human diseases. Mol Cancer. 2010;9:232.
19. Johnnidis JB, Harris MH, Wheeler RT, Stehling-Sun S, Lam MH, Kirak O, Brummelkamp TR, Fleming MD, Camargo FD. Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. Nature. 2008;451:1125–1129.
20. Jovanovic M, Hengartner MO. miRNAs and apoptosis: RNAs to die for. Oncogene. 2006;25:6176–6187.
21. Li YH, Tavallaee G, Tokar T, Nakamura A, Sundararajan K, Weston A, Sharma A, Mahomed NN, Gandhi R, Jurisica I, Kapoor M. Identification of synovial fluid microRNA signature in knee osteoarthritis: differentiating early- and late-stage knee osteoarthritis. Osteoarthritis Cartilage. 2016; 24:1577–1586.
22. Liu S, Liu C, Wang Z, Huang J, Zeng Q. microRNA-23a-5p acts as a potential biomarker for sepsis-induced acute respiratory distress syndrome in early stage. Cell Mol Biol (Noisy-le-grand). 2016;62:31–37.
23. Meng F, Henson R, Lang M, Wehbe H, Maheshwari S, Mendell JT, Jiang J, Schmittgen TD, Patel T. Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. Gastroenterology. 2006;130:2113–2129.
24. Murata K., Yoshitomi H., Tanida S., Ishikawa M., Nishitani K., Ito H., Nakamura T. Plasma and synovial fluid microRNAs as potential biomarkers of rheumatoid arthritis and osteoarthritis. Arthritis Res Ther. 2010;12:86.
25. Nahid MA, Pauley KM, Satoh M, Chan EK. miR-146a is critical for endotoxin-induced tolerance: implication in innate immunity. J Biol Chem. 2009;284:34590–34599.
26. Nahid MA, Satoh M, Chan EK. Mechanistic role of microRNA-146a in endotoxin-induced differential cross-regulation of TLR signaling. J Immunol. 2011;186:1723–1734.
27. Nakasa T, Miyaki S, Okubo A, Hashimoto M, Nishida K, Ochi M, Asahara H. Expression of microRNA-146 in rheumatoid arthritis synovial tissue. Arthritis Rheum. 2008;58:1284–1292.
28. Nziza N, Jeziorski E, Delpont M, Cren M, Chevassus H, Carbasse A, Mahe P, Abassi H, Joly-Monrigal P, Schordan E, Mangé A, Jorgensen C, Apparailly F, Duroux-Richard I. Synovial-fluid miRNA signature for diagnosis of juvenile idiopathic arthritis. Cells. 2019;8:1521.
29. O'Brien J, Hayder H, Zayed Y, Peng C. Overview of microRNA biogenesis, mechanisms of actions, and circulation. Front Endocrinol (Lausanne). 2018;9:402.
30. O'Connell RM, Rao DS, Baltimore D. microRNA regulation of inflammatory responses. Annu Rev Immunol. 2012;30:295–312.
31. Parvizi J, Tan TL, Goswami K, Higuera C, Della Valle C, Chen AF, Shohat N. The 2018 definition of periprosthetic hip and knee infection: an evidence-based and validated criteria. J Arthroplasty. 2018;33:1309–1314.e2.
32. Parvizi J, Zmistowski B, Berbari EF, Bauer TW, Springer BD, Della Valle CJ, Garvin KL, Mont MA, Wongworawat MD, Zalavras CG. New definition for periprosthetic joint infection: from the Workgroup of the Musculoskeletal Infection Society. Clin Orthop Relat Res. 2011;469:2992–2994.
33. Pauley KM, Cha S, Chan EK. MicroRNA in autoimmunity and autoimmune diseases. J Autoimmun. 2009;32:189–194.
34. Pauley KM, Cha S. miRNA-146a in rheumatoid arthritis: a new therapeutic strategy. Immunotherapy. 2011;3:829–831.
35. Perry MM, Moschos SA, Williams AE, Shepherd NJ, Larner-Svensson HM, Lindsay MA. Rapid changes in microRNA-146a expression negatively regulate the IL-1beta-induced inflammatory response in human lung alveolar epithelial cells. J Immunol. 2008;180:5689–5698.
36. Randau TM, Friedrich MJ, Wimmer MD, Reichert B, Kuberra D, Stoffel-Wagner B, Limmer A, Dieter C Wirtz DC, Gravius G. Interleukin-6 in serum and in synovial fluid enhances the differentiation between periprosthetic joint infection and aseptic loosening. PLoS One. 2014;9:e89045.

37. Rottiers V, Näär AM. MicroRNAs in metabolism and metabolic disorders. Nat Rev Mol Cell Biol. 2012;13:239–250.
38. Saumet A, Vetter G, Bouttier M, Portales-Casamar E, Wasserman WW, Maurin T, Mari B, Barbry P, Vallar L, Friederich E, Arar K, Cassinat B, Chomienne C, Lecellier CH. Transcriptional repression of microRNA genes by PML-RARA increases expression of key cancer proteins in acute promyelocytic leukemia. Blood. 2009;113:412–421.
39. Shenoy A, Blelloch RH. Regulation of microRNA function in somatic stem cell proliferation and differentiation. Nat Rev Mol Cell Biol. 2014;5:565–576.
40. Si X, Cao D, Chen J, Nie Y, Jiang Z, Chen MY, Wu JF, Guan XD. miR23a downregulation modulates the inflammatory response by targeting ATG12mediated autophagy. Mol Med Rep. 2018;18:1524–1530.
41. Stanczyk J, Pedrioli DM, Brentano F, Sanchez-Pernaute O, Kolling C, Gay RE, Detmar M, Gay S, Kyburz D. Altered expression of MicroRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis. Arthritis Rheum. 2008;58:1001–1009.
42. Stata Version 13, Stata Press Publication, Texas, 2013.
43. Taganov KD, Boldin MP, Chang KJ, Baltimore D. NF-B-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci U S A. 2006;103:12481–12486.
44. Tavallaee G, Rockel JS, Lively S, Kapoor M. MicroRNAs in Synovial Pathology Associated With Osteoarthritis. Front Med (Lausanne). 2020;7:376..
45. Wada S, Kato Y, Okutsu M, Miyaki S, Suzuki K, Asahara H, Ushida T, Akimoto T. miR-23a attenuates skeletal muscle atrophy by targeting MAFbx/atrogin-1 and MuRF1. J Physiol Sci. 2009;59:216–216.
46. Wang HJ, Zhang PJ, Chen WJ, Feng D, Jia YH, Xie LX. Four serum microRNAs identified as diagnostic biomarkers of sepsis. J Trauma Acute Care Surg. 2012;73:850–854.
47. Wang J, Chen J, Sen S. MicroRNA as biomarkers and diagnostics. J Cell Physiol. 2016;231:25–30.
48. Wang JF, Yu ML, Yu G, Bian JJ, Deng XM, Wan XJ, Zhu KM. Serum miR-146a and miR-223 as potential new biomarkers for sepsis. Biochem Biophys Res Commun. 2010;394:184–188.
49. Xi X, Zhang C, Han W, Zhao H, Zhang H, Jiao J. MicroRNA-223 is upregulated in active tuberculosis patients and inhibits apoptosis of macrophages by targeting FOXO3. Genet Test Mol Biomarkers. 2015;19:650–656.

Corresponding author:
Pavlína Kušnierová
Institute of Laboratory Medicine, Department of Clinical Biochemistry
University Hospital Ostrava
tř. 17. listopadu 1790
708 52 Ostrava
E-mail: pavlina.kusnierova@fno.cz